IMMUNE EVASION STRATEGIES IN PROTOZOAN-HOST INTERACTIONS, 2nd Edition

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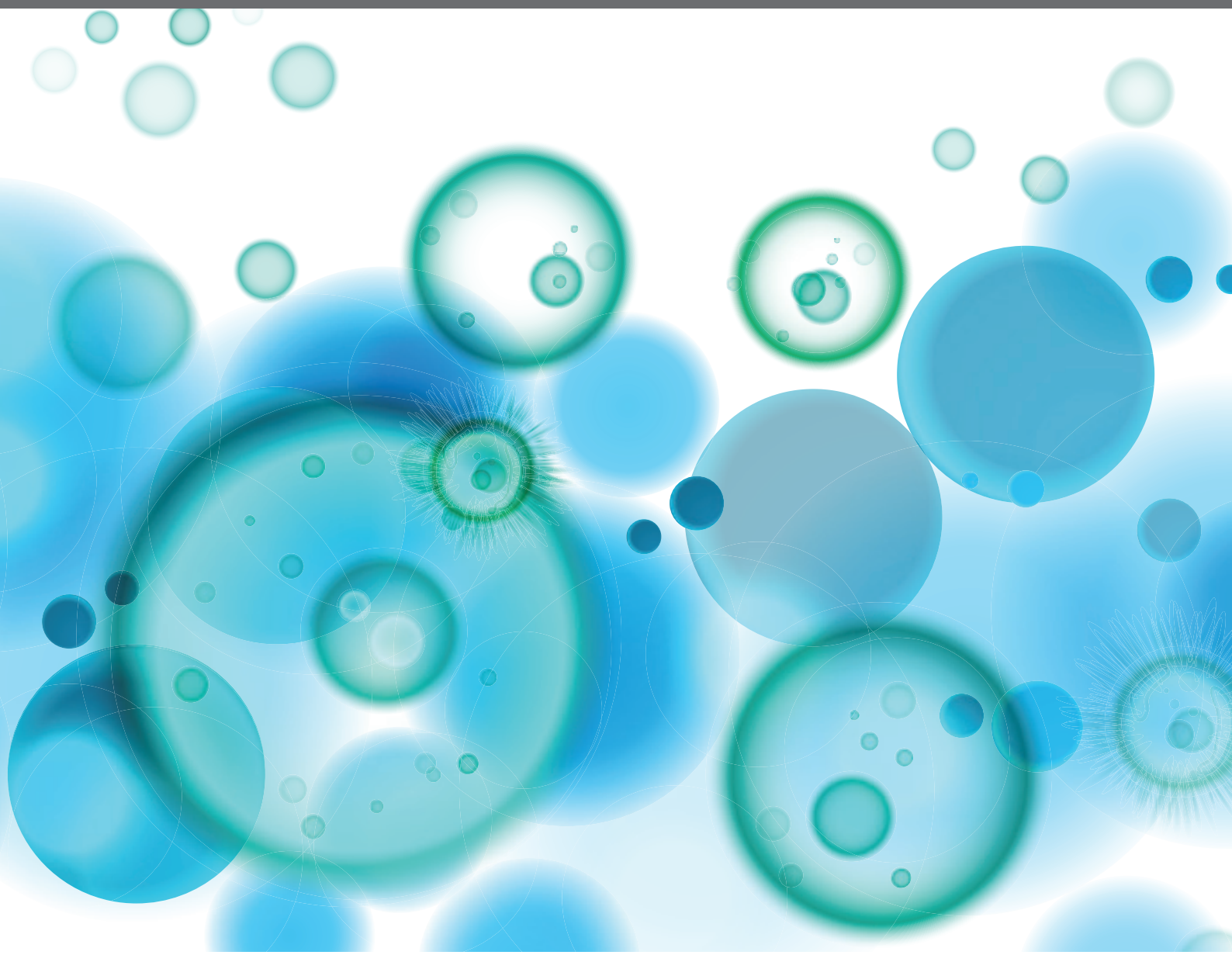

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IMMUNE EVASION STRATEGIES IN PROTOZOAN-HOST INTERACTIONS, 2nd Edition

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EDITORIAL
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Editorial: Immune Evasion Strategies in Protozoan-Host Interactions

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Editorial on the Research Topic

Immune Evasion Strategies in Protozoan-Host Interactions

One of the most successful protozoans is the malaria parasite, which has evolved key mechanisms to avoid the host immune system. This theme was recently reviewed with a focus on their capacity to overcome both innate responses, and the induction and maintenance of adaptive immune responses (Gomes, Bhardwaj et al.; Rénia and Goh; Gomes, Feijó et al.). With regards to this topic, the authors highlighted the importance of carbohydrate-mediated interactions that directly affect *Plasmodium* survival and host resistance. *Plasmodium* parasites have a complex life cycle in the vertebrate host. Initial stages comprise infection of hepatocytes, in which the CSP and TRAP domains of the sporozoite form of the parasite mediate an adhesive interaction with sulfated glycoconjugates on the surface of hepatocytes, initiating the intracellular parasitism stage in the host. The dependency on carbohydrate-mediated interactions for the parasitism also continues in the bloodstream form of the parasite *via* carbohydrates expressed by red blood cells, such as ABO, Lewis, and Duffy, which thereby influences erythrocyte parasitism (Gomes, Feijó et al.).

The glycobiology of parasite-host interactions has been proposed as a potential drug target. This is of particular importance for Chagas disease, a chronic infection caused by *Trypanosoma cruzi*. *T. cruzi* infection induces CD8 T cell responses able to control but not eliminate the parasite, which is then able to subvert the host defenses *via* immuno-endocrine regulation (Cardoso et al.; Morrot et al.; Mendonça et al.; Silva-Neto et al.; Decote-Ricardo et al.; Sanmarco et al.; Gil-Jaramillo et al.). Drugs designed to interfere with carbohydrate-mediated interactions during host-pathogen interplay have been shown to block the ability of *T. cruzi* to evade host immune surveillance. The parasite expresses a multifunctional enzyme called trans-sialidase responsible for catalyzing the transfer of sialic acid domains from the host glycoconjugates to mucin-like molecules on the *T. cruzi* cell surface. The sialylated domains of parasite mucins are shown to jeopardize host defenses, compromising both B and T cell-adaptive responses during infection (Nardy et al.; Freire-de-Lima et al.).

The evasion strategies employed by Trypanosomadidae involve the modulation of components that make up the large arsenal of defense mechanisms. This is seen for *Leishmania* parasites in which upregulation of L-arginine and polyamine production correlate with increased levels of immunosuppressive IL-10 and concomitant reductions in the expression of the inflammatory cytokines, IL-12, and TNF-α, in *L. donovani*-infected macrophages (Mandal et al.). The maintenance of an immunosuppressive cytokine milieu by the parasite reservoir is also demonstrated in visceral leishmaniasis in which B-1 regulatory cells contribute to sustained levels of splenic IL-10 and impairment of macrophage resistance (Arcanjo et al.). Furthermore,

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the manipulation of host cytokine networks by *Leishmania* parasites (Mahanta et al.) has been shown to be a determinant factor for the establishment of immune evasion mechanisms that favor parasite persistence in *Leishmania* infection (Pérez-Cabezas et al.; Falcão et al.; de Freitas et al.).

Trypanosomatids serve as good examples of parasites that modulate the contribution made by activated macrophages in controlling infection. *T. brucei*-derived trypanosome suppression immunomodulating factor (TSIF) plays a role in triggering both macrophage and T cell suppressive states thus leading to pathogenicity (Stijlemans et al.). Other host immune evasion mechanisms rely on the suppression of the macrophage respiratory burst. This is well characterized for *Entamoeba histolytica* parasitism as the parasite can induce neutrophil apoptosis and suppress nitric oxide (NO) production by macrophages (Begum et al.). In fact, reactive oxygen species (ROS) production seems to be a major component in the ability of macrophages to resolve protozoan parasite infections (Moreira-Souza et al.). The control of cell-mediated innate responses may impair the development of effective immune mechanisms thus allowing parasite survival (Geiger et al.). Modulation of lipid pathways is another critical step in guaranteeing protozoan parasite survival by interfering with sentinel cell signaling, clearance, and trafficking mechanisms (Toledo et al.; Totino and Lopes). This is exemplified by *Toxoplasma gondii* infection, where parasites reach immune-privileged host sites and persist in quiescence (Nakada-Tsukui and Nozaki; Chakraborty et al.; Brasil et al.).

Understanding host-protozoan interactions requires interdisciplinary study focused on these complex interactions within biological systems. In malaria, determinants of hepatocyte liver stage infection have been elucidated, and new systems biology approaches have resulted in the development of methodologies that could ultimately lead to advanced strategies to eradicate malaria (Zuck et al.). Following this approach, the characterization of the malaria parasite secretome has provided knowledge on immune evasion and virulence targets that can be used to optimize intervention strategies to control infection (Soni et al.). Finally, the outcome of protozoan-host interactions can also be influenced by co-infections (Pinna et al.), the ability of parasites to manipulate host resources and defense responses for their survival (Ramírez-Toloza et al.; Kakani et al.; Linhares-Lacerda and Morrot), and polymorphisms (Lima-Junior and Pratt-Riccio).

The eradication of protozoan infections is complex and must involve the integration of new approaches as control strategies. Successful health management policies to contain and eradicate such tropical diseases must include the integration of diverse intervention strategies (Filardy et al.). Applying geographic information systems technologies to monitor the spatial distribution of drug resistance and parasite polymorphisms (Filardy et al.), together with effective disease biomarkers for early parasite diagnosis and that distinguish clinical phases of infections (Linhares-Lacerda et al.; Pinho et al.), optimized vaccine design (Wilson et al.; Luca and Macedo; Furtado-de-Mendonça et al.; Minigo et al.; Stijlemans et al.), the use of next generation drugs and alternative therapies for progressive diseases (Pandey et al.), as well as a better understanding of the vector biology for controlling the transmission cycles (Soumana et al.; Ngoune et al.; Jacob et al.), will, together, permit us to develop effective protozoan disease elimination programs.

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The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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Kinetoplastid membrane protein-11 as a vaccine candidate and a virulence factor in *Leishmania*

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Kinetoplastid membrane protein-11 (KMP-11), a protein present in all kinetoplastid protozoa, is considered a potential candidate for a leishmaniasis vaccine. In *Leishmania amazonensis*, KMP-11 is expressed in promastigotes and amastigotes. In both stages, the protein was found in association with membrane structures at the cell surface, flagellar pocket, and intracellular vesicles. More importantly, its surface expression is higher in amastigotes than in promastigotes and increases during metacyclogenesis. The increased expression of KMP-11 in metacyclic promastigotes, and especially in amastigotes, indicates a role for this molecule in the parasite relationship with the mammalian host. In this connection, we have shown that addition of KMP-11 exacerbates *L. amazonensis* infection in peritoneal macrophages from BALB/c mice by increasing interleukin (IL)-10 secretion and arginase activity while reducing nitric oxide production. The doses of KMP-11, the IL-10 levels, and the intracellular amastigote loads were strongly, positively, and significantly correlated. The increase in parasite load induced by KMP-11 was inhibited by anti-KMP-11 or anti-IL-10-neutralizing antibodies, but not by isotype controls. The neutralizing antibodies, but not the isotype controls, were also able to significantly decrease the parasite load in macrophages cultured without the addition of KMP-11, demonstrating that KMP-11-induced exacerbation of the infection is not dependent on the addition of exogenous KMP-11 and that the protein naturally expressed by the parasite is able to promote it. All these data indicate that KMP-11 acts as a virulence factor in *L. amazonensis* infection.

Keywords: *Leishmania*, leishmaniasis, KMP-11, virulence factor, vaccine, *Leishmania amazonensis*, *Leishmania braziliensis*

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THE LEISHMANIASES

Diversity is the key word for defining the leishmaniases, a group of diseases caused by the infection with parasitic protozoa of the genus *Leishmania* and transmitted by sandfly (Phlebotominae) vectors (1): diversity of parasite species, diversity of vector species, diversity of eco-epidemiological conditions involved in transmission, and diversity of clinical presentations. The leishmaniasis can be broadly classified as tegumentary (2), in which the parasitism is restricted to the integument (skin

or mucosa) and visceral leishmaniasis (VL), in which internal organs like spleen, liver, bone marrow, and lymph nodes are infected. The former can be further divided into cutaneous (CL), diffuse cutaneous (DCL), and mucosal (or mucocutaneous, ML) leishmaniasis (1), according to clinical and immunopathological patterns. CL is the primary clinical form in all cases. It can be caused by all the dermotropic *Leishmania* parasites and it is, by far, the most common presentation of tegumentary leishmaniasis. ML and DCL are less frequent and more severe clinical forms, associated with distinct species and particular patterns of immune response. VL is caused by only two *Leishmania* species: *Leishmania donovani* and *Leishmania infantum* (3), but many species, belonging to two different subgenera (*Leishmania* and *Viannia*), can produce tegumentary leishmaniasis (1). While Old World CL is caused by three species, all of them of the *Leishmania* subgenus, American tegumentary leishmaniasis, so called because it encompasses CL, DCL, and ML, can be caused by various species of the *Leishmania* and the *Viannia* subgenera, the latter been exclusive of the American continent. It is currently estimated an annual incidence of 0.2–0.4 and 0.7–1.2 million cases for VL and CL cases, respectively, with a tentative estimate of 20,000–40,000 deaths per year due to VL. However, all these numbers are probably underestimated. Six countries (India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil) account for more than 90% of global VL cases. CL has a wider geographical distribution, with the Americas, the Mediterranean basin, and western Asia being the most affected regions (4).

CONTROL OF *LEISHMANIA* INFECTION BY THE MAMMALIAN IMMUNE SYSTEM

There are two major morphological stages in the life cycle of *Leishmania*: the promastigote and the amastigote. The promastigotes are the 15–20 μm long flagellated and motile forms found within the insect vectors, while the 3–5 μm long amastigotes, which lack the external flagellum, are found inside mononuclear phagocytic cells of the mammalian hosts (5).

The promastigotes undergo a differentiation process termed metacyclogenesis within the gut of the insect vector (6). Metacyclic promastigotes are the infective form for the mammalian host. They have been shown to be far more resistant to complement-mediated lysis than the procyclic promastigotes, which divide attached to the vector's midgut epithelial cells (7). After the inoculation of the infective promastigotes by the sandfly bite, the establishment of the intracellular infection depends on a number of factors: size of inoculum (8); the phlebotomine saliva, which contains immunomodulatory molecules (9); presence of apoptotic promastigotes (10), and, especially, the ability of the parasites to survive the innate immune response of the host, which includes, among other factors, complement-mediated lysis and opsonization, phagocytosis by neutrophils and macrophages, Toll-like receptors, the NLRP3 inflammasome, and many cytokines and chemokines (11). The successful establishment of the infection results in the amastigotes dividing in phagolysosomes of macrophages, where they inhibit or subvert the killing mechanisms of these cells, making them permissive to the infection (12).

At this point, the control of the infection will depend on the adaptive immune response. Th1 $CD4^+$ cells induce the activation of the parasitized macrophages through the secretion of interferon-gamma (IFN-γ) (13), with the help of other proinflammatory cytokines, such as tumor necrosis factor-alpha (14), rendering these cells capable to kill the amastigotes by producing nitric oxide (NO) and/or reactive oxygen species (ROS) (15). The generation of an effective memory T-cell response is the goal of vaccination.

THE SEARCH FOR A VACCINE AGAINST LEISHMANIASIS

The transmission of pathogenic *Leishmania* species is characterized by a high degree of parasite-vector specificity (16) and ~30 sandfly species are believed to be competent vectors (17). Each species has a particular ecology (18), which determines the transmission conditions and the risk factors for acquiring the disease. This diversity makes the design of control strategies extremely difficult. Moreover, the current control measures directed toward vectors and animal reservoirs have not been reliably effective (19). As a result, the geographical distribution of leishmaniasis is expanding, even to urban areas (20). On the other hand, the currently used chemotherapy regimens are toxic and expensive. Most of them have to be used parenterally for long periods (21), making adherence to therapy difficult to achieve (22). In addition, resistance to standard therapy, as pentavalent antimonials, is becoming more frequent (23). Therefore, an effective and safe vaccine could be the most comprehensive and cost-effective tool for the prevention of leishmaniasis (24).

There is no effective vaccine against any form of human leishmaniasis (24). However, during the last four decades, there have been many approaches for the development of a vaccine against leishmaniasis. Most of them stopped at the experimental level. Only a few have reached clinical trials. The majority of these were the so-called first-generation vaccine (25) candidates, composed of killed promastigotes. A major advantage of these vaccines is that they could be manufactured at low technological level and relative low cost in endemic countries (25). However, standardization of vaccines derived from cultured parasites would be impossible. Furthermore, after the various clinical trials performed with these vaccines, their efficacy has not been clearly demonstrated (26). The second-generation vaccine candidates encompass a variety of approaches: recombinant proteins, DNA, and genetically engineered organisms, such as vectored vaccines and attenuated *Leishmania*. As a rule, recombinant DNA technology is involved in their production. Their main advantages relate to safety and standardization because in this kind of vaccine, the content is precisely known. The immunization strategies mentioned above represent different modes of delivery of defined immunogens, which are, in general, parasite molecules. A number of them have been proposed as vaccine candidates, such as glycoprotein gp63, *Leishmani*a homolog of receptors for activated C kinase (LACK), kinetoplastid membrane protein-11 (KMP-11), histone H1, sterol 24-c-methyltranferase, amastigote-specific protein A2, cysteine proteinases, nucleoside hydrolase,

thiol-specific antioxidant, *Leishmania major* stress-inducible protein 1, *Leishmania* elongation initiation factor, among others (27, 28). The latter three constitute a multi-subunit candidate vaccine, Leish-111F, the only recombinant candidate vaccine against leishmaniasis already tested in humans (27, 28), so far without evidence of efficacy.

KINETOPLASTID MEMBRANE PROTEIN-11 AS A VACCINE CANDIDATE

Kinetoplastid membrane protein-11 was discovered as a T cell-reactive contaminant (29) in preparations of lipophosphoglycan, the most abundant macromolecule on the surface of the promastigote stage of *Leishmania* spp. (30). Since then, it has been considered as a promising candidate antigen for a vaccine against leishmaniasis. It has shown an immunoprotective effect in a variety of immunization protocols (31–34).

Kinetoplastid membrane protein-11 is a protein characteristic and specific of kinetoplastid protozoa (35). The KMP-11 coding genes and their products show a remarkably high degree of sequence homology among all *Leishmania* species of both subgenera. When KMP-11 gene sequences of *L. (Viannia) panamensis*, *L. (Leishmania) infantum*, and *L. (L) donovani* were compared, a homology of more than 95% was found among them, and only three amino acid changes were found when the corresponding deduced amino acid sequences were compared (36). On the other hand, this protein shows very low homology with human proteins (37). KMP-11 has a strong antigenicity for murine (31) and human T cells (38) and is capable of stimulating both innate (39) and adaptive (38) immune responses. All these are characteristics of an ideal leishmaniasis vaccine candidate.

Another fundamental aspect for a candidate antigen for a leishmaniasis vaccine is its expression in the amastigote, the infective stage for mammals. Concerning this subject, there are interesting reports on the variability of KMP-11 expression among different species of *Leishmania*. This protein was found to be expressed at higher levels in *L. infantum* promastigotes than in amastigotes (40, 41), whereas its expression is up-regulated in amastigotes of *Leishmania amazonensis* (42) and *Leishmania mexicana* (41). It is interesting to notice that these three species belong to the *Leishmania* subgenus. To our knowledge, a similar investigation on differential expression of KMP-11 in species belonging to the *Viannia* subgenus has never been performed. Recognizing this variability is necessary for the understanding of the diversity found in the infections with different *Leishmania* species with regard to host–parasite relationship and pathogenesis. Unfortunately, this aspect has been largely neglected in leishmaniasis research. It is possible that a molecule, which plays a key role in the infection with a given *Leishmania* species would have no relevance at all for another. In this sense, it is surprising that the genomes of species causing so diverse diseases in humans like *L. major*, *L. infantum* (both from the *Leishmania* subgenus) and *Leishmania braziliensis* (*Viannia* subgenus) contain <1% species-specific genes (43). A possible explanation for this unexpected finding is that, in spite of the high similarity in their genome sequences, important differences were found between different *Leishmania* species with regard to stage-regulated gene expression (44). These differences may represent the adaptation to different vector species or the development of different strategies for survival in the mammalian host.

IMMUNOLOGICAL BASIS FOR VIRULENCE FACTORS AS VACCINES AGAINST LEISHMANIASIS

During several decades, a reductionist vision has oversimplified the understanding of immunopathology of the leishmaniases. This was based in conclusions drawn from the mouse model of *L. major* infection. In this model, there is an association of resistance or susceptibility to infection with the predominance of Th1 or Th2 $CD4^+$ T cell-mediated responses, respectively (45). Although this model has contributed to demonstrate the key role played by IFN-γ and Th1 cells in the control of *Leishmania* infection, it has become clear that the resistance/susceptibility to other *Leishmania* species do not fit into the so-called Th1/Th2 paradigm (46). Nevertheless, it has long guided the efforts of immunoparasitologists and vaccinologists toward the development of an anti-*Leishmania* vaccine. During this period, the Th1/Th2 paradigm was the conceptual basis for the search of potentially protective candidate antigens for a vaccine against leishmaniasis. However, this strategy eventually proved to be ineffective. *Leishmania* antigens that stimulate a Th1 immune response during the disease or even after cure were not able to induce protection when used as vaccines. On the other hand, antigens associated with disease-promoting immune responses in the early infection have been found to be highly protective if a Th1 response to them is generated by vaccination before infection (47). Probably, the best example of this is the LACK antigen which stimulates a strong Th2 response soon after infection of BALB/c mice (48) that is responsible for their extreme susceptibility to this parasite (49). However, the same antigen, when administered with adjuvants that stimulate Th1 responses (50) or as a DNA vaccine (51) protects BALB/c mice from subsequent infections with *L. major*. During coevolution, parasites have learned how to manipulate the host immune system to their own advantage by developing particular ways of antigen presentation and delivery during infection. Based on accumulating evidence, it is reasonable to believe that those evasion strategies can be overcome by defined immunization protocols using disease-promoting parasite antigens. Thus, at present, virulence factors are considered as potential drug targets and vaccine candidates for the control of leishmaniasis (52) and other infectious diseases (53).

KINETOPLASTID MEMBRANE PROTEIN-11 AS A VIRULENCE FACTOR IN *LEISHMANIA* SPP.

It has been shown that KMP-11 is a potent inducer of interleukin-10 (IL-10) production in peripheral blood mononuclear

cells from patients with American CL and it is also able to inhibit the IFN-γ response of these cells to soluble *L. braziliensis* antigen extract (54, 55). IL-10 is a cytokine with anti-inflammatory properties produced by T cells, B cells, macrophages/monocytes, and keratinocytes. It can inhibit the synthesis of proinflammatory cytokines and chemokines as well as the production of NO and ROS by macrophages (56–58), restraining their ability to kill intracellular organisms (59–62).

Mukhopadhyay et al. suggested that KMP-11 may play a role in the virulence of *L. donovani* promastigotes because the loss of infective power obtained by successive sub-culturing was associated with a down-regulation of its expression (63).

Moreover, the increased expression of KMP-11 in metacyclic promastigotes, and especially in amastigotes, indicates a role for this molecule in the parasite relationship with the mammalian host, at least in members of the *L. mexicana* complex (64): *L. amazonensis* (42) and *L. mexicana* (41).

All these observations have prompted us to investigate a possible role for KMP-11 as a virulence factor in *Leishmania*. By using an *in vitro* model, we showed an exacerbating effect of KMP-11 on the infection of peritoneal macrophages from BALB/c mice with *L. amazonensis*, implicating this protein as a virulence factor for this species. This effect was higher when KMP-11 was added to the cultures 4 h after infection (and after the removal of the remaining extracellular promastigotes), as compared to simultaneously or 4 h before infection, demonstrating that the infection-promoting effect of the protein was on amastigote proliferation rather than on the internalization of promastigotes. The increase in amastigote loads was associated to an increase in IL-10 secretion and arginase activity and to an inhibition of NO production. More importantly, anti KMP-11 and anti-IL-10 antibodies were able to significantly decrease the parasite load in macrophages cultured without the addition of KMP-11, demonstrating that KMP-11-induced exacerbation of the infection is not dependent on the addition of exogenous KMP-11 and that the protein naturally expressed by the parasite is able to promote it (65).

It was recently demonstrated that poly(lactide-co-glycolide acid) nanoparticles loaded with KMP-11 induce of potent innate responses in BALB/c macrophages infected with *L. braziliensis*, promoting amastigote killing. These responses involve increased production of NO, superoxide, TNF-α and IL-6; release of CCL2/MCP-1 and CXCL1/KC; recruitment of macrophages and neutrophils *in vitro*; activation of caspase-1 and the secretion of IL-1β and IL-18 (39). Interestingly, the recombinant protein alone did not show such an effect. In contrast to our work with *L. amazonensis*, which was performed with resident peritoneal macrophages (65), thioglycolate-activated macrophages were used in this study.

The results described obtained with soluble or PLGA-coupled KMP-11 in *in vitro* infections of resident or thioglycolate-activated BALB/c peritoneal macrophages infected with *L. amazonensis* or *L. braziliensis* pose interesting questions concerning antigen delivery, macrophage activation, and differences in patterns of host–parasite relationship between different *Leishmania* species.

Leishmania amazonensis or *L. braziliensis* belong to different subgenera, *Leishmania* and *Viannia*, respectively (66), which are thought to have diverged 90 million years ago, when South America and Africa separated (67). Thus, New World CL is a disease caused by parasites that are quite different from each other. From the human health point of view, one of their most significant differences is the way that species from different subgenera interact with the mammalian host immunity (66).

Leishmania amazonensis and other members of the *L. mexicana* complex possess a remarkable ability to subvert or modulate innate and adaptive immune responses of the vertebrate host (68, 69). As a result of this, these parasites cause non-healing cutaneous lesions in most inbred strains of mice (68), although differences in susceptibility can be observed among them (69). In humans, *L. amazonensis* and *L. mexicana* are responsible for DCL, the only incurable form of human leishmaniasis, characterized by complete absence of specific type 1 response (proinflammatory, parasiticidal) to leishmanial antigens and unrestrained parasite growth (70).

Leishmania braziliensis and other species of the *Viannia* subgenus are not as able as the species of the *L. mexicana* complex to suppress proinflammatory and parasiticidal type 1 responses. Instead, the disease occurs in presence of an established Th1 response and IFN-γ production. Nevertheless, this response has some inhibitory effect on parasite growth. That is why parasites are less numerous in cutaneous lesions caused by *L. braziliensis* than in those produced by infection with *L. amazonensis* (66). The severe clinical form resulting from *L. braziliensis* is ML, which is associated with up-regulated Th1 responses (71). *L. braziliensis* is much less pathogenic for mice than *L. amazonensis*. Experimental infection with *L. braziliensis* can only be achieved in the BALB/c strain (72, 73).

CONCLUSION AND PERSPECTIVES

The presented data indicate that KMP-11 can act as a virulence factor for *L. amazonensis*, although this may not be the case for other *Leishmania* species. Future research on this subject should include the demonstration of an *in vivo* disease-exacerbating effect of KMP-11 in leishmanial infection and the evaluation of the role played by this molecule in the infection with other *Leishmania* species.

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REFERENCES

1. Kevric I, Cappel MA, Keeling JH. New world and old world *Leishmania* infections: a practical review. *Dermatol Clin* (2015) **33**(3):579–93. doi:10.1016/j.det.2015.03.018
2. Escobar MA, Martinez F, Scott Smith D, Palma GI. American cutaneous and mucocutaneous leishmaniasis (tegumentary): a diagnostic challenge. *Trop Doct* (1992) **22**(Suppl 1):69–78;63–4
3. Roatt BM, Aguiar-Soares RD, Coura-Vital W, Ker HG, Moreira ND, Vitoriano-Souza J, et al. Immunotherapy and immunochemotherapy in visceral leishmaniasis: promising treatments for this neglected disease. *Front Immunol* (2014) **5**:272. doi:10.3389/fimmu.2014.00272
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. WHO leishmaniasis control team. leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
5. Pace D. Leishmaniasis. *J Infect* (2014) **69**(Suppl 1):S10–8. doi:10.1016/j.jinf.2014.07.016
6. Sacks DL. Metacyclogenesis in *Leishmania* promastigotes. *Exp Parasitol* (1989) **69**(1):100–3. doi:10.1016/0014-4894(89)90176-8
7. Descoteaux A, Turco SJ. Glycoconjugates in *Leishmania* infectivity. *Biochim Biophys Acta* (1999) **1455**(2–3):341–52. doi:10.1016/S0925-4439(99)00065-4
8. Kimblin N, Peters N, Debrabant A, Secundino N, Egen J, Lawyer P, et al. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proc Natl Acad Sci U S A* (2008) **105**(29):10125–30. doi:10.1073/pnas.0802331105
9. Gillespie RD, Mbow ML, Titus RG. The immunomodulatory factors of bloodfeeding arthropod saliva. *Parasite Immunol* (2000) **22**(7):319–31. doi:10.1046/j.1365-3024.2000.00309.x
10. Wanderley JL, Pinto da Silva LH, Deolindo P, Soong L, Borges VM, Prates DB, et al. Cooperation between apoptotic and viable metacyclics enhances the pathogenesis of Leishmaniasis. *PLoS One* (2009) **4**(5):e5733. doi:10.1371/journal.pone.0005733
11. Gurung P, Kanneganti TD. Innate immunity against *Leishmania* infections. *Cell Microbiol* (2015) **17**(9):1286–94. doi:10.1111/cmi.12484
12. Vannier-Santos MA, Martiny A, de Souza W. Cell biology of *Leishmania* spp.: invading and evading. *Curr Pharm Des* (2002) **8**(4):297–318. doi:10.2174/1381612023396230
13. Santos Cda S, Boaventura V, Ribeiro Cardoso C, Tavares N, Lordelo MJ, Noronha A, et al. CD8(+) granzyme B(+)-mediated tissue injury vs. CD4(+) IFNγ(+)-mediated parasite killing in human cutaneous leishmaniasis. *J Invest Dermatol* (2013) **133**(6):1533–40. doi:10.1038/jid.2013.4
14. Green SJ, Crawford RM, Hockmeyer JT, Meltzer MS, Nacy CA. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *J Immunol* (1990) **145**(12):4290–7.
15. Mukbel RM, Patten C Jr, Gibson K, Ghosh M, Petersen C, Jones DE. Macrophage killing of *Leishmania amazonensis* amastigotes requires both nitric oxide and superoxide. *Am J Trop Med Hyg* (2007) **76**(4):669–75.
16. Killick-Kendrick R. Some epidemiological consequences of the evolutionary fit between leishmaniae and their phlebotomine vectors. *Bull Soc Pathol Exot* (1985) **78**:747–55.
17. Desjeux P. Public health aspects and control. *Clin Dermatol* (1996) **14**:417–23. doi:10.1016/0738-081X(96)00057-0
18. Rotureau B, Gaborit P, Issaly J, Carinci R, Fouque F, Carme B. Diversity and ecology of sand flies (Diptera: Psychodidae: Phlebotominae) in coastal French Guiana. *Am J Trop Med Hyg* (2006) **75**(1):62–9.
19. Stockdale L, Newton R. A review of preventative methods against human leishmaniasis infection. *PLoS Negl Trop Dis* (2013) **7**(6):e2278. doi:10.1371/journal.pntd.0002278
20. Martins-Melo FR, Lima Mda S, Ramos AN Jr, Alencar CH, Heukelbach J. Mortality and case fatality due to visceral leishmaniasis in Brazil: a nationwide analysis of epidemiology, trends and spatial patterns. *PLoS One* (2014) **9**(4):e93770. doi:10.1371/journal.pone.0093770
21. Sharifi I, FeKri AR, Aflatonian MR, Khamesipour A, Nadim A, Mousavi MR, et al. Randomised vaccine trial of single dose of killed *Leishmania* major plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. *Lancet* (1998) **351**(9115):1540–3. doi:10.1016/S0140-6736(98)09552-X
22. Ribeiro MN, Pimentel MI, Schubach Ade O, Oliveira Rde V, Teixeira JL, Leite MP, et al. Factors associated to adherence to different treatment schemes with meglumine antimoniate in a clinical trial for cutaneous leishmaniasis. *Rev Inst Med Trop Sao Paulo* (2014) **56**(4):291–6. doi:10.1590/S0036-46652014000400004
23. Sundar S, Rai M. Advances in the treatment of leishmaniasis. *Curr Opin Infect Dis* (2002) **15**(6):593–8. doi:10.1097/00001432-200212000-00007
24. Modabber F. Leishmaniasis vaccines: past, present and future. *Int J Antimicrob Agents* (2010) **36**(Suppl 1):S58–61. doi:10.1016/j.ijantimicag.2010.06.024
25. Evans KJ, Kedzierski L. Development of vaccines against visceral leishmaniasis. *J Trop Med* (2012) **2012**:892817. doi:10.1155/2012/892817
26. Noazin S, Khamesipour A, Moulton LH, Tanner M, Nasseri K, Modabber F, et al. Efficacy of killed whole-parasite vaccines in the prevention of leishmaniasis: a meta-analysis. *Vaccine* (2009) **27**(35):4747–53. doi:10.1016/j.vaccine.2009.05.084
27. Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. *Clin Transl Immunology* (2014) **3**(3):e13. doi:10.1038/cti.2014.4
28. Coler RN, Reed SG. Second-generation vaccines against leishmaniasis. *Trends Parasitol* (2005) **21**(5):244–9. doi:10.1016/j.pt.2005.03.006
29. Jardim A, Tolson DL, Turco SJ, Pearson TW, Olafson RW. The *Leishmania donovani* lipophosphoglycan T lymphocyte-reactive component is a tightly associated protein complex. *J Immunol* (1991) **147**:3538–44.
30. McConville MJ, Schnur LF, Jaffe C, Schneider P. Structure of *Leishmania* lipophosphoglycan: inter- and intra-specific polymorphism in old world species. *Biochem J* (1995) **310**:807–18. doi:10.1042/bj3100807
31. Ramírez JR, Gilchrist K, Robledo S, Sepúlveda JC, Moll H, Soldati D, et al. Attenuated *Toxoplasma gondii* ts-4 mutants engineered to express the *Leishmania* antigen KMP-11 elicit a specific immune response in BALB/c mice. *Vaccine* (2001) **20**(3–4):455–61. doi:10.1016/S0264-410X(01)00341-3
32. Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *J Immunol* (2005) **174**(11):7160–71. doi:10.4049/jimmunol.174.11.7160
33. Bhaumik S, Basu R, Sen S, Naskar K, Roy S. KMP-11 DNA immunization significantly protects against *L. donovani* infection but requires exogenous IL-12 as an adjuvant for comparable protection against *L. major*. *Vaccine* (2009) **27**(9):1306–16. doi:10.1016/j.vaccine.2008.12.053
34. Guha R, Das S, Ghosh J, Naskar K, Mandala A, Sundar S, et al. Heterologous priming-boosting with DNA and vaccinia virus expressing kinetoplastid membrane protein-11 induces potent cellular immune response and confers protection against infection with antimony resistant and sensitive strains of *Leishmania (Leishmania) donovani*. *Vaccine* (2013) **31**(15):1905–15. doi:10.1016/j.vaccine.2013.02.025
35. Stebeck CE, Beecroft RP, Singh BN, Jardim A, Olafson RW, Tuckey C, et al. Kinetoplastid membrane protein-11 (KMP-11) is differentially expressed during the life cycle of African trypanosomes and is found in a wide variety of kinetoplastid parasites. *Mol Biochem Parasitol* (1995) **71**:1–13. doi:10.1016/0166-6851(95)00022-S
36. Ramírez JR, Berberich C, Jaramillo A, Alonso C, Vélez IV. Molecular and antigenic characterization of the *Leishmania (Viannia) panamensis* kinetoplastid membrane protein-11. *Mem Inst Oswaldo Cruz* (1998) **93**(2):247–54. doi:10.1590/s0074-02761998000200022
37. Jardim A, Hanson S, Ullman B, Mccubbin WD, Kayt CM, Olafson RW. Cloning and structure-function analysis of the *Leishmania donovani* kinetoplastid membrane protein-11. *Biochem J* (1995) **305**:315–20. doi:10.1042/bj3050315
38. Russo DM, Turco SJ, Burns JM Jr, Reed SG. Stimulation of human T lymphocytes by *Leishmania* lipophosphoglycan-associated proteins. *J Immunol* (1992) **148**(1):202–7.
39. Santos DM, Carneiro MW, de Moura TR, Soto M, Luz NF, Prates DB, et al. PLGA nanoparticles loaded with KMP-11 stimulate innate immunity and induce the killing of *Leishmania*. *Nanomedicine* (2013) **9**(7):985–95. doi:10.1016/j.nano.2013.04.003
40. Berberich C, Machado G, Morales G, Carrillo G, Jiménez-Ruiz A, Alonso C. The expression of the *Leishmania infantum* KMP-11 protein is developmentally regulated and stage specific. *Biochim Biophys Acta* (1998) **1442**:230–7. doi:10.1016/S0167-4781(98)00176-6

41. Lynn MA, Marr AK, McMaster WR. Differential quantitative proteomic profiling of *Leishmania infantum* and *Leishmania mexicana* density gradient separated membranous fractions. *J Proteomics* (2013) **82**:179–92. doi:10.1016/j.jprot.2013.02.010
42. Matos DC, Faccioli LA, Cysne-Finkelstein L, Luca PM, Corte-Real S, Armoa GR, et al. Kinetoplastid membrane protein-11 is present in promastigotes and amastigotes of *Leishmania amazonensis* and its surface expression increases during metacyclogenesis. *Mem Inst Oswaldo Cruz* (2010) **105**:341–7. doi:10.1590/S0074-02762010000300018
43. Lynn MA, McMaster WR. *Leishmania*: conserved evolution – diverse diseases. *Trends Parasitol* (2008) **24**(3):103–5. doi:10.1016/j.pt.2007.11.006
44. Rochette A, Raymond F, Ubeda JM, Smith M, Messier N, Boisvert S, et al. Genome-wide gene expression profiling analysis of *Leishmania major* and *Leishmania infantum* developmental stages reveals substantial differences between the two species. *BMC Genomics* (2008) **9**:255. doi:10.1186/1471-2164-9-25
45. Reiner SL, Locksley RM. Lessons from *Leishmania*: a model for investigations of CD4+ subset differentiation. *Infect Agents Dis* (1992) **1**(1):33–42.
46. McMahon-Pratt D, Alexander J. Does the *Leishmania major* paradigm of pathogenesis and protection hold for new world cutaneous leishmaniases or the visceral disease? *Immunol Rev* (2004) **201**:206–24. doi:10.1111/j.0105-2896.2004.00190.x
47. Campos-Neto A. What about Th1/Th2 in cutaneous leishmaniasis vaccine discovery? *Braz J Med Biol Res* (2005) **38**(7):979–84. doi:10.1590/S0100-879X2005000700001
48. Launois P, Maillard I, Pingel S, Swihart KG, Xénarios I, Acha-Orbea H, et al. IL-4 rapidly produced by V beta 4 V alpha 8 CD4+ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. *Immunity* (1997) **6**(5):541–9. doi:10.1016/S1074-7613(00)80342-8
49. Julia V, Rassoulzadegan M, Glaichenhaus N. Resistance to *Leishmania major* induced by tolerance to a single antigen. *Science* (1996) **274**:421–3. doi:10.1126/science.274.5286.421
50. Mougneau E, Altare F, Wakil AE, Zheng S, Coppola T, Wang ZE, et al. Expression cloning of a protective leishmania antigen. *Science* (1995) **268**:563–6. doi:10.1126/science.7725103
51. Gurunathan S, Sacks DL, Brown DR, Reiner SL, Charest H, Glaichenhaus N, et al. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *J Exp Med* (1997) **186**(7):1137–47. doi:10.1084/jem.186.7.1137
52. Mottram JC, Coombs GH, Alexander J. Cysteine peptidases as virulence factors of *Leishmania*. *Curr Opin Microbiol* (2004) **7**(4):375–81. doi:10.1016/j.mib.2004.06.010
53. Li S, Huang H, Rao X, Chen W, Wang Z, Hu X. Phenol-soluble modulins: novel virulence-associated peptides of staphylococci. *Future Microbiol* (2014) **9**(2):203–16. doi:10.2217/fmb.13.153
54. de Carvalho LP, Soto M, Jerônimo S, Dondji B, Bacellar O, Luz V, et al. Characterization of the immune response to *Leishmania infantum* recombinant antigens. *Microbes Infect* (2003) **5**(1):7–12. doi:10.1016/S1286-4579(02)00051-5
55. Carvalho LP, Passos S, Dutra WO, Soto M, Alonso C, Gollob KJ, et al. Effect of LACK and KMP11 on IFN-gamma production by peripheral blood mononuclear cells from cutaneous and mucosal leishmaniasis patients. *Scand J Immunol* (2005) **61**(4):337–42. doi:10.1111/j.1365-3083.2005.01581.x
56. Spits H, de Waal Malefyt R. Functional characterization of human IL-10. *Int Arch Allergy Immunol* (1992) **99**(1):8–15. doi:10.1159/000236329
57. Opal SM, Wherry JC, Grint P. Interleukin-10: potential benefits and possible risks in clinical infectious diseases. *Clin Infect Dis* (1998) **27**(6):1497–507. doi:10.1086/515032
58. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* (2001) **19**:683–765. doi:10.1146/annurev.immunol.19.1.683
59. Frei K, Nadal D, Pfister HW, Fontana A. *Listeria meningitis*: identification of a cerebrospinal fluid inhibitor of macrophage listericidal function as interleukin 10. *J Exp Med* (1993) **178**(4):1255–61. doi:10.1084/jem.178.4.1255
60. Reed SG, Brownell CE, Russo DM, Silva JS, Grabstein KH, Morrissey PJ. IL-10 mediates susceptibility to *Trypanosoma cruzi* infection. *J Immunol* (1994) **153**(7):3135–40.
61. Murray PJ, Wang L, Onufryk C, Tepper RI, Young RA. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. *J Immunol* (1997) **158**(1):315–21.
62. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol* (2001) **166**(2):1141–7. doi:10.4049/jimmunol.166.2.1141
63. Mukhopadhyay S, Sen P, Majumder HK, Roy S. Reduced expression of lipophosphoglycan (LPG) and kinetoplastid membrane protein (KMP)-11 in *Leishmania donovani* promastigotes in axenic culture. *J Parasitol* (1998) **84**:644–7. doi:10.2307/3284745
64. Tschoeke DA, Nunes GL, Jardim R, Lima J, Dumaresq AS, Gomes MR, et al. The comparative genomics and phylogenomics of *Leishmania amazonensis* parasite. *Evol Bioinform Online* (2014) **10**:131–53. doi:10.4137/EBO.S13759
65. Lacerda DI, Cysne-Finkelstein L, Nunes MP, De-Luca PM, Genestra Mda S, Leon LL, et al. Kinetoplastid membrane protein-11 exacerbates infection with *Leishmania amazonensis* in murine macrophages. *Mem Inst Oswaldo Cruz* (2012) **107**(2):238–45. doi:10.1590/S0074-02762012000200014
66. Silveira FT, Lainson R, Corbett CE. Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil: a review. *Mem Inst Oswaldo Cruz* (2004) **99**(3):239–51. doi:10.1590/S0074-02762004000300001
67. Fernandes AP, Nelson K, Beverley SM. Evolution of nuclear ribosomal RNAs in kinetoplastid protozoa: perspectives on the age and origins of parasitism. *Proc Natl Acad Sci Evol* (1993) **90**:11608–12. doi:10.1073/pnas.90.24.11608
68. Soong L. Subversion and utilization of host innate defense by *Leishmania amazonensis*. *Front Immunol* (2012) **3**:58. doi:10.3389/fimmu.2012.00058
69. Pereira BA, Alves CR. Immunological characteristics of experimental murine infection with *Leishmania (Leishmania) amazonensis*. *Vet Parasitol* (2008) **158**(4):239–55. doi:10.1016/j.vetpar.2008.09.015
70. Azeredo-Coutinho RB, Conceição-Silva F, Schubach A, Cupolillo E, Quintella LP, Madeira MF, et al. First report of diffuse cutaneous leishmaniasis and *Leishmania amazonensis* infection in Rio de Janeiro State, Brazil. *Trans R Soc Trop Med Hyg* (2007) **101**(7):735–7. doi:10.1016/j.trstmh.2007.01.005
71. Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. *Infect Immun* (2002) **70**(12):6734–40. doi:10.1128/IAI.70.12.6734-6740.2002
72. Samuelson J, Lerner E, Tesh R, Titus R. A mouse model of *Leishmania braziliensis braziliensis* infection produced by coinjection with sand fly saliva. *J Exp Med* (1991) **173**(1):49–54. doi:10.1084/jem.173.1.49
73. de Moura TR, Novais FO, Oliveira F, Clarêncio J, Noronha A, Barral A, et al. Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. *Infect Immun* (2005) **73**(9):5827–34. doi:10.1128/IAI.73.9.5827-5834.2005

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Immune Evasion Mechanisms of *Entamoeba histolytica*: Progression to Disease

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Entamoeba histolytica (*Eh*) is a protozoan parasite that infects 10% of the world's population and results in 100,000 deaths/year from amebic dysentery and/or liver abscess. In most cases, this extracellular parasite colonizes the colon by high affinity binding to MUC2 mucin without disease symptoms, whereas in some cases, *Eh* triggers an aggressive inflammatory response upon invasion of the colonic mucosa. The specific host-parasite factors critical for disease pathogenesis are still not well characterized. From the parasite, the signature events that lead to disease progression are cysteine protease cleavage of the C-terminus of MUC2 that dissolves the mucus layer followed by *Eh* binding and cytotoxicity of the mucosal epithelium. The host mounts an ineffective excessive host pro-inflammatory response following contact with host cells that causes tissue damage and participates in disease pathogenesis as *Eh* escapes host immune clearance by mechanisms that are not completely understood. Ameba can modulate or destroy effector immune cells by inducing neutrophil apoptosis and suppressing respiratory burst or nitric oxide (NO) production from macrophages. *Eh* adherence to the host cells also induce multiple cytotoxic effects that can promote cell death through phagocytosis, apoptosis or by trogocytosis (ingestion of living cells) that might play critical roles in immune evasion. This review focuses on the immune evasion mechanisms that *Eh* uses to survive and induce disease manifestation in the host.

Keywords: *Entamoeba histolytica*, immune evasion, phagocytosis, apoptosis, trogocytosis

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INTRODUCTION

Entamoeba histolytica (*Eh*), the causative agent of amebiasis, is an intestinal protozoan parasite that colonizes the intestinal lumen asymptomatically (known as noninvasive disease) in approximately 90% of cases. However, in 10% of individuals, this asymptomatic relationship breaks down and the parasite breaches the innate mucosal barrier and invades the underlying lamina propria resulting in 100,000 death per year globally (Stanley, 2003). Parasite cysts are acquired through the ingestion of contaminated food and water mostly in areas of poor sanitation (Ralston and Petri, 2011a). Birth cohort studies done in an urban slum in Dhaka, Bangladesh found that approximately 50% of infants are infected in the first year of life, with repeated infections that are connected with malnourishment and stunting (Mondal et al., 2006; Korpe et al., 2013). The exact reasons why *Eh* occasionally invade the mucosal epithelium and what host-parasite factors are involved in parasite invasion are not clear. The outcome of invasive *Eh* infection is variable; it can result in amebic diarrhea, amebic colitis, and/or dissemination of the parasites through the portal circulation to cause liver abscess (Ralston and Petri, 2011b). *Eh* invasion induces a robust pro-inflammatory

response and host tissue destruction that exacerbates disease (Moonah et al., 2013; Mortimer et al., 2015). Currently, there is no effective vaccine for this disease; however nitroimidazoles (such as metronidazole) are effective to treat this tissue dwelling parasites. Metronidazole treatment can cause toxic side effects and enhance the probability of developing drug resistant (Petri, 2003; Ralston and Petri, 2011b). Our host immune system sets up a series of defensive responses against the parasite. However, continued morbidity and mortality point out that this parasite is capable of escaping host defense responses to maintain its own survival (Moonah et al., 2013). Thus, an understanding of the human immune response to the parasite and the strategies used by the parasite to evade host defense will deeply improve the development of effective immunotherapies. In this review, we will focus on the host immune responses against *Eh* and the complex strategies the parasite uses to evade host immunity.

HOST IMMUNITY AGAINST *E. histolytica*

Innate and Adaptive Immune Response

For any ingested parasite, immunity begins from the stomach acid as it has the ability to kill acid-sensitive microorganisms. However, the mucus barrier of the intestine serves as the first protective layer that prevents *Eh* from making contact with the underlying intestinal epithelial cells (IECs; Moonah et al., 2013). There are three major virulent factors of *Eh* known to mediate pathogenicity: (1) galactose/N-acetylgalactosamine-inhibitable lectin (Gal-lectin) is responsible for binding colonic mucin in colonization and host cell adhesion in disease pathogenesis, (2) pore forming peptide amoebapore used for host cell killing and, (3) cysteine proteases that lyse host extracellular matrix (Campos-Rodríguezp and Jarillo-Luna, 2005) and stimulate pro-inflammatory responses. The mucus barrier in the colon is composed of MUC2 mucin, which is a glycoprotein secreted from goblet cells. Mucin binds with high affinity to *Eh* Gal-lectin allowing the parasite to colonize the gut and at the same time it acts as a physical barrier to inhibit parasite invasion of the underlying epithelium (Chadee et al., 1987; Moonah et al., 2013). When *Eh* overcomes innate host defenses and contact IECs they produce a variety of pro-inflammatory mediators/chemokines including interleukin-1 (IL-1β), interleukin-8 (IL-8), and TNF-α. Several of these mediators trigger the recruitment of immune cells including neutrophils and macrophages to the site of parasite invasion (Yu and Chadee, 1997). The main amebicidal activity of neutrophils is the release of reactive oxygen species (ROS; Guerrant et al., 1981; Denis and Chadee, 1989). In macrophages *Eh* Gal-lectin up-regulates the mRNA expression of different cytokines. Gal-lectin induces pattern recognition receptor (PRRs) such as TLR-2 and TLR-4 mRNA expression in macrophages which is controlled by nuclear factor NF-κB and MAPK pathway. Recognition of parasite molecules by surface PRRs are crucial for the up-regulation of pro-inflammatory cytokine expression via NF-κB (Kammanadiminti et al., 2004). Macrophages activated with cytokines such as IFN-γ or TNF-α kills *Eh* trophozoites *in vitro* by producing nitric oxide (NO) from L-arginine mediated by inducible nitric oxide synthase (iNOS) (Lin et al., 1994). NO is critical for macrophage-mediated killing as iNOS deficient mice are more vulnerable to amebic liver abscess (ALA) and hepatocytic apoptosis (Seydel et al., 2000). Interferon gamma (IFN-γ) is the major cytokine that activates neutrophils and macrophages to exert amebicidal activity. Higher levels of IFN-γ are related to a lower incidence of *Eh* infection (Denis and Chadee, 1989; Ghadirian and Denis, 1992; Haque et al., 2007).

Humoral immune responses against *Eh* are well characterized and it was been found that 81–100% of invasive amebiasis patients develop circulatory antibodies within 7 days of infection (Kaur et al., 2004). A prospective cohort study of pre-school children in Dhaka, Bangladesh, showed that mucosal IgA antibodies against the carbohydrate recognition domain (CRD) of the Gal-lectin heavy chain provided protection against *Eh* infection and disease (Haque et al., 2001, 2006). In contrast, serum anti-lectin IgG was not associated with protection but mainly with the frequency of new infection (Haque et al., 2001). Higher levels of anti-lectin IgG was found in ALA and intestinal amebiasis as compared to asymptomatic patients (Kaur et al., 2004). These findings indicate that systematic anti-lectin antibodies are not involved in direct protection against amebiasis.

Inflammasome Activation by *E. histolytica*

Eh imposes damage through the adherence to host cells, which plays a critical role in killing or ingesting host target (Mortimer and Chadee, 2010). Thus, amebae adherence to host cell is one of the major characteristics of *Eh* pathogenicity. At present, the pattern recognition receptors (PRRs) that bind *Eh* Gal-lectin are not known. We recently identified that the inflammasome pathway is only activated on contact with live *Eh* and distinguishes between different physical forms of *Eh* (Mortimer et al., 2014). Inflammasome is a cytosolic multiprotein complex, which acts as a sensor for pathogens and cellular damage. This multimeric complex consists of an inflammasome sensor molecule (NOD-like receptor), the adaptor protein ASC and caspase-1. Activation of inflammasome leads to rapid and robust secretion of IL-1β, IL-18, IL-1α, FGF-2, IP-10 (Mortimer et al., 2014). Interestingly, when *Eh* activates the inflammasome, it does not trigger caspase-1 dependent cell death (known as pyroptosis) (Mortimer et al., 2014). It is unclear if inflammasome-activated macrophages are amebicidal and whether it plays other protective roles in amebic infection. On the other hand, if inflammasome activation triggers cell death in macrophages (pyroptotic cell death), *Eh* can use it as an advantage to limit immune elimination that can become detrimental to host defense.

IMMUNE EVASION MECHANISMS OF *E. histolytica*

Eh has a two-phase life cycle: it can survive as an infective cyst in the environment or it can be found as trophozoites, the feeding and tissue dwelling stage in the human colon. After excystation in the colon, *Eh* trophozoites usually establishes harmless colonization where the parasites reside in the gut lumen and feed on enteric bacteria by phagocytosis (Voigt et al., 1999;

Wilson et al., 2012). However, for unknown reasons trophozoites can become invasive, where parasite virulence factors allow it to degrade colonic mucin and other innate epithelial barrier functions (Wilson et al., 2012). Host immune responses, both innate and adaptive, are robust against invasive *Eh* but still this parasite is able to survive by developing immune evasion strategies. In particular, *Eh* cysteine proteases can cleave MUC2 mucin abrogating its protective functions allowing the parasite to breach the mucus layer and attach to the underlying epithelial cells (Lidell et al., 2006). Intestinal antimicrobial peptides are also an important component of host innate immune defense. Even though human LL-37 and murine CRAMP (cathelin-related antimicrobial peptide) cathelicidins are induced by *Eh* trophozoites both at the mRNA and protein level in IECs, *Eh* cysteine proteases can cleave these antimicrobial peptides (Cobo et al., 2012). Thus, *Eh* is resistant to both intact and cleaved antimicrobial cathelicidins in the intestine (Cobo et al., 2012).

After amebic invasion, neutrophils are the earliest infiltrating cells but virulent *Eh* are effective in killing, lysing, and phagocytosing neutrophils. *In vitro*, one trophozoite was shown to kill approximately 3000 neutrophils (Guerrant et al., 1981; Guo et al., 2007). There are several conflicting mechanisms by which ameba interfere with neutrophil functions. *Eh* can disrupt NADPH oxidase activities and inhibit the respiratory burst of neutrophils to avoid oxidative stress. *Eh* iron-containing superoxide dismutase and NADPH:flavin oxidoreductase (Elnekave et al., 2003) are able to detoxify ROS by forming H_2O_2 (Bruchhaus et al., 1998; Sim et al., 2005). *Eh* trophozoites can protect themselves from neutrophil reactive oxygen properties with a 29-kDa surface protein, peroxiredoxin that has potent antioxidant activity (Davis et al., 2006). Studies have shown (Sim et al., 2005) that *Eh* can induce host cells (neutrophil) apoptosis through the activation of ERK1/2 by the generation of NADPH oxidase-derived ROS.

Macrophages, another effector cell present during amebic infection also show suppressed cell mediated immunity due to *Eh*-induced strategic immune modulation. *Eh* trophozoites inhibit respiratory burst (ROS: H_2O_2, O^{2-}, OH^-) and NO production by macrophages (Lin et al., 1993; Wang et al., 1994). NOS substrate L-arginine is competitively converted to L-ornithine by ameba arginase that limit NO production by macrophages (Elnekave et al., 2003). When *Eh* and macrophages are exposed to each other, ameba produces the immunoregulatory molecule prostaglandin E2 (PGE_2), synthesized by a cyclooxygenase (COX)-like enzyme by the parasite (Dey et al., 2003). Coupling through EP2/4 receptors, PGE_2 increases cyclic adenosine monophosphate (cAMP) levels in macrophages that inhibits Th1 cytokine release, NADPH-mediated oxidative burst, and NO synthesis through the protein kinase C (PKC) pathway (Wang and Chadee, 1995). Another immunosuppressive pentapeptide, monocyte locomotion inhibitory factor (MLIF) produced by *Eh* showed anti-inflammatory activities by inhibiting NO production (Rico et al., 2003).

The complement system of the host is able to prevent trophozoite dissemination into the extra intestinal space. Activated complement forms the membrane attack complex (MAC) that can potentially lyse the parasite. *Eh* resists complement activation by the Gal-lectin which have sequence resemblance and antigenic cross reactivity with the MAC-inhibitory protein CD59 and thus inhibit MAC-mediated lysis (Braga et al., 1992). Cysteine proteases can also cleave complement components (Reed et al., 1995). The potent pro-inflammatory activities of the complement component C3a and C5a are degraded by *Eh* secreted extracellular cysteine proteases (Zambrano-Villa et al., 2002). Secretory IgA and serum IgG mediate adaptive immunity against *Eh* and ameba can degrade these immunoglobulins *in vitro*. *Eh* extracellular cysteine proteases play a key role in the disruption of host adaptive defenses. For successful invasion, *Eh* secreted and membrane-bound cysteine proteases cleave extracellular matrix proteins, fibronectin, and laminin and avoid host defenses by cleaving gut lumen sIgA and circulatory IgG (Que and Reed, 1997; Zambrano-Villa et al., 2002).

E. histolytica-induced Cell Death: Immune Evasion Strategy

Eh uses different strategies to evade host immune defense but one striking mechanism is the induction of host cell death. The term "histolytica" refers to *Eh* ability to destroy host tissues by potent cytotoxicity/cell killing activity toward different host cells including neutrophils, macrophages, T-lymphocytes; though the exact mechanism of host cell killing is not clear. *Eh* has several cytotoxic effector molecules (**Table 1**) but how ameba deliver this deadly action is not clear. *Eh* can induce host cell apoptosis, phagocytosis and amebic trogocytosis; the latter involving a recently described mechanism of ameba-induced host cell killing (Ralston et al., 2014).

Apoptotic Cell Death

Host cell killing is usually a stepwise process mediated by parasite adherence to the target cell, elevation of intracellular calcium level, dephosphorylation of host proteins which all contribute to cell death via activation of caspase-3 (Ralston and Petri, 2011b). Caspase-3 activation is the signature event of apoptosis. The first step of adherence is mediated by the parasite surface Gal-lectin to host cell carbohydrate determinants containing Gal and/or GalNAc residues (**Figure 1A**). Gal-lectin mediated adherence to target cell is a prerequisite for parasite cytotoxicity as the addition of excess Gal or GalNAc monomers inhibit *Eh* adherence and target cell killing (Ravdin and Guerrant, 1981; Saffer and Petri, 1991). Adherence to the target cell induces calcium flux which also contribute to the cell killing proved by the inhibition of calcium channel or by using calcium chelators (Ravdin et al., 1982; Ralston and Petri, 2011b). Amebic cytotoxicity both *in vitro* and *in vivo* occurs via the caspase-3 dependent apoptotic cell death pathway in Jurkat cells after contact with *Eh* (**Figures 1A,C**) (Seydel and Stanley, 1998; Ralston and Petri, 2011b). Caspase-3 knockout mice are resistant to amebiasis and a pharmacological inhibitor of caspase-3, Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-aldehyde) reduces parasite cytotoxicity to host cells (Huston et al., 2000; Becker et al., 2010). Studies illustrate that *Eh*-induced apoptosis is independent of caspase-8 or caspase-9 (Huston et al., 2000). Another study showed that

TABLE 1 | Potential cytotoxic effector molecules identified in *Entamoeba histolytica.*

Eh effectors molecules	Identified effect on host cell	References
Amebapores	Pore forming proteins	Leippe et al., 1991
	All three types induce pore formation in synthetic liposomes	Andrä et al., 2003
Amebapore A	Active at low pH 5.2. May play a role in host cell killing before ingestion	Andrä et al., 2003
Amebapore B Amebapore C	*In vitro*, purified amebapores showed bactericidal activity against Gram-positive bacteria at nanomolar concentration Purified amebapores are cytotoxic to Jurkat or U937 cells at micromolar concentration	Leippe et al., 1994; Andrä et al., 2003
Cysteine proteinases (CPs)	At least 50 CP genes are encoded and some of them are secretory	Tillack et al., 2007
	Proteinases act on a variety of host substrates such as mucin, villin, laminin, collagen, proteoglycan, and extracellular matrix (ECM). It plays a role in pathogenesis by cleaving MUC mucin and ECM degradation	Li et al., 1995; Lidell et al., 2006
	In vivo, overexpression of *Eh*CP5 (*Eh*CP5) increases liver abscess formation compared to wild-type controls. Other CPs like *Eh*CP1 or *Eh*CP2 overexpression had no effect	Hellberg et al., 2001; Tillack et al., 2006
Membrane proteins: Gal/GalNAc lectin associated	35kDa light subunit of the Gal/GalNAc lectin and surface-localized thiol-dependent peroxidase	Ankri et al., 1999; Sen et al., 2007
	Antisense inhibition of both of these prevents cell killing and deceased liver abscess	Ankri et al., 1999; Sen et al., 2007
*Eh*STIRPs (*Eh* serine, threonine and isoleucine rice proteins)	Silenced by dsRNA resulted in defects in both adhesion and cytotoxicity	MacFarlane and Singh, 2007
*Eh*TMKB1-9 (*Eh* transmembrane kinase B1-9)	Antisense inhibition of this protein showed defects in both adhesion and host cell killing	Shrimal et al., 2010
KERP1 (Lysine and glutamic acid rice protein 1)	Parasite membrane protein binds to host cell membrane	Santi-Rocca et al., 2008
	Antisense inhibition of this protein failed to decrease mRNA but due to affinity for host cell membranes, it might have role in cytotoxicity	

Eh killing of hepatocyte and immune cell was not dependent or mediated by the classical Fas/Fas ligand or TNFα receptor pathway (Seydel and Stanley, 1998). Mice lacking Fas or producing a mutated non-functional Fas protein developed amebic ALA similar to wild type mice. Similarly, TNF receptor knockout mice and wild type mice develop comparable ALA (Seydel and Stanley, 1998). *Eh* induces a non-classical pathway of apoptosis that may have an important role in pathogenesis (Seydel and Stanley, 1998; Huston et al., 2000). Apoptotic cell death is immunologically silent, thus induction of host cell apoptosis by *Eh* tricks the host cell to kill itself without evoking an inflammatory response to avoid being detected by other immune cells. The importance of apoptosis in regards to amebic virulence is brought to light by studies of the leptin-signaling pathway. The hormone leptin is linked to malnutrition (signals satiety) and regulates the immune response to infection through the Th1 inflammatory response and by preventing apoptosis (Wilson et al., 2012). Leptin signaling provides protection from mucosal destruction and experiments in mice showed the anti-apoptotic role of leptin in gut epithelia (Guo et al., 2011). Polymorphism (even a single amino acid substitution) in the leptin receptor was found to be associated with increased *Eh* infection susceptibility (Duggal et al., 2011).

Phagocytosis by *E. histolytica*

The fate of *Eh* induced apoptotic host cells (Huston et al., 2003) and subsequent phagocytosis may play an important role in the host-parasite relationship in amebiasis. Amebae induced phagocytosis of erythrocyte is one of the possible distinctive feature of *Eh* from the commensal *E. dispar* (González-Ruiz et al., 1994). In multicellular organisms, phagocytosis is the last step of the apoptotic pathway to clear dead cells without provoking an inflammatory response by the toxic components of dead cells (**Figure 1D**; Savill and Fadok, 2000; Fadok et al., 2001). Similarly, apoptotic host cells are phagocytosed by *Eh* with the help of exposed phosphatidylserine (Huston et al., 2003). As *Eh* rapidly clears apoptotically killed host cells by phagocytosis, this limits the spillage of toxic intracellular contents from killed cells. Through this strategy *Eh* restrains host inflammatory responses and build up prolonged infection.

Eh-induced phagocytosis is important for pathogenicity but very little is known about the amebic receptors and the corresponding ligands that they bind to. Adherence with host cells by parasite Gal-lectin is critical for cell death but blocking of adherence does not prevent phagocytosis (Teixeira et al., 2008). This suggests the sequential exposure of new ligands on dying cells as well as the recruitment of new receptors on *Eh* in addition to the Gal-lectin following host cell killing (Teixeira et al., 2008). From an evolutionary concept, *Eh* phagocytoses bacteria for nutrient acquisition; therefore amebae preferentially recognize and phagocytose apoptotic cells that have surface similarities with bacteria (Teixeira et al., 2008).

Ameba Trogocytosis

Recently, a new mechanism of *Eh* killing came to light. Using live cell imaging technology it was discovered that following host

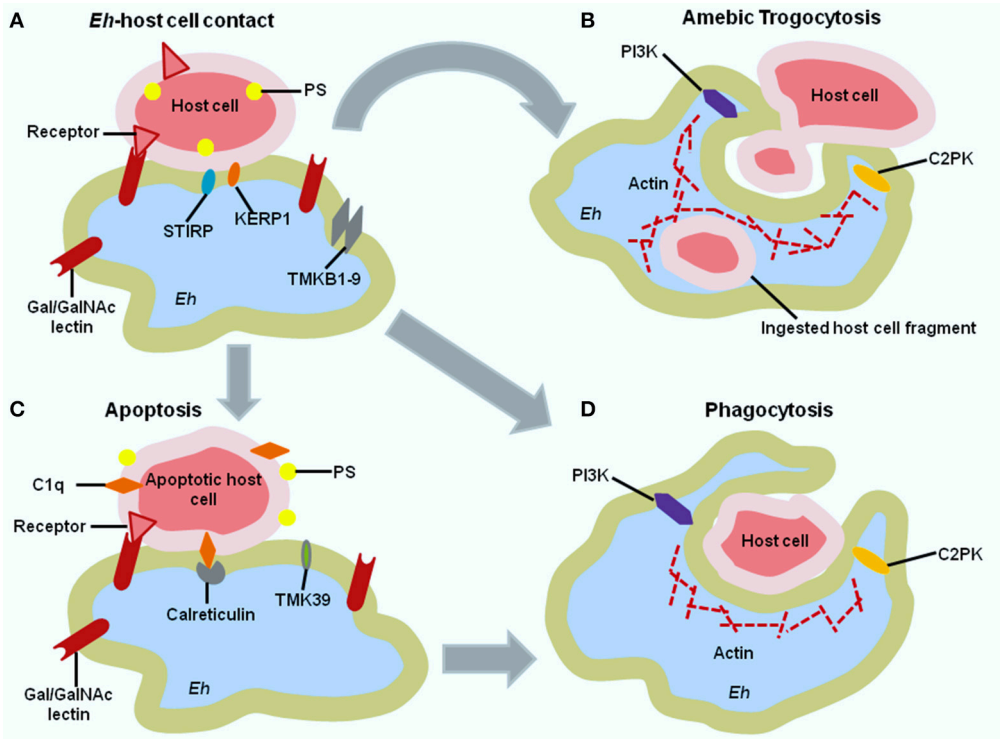

FIGURE 1 | Immune evasion of *E. histolytica* (*Eh*) by host cell killing. (A) Contact between *Eh* and host cell is mediated by the Gal-lectin and host cell surface Gal and GalNAc receptors. Other amebic proteins involved in host cell attachment include the trans-membrane serine, threonine, and isoleucine proteins (STIRP) and transmembrane kinase family member TMKb1-9. After *Eh*-host contact, three events can take place-**(B)** amebic trogocytosis **(C)** apoptotic cell death **(D)** phagocytosis. In **(B)** larger cells undergo amebic trogocytosis. Amebic trogocytosis require PI3K and C2PK signal transduction for actin polymerization. **(C)** Host cells that have been induced to undergo apoptosis express phosphatidylserine (PS) and C1q complement protein that induce opsonization by ameba involving amebic calreticulin receptor. Finally, in **(D)** both apoptotically death cell and smaller cell undergo phagocytosis. Signal transduction for phagocytosis is also mediated by PI3K and EhC2PK and influences actin polymerization. PI3K-phosphoinositide 3 kinase, C2PK-C2 domain containing protein kinase, TMK39-Transmembrane kinase family member.

cell attachment, *Eh* trophozoites ingest separate parts (bites) of host cells which was termed "amebic trogocytosis" (**Figure 1B**; Ralston et al., 2014). This was a very rapid process as within 1 min of attachment amebic trogocytosis is initiated. Due to biting off and ingestion of separate pieces of host cells, intracellular calcium levels were elevated and this triggered ultimate cell death, evidenced by the loss of cell membrane integrity (Ralston et al., 2014). Interestingly, when trophozoites were incubated with either live or pre-killed host cells, only live cells were seen to be trogocytosed by ameba and pre-killed cells were ingested whole (phagocytosed; Ralston et al., 2014; Ralston, 2015). The cell surface characteristics of pre-killed host cells might be different from the directly killed cells and *Eh* might use this surface difference to determine the type of ingestion (Ralston, 2015). After ingestion of bites, amebae detach from the host cell, and the ingested cell eventually dies. It was speculated that this process contributes to amebic invasion in the colon as *Eh* can also trogocytose mucosal epithelial cells. Though amebic trogocytosis is a rapid process, this depends on specific conditions like physiological temperature, amoebic actin rearrangements, Gal-lectin, EhC2PK (*Eh* C2-domain-containing protein kinase), and PI3K (phosphoinositide 3-kinase) signaling (Ralston et al., 2014). Interference with any of these protein was shown to reduce *Eh* trogocytosis and subsequent decrease in host cell death (Ralston, 2015). It should be noted that trogocytosis also occurs in multicellular organism and different immune cell types but this trogocytosis does not trigger cell death. The exact reason for this distinction is not clear but trogocytosis in multicellular organisms mainly involves the exchange of cell membrane fragments; whereas *Eh* trogocytosis contains target cell cytoplasm and sometimes organelles (Joly and Hudrisier, 2003; Ralston et al., 2014).

The concept of trogocytosis raises an important question, whether amebic trogocytosis is different from phagocytosis. The signaling proteins involved in amebic trogocytosis also play important roles during *Eh* phagocytosis but amebic trogocytosis is predominant in living cells (Ralston, 2015). Some factors might take part in the distinction of trogocytosis and phagocytosis like target cell deformability, target cell viability, target cell size etc. (Ralston, 2015). However, the specific signaling pathways that define amebic trogocytosis and phagocytosis in *Eh* are not identified; the relationship or differences between these two processes are not well understood.

CONCLUSION

Eh is an enteric dwelling protozoan parasite that causes significant morbidity and mortality in developing countries. This parasite can develop a harmless colonization in the colon and for unknown reasons it can become a pathogenic phenotype. With the pathogenic phenotype, *Eh* disrupts innate mucosal barriers and penetrates the underlying lamina propria where the parasite develops potent cytotoxic activity and extensive tissue destruction. It is still not clear how or what factors induce this pathogenic phenotype. Human and parasite genetics along with environmental factors might have a role as it has been found that not all children are equally susceptible to infection. Malnutrition extensively increases disease susceptibility. Gut microbiome also influence *Eh* infection susceptibility. Both host innate and adaptive immune response take part in the elimination of invasive *Eh*. The host immune system builds up a rapid inflammatory response by the secretion of cytokines/chemokines, recruitment of immune cells (neutrophils, macrophages), and the activation of inflammasome to control invasive parasites. This parasite also develops multiple strategies to subvert host immune responses and to promote its own survival. *Eh* induces host cell killing primarily by apoptosis, which is a non-inflammatory cell death mechanism. Induction of apoptotic cell death is an active and stepwise process. After inducing cell death, the parasite also clears the corpse by phagocytosis to inhibit further inflammatory responses. Another mechanism is amebic trogocytosis where *Eh* bites live cells very rapidly and induces cell death. The proper characterization of proteins, receptor/ligand interaction involved in parasite adherence, cell killing, phagocytosis, and amebic trogocytosis will provide promise of future vaccine candidates.

AUTHOR CONTRIBUTIONS

SB, JQ, and KC wrote and edit the manuscript.

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REFERENCES

Andrä, J., Herbst, R., and Leippe, M. (2003). Amoebapores, archaic effector peptides of protozoan origin, are discharged into phagosomes and kill bacteria by permeabilizing their membranes. *Dev. Comp. Immunol.* 27, 291–304. doi: 10.1016/S0145-305X(02)00106-4

Ankri, S., Padilla-Vaca, F., Stolarsky, T., Koole, L., Katz, U., and Mirelman, D. (1999). Antisense inhibition of expression of the light subunit (35 kDa) of the Gal/GalNac lectin complex inhibits *Entamoeba histolytica* virulence. *Mol. Microbiol.* 33, 327–337. doi: 10.1046/j.1365-2958.1999.01476.x

Becker, S. M., Cho, K.-N., Guo, X., Fendig, K., Oosman, M. N., Whitehead, R., et al. (2010). Epithelial cell apoptosis facilitates *Entamoeba histolytica* infection in the gut. *Am. J. Pathol.* 176, 1316–1322. doi: 10.2353/ajpath.2010.090740

Braga, L. L., Ninomiya, H., McCoy, J. J., Eacker, S., Wiedmer, T., Pham, C., et al. (1992). Inhibition of the complement membrane attack complex by the galactose-specific adhesion of *Entamoeba histolytica*. *J. Clin. Invest.* 90, 1131. doi: 10.1172/JCI115931

Bruchhaus, I., Richter, S., and Tannich, E. (1998). Recombinant expression and biochemical characterization of an NADPH: flavin oxidoreductase from *Entamoeba histolytica*. *Biochem. J.* 330, 1217–1221. doi: 10.1042/bj3301217

Campos-Rodríguezp, R., and Jarillo-Luna, A. (2005). The pathogenicity of *Entamoeba histolytica* is related to the capacity of evading innate immunity. *Parasite Immunol.* 27, 1–8. doi: 10.1111/j.1365-3024.2005.00743.x

Chadee, K., Petri, W. A. Jr., Innes, D. J., and Ravdin, J. I. (1987). Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. *J. Clin. Invest.* 80, 1245. doi: 10.1172/JCI113199

Cobo, E. R., He, C., Hirata, K., Hwang, G., Tran, U., Eckmann, L., et al. (2012). *Entamoeba histolytica* induces intestinal cathelicidins but is resistant to cathelicidin-mediated killing. *Infect. Immun.* 80, 143–149. doi: 10.1128/IAI.05029-11

Davis, P. H., Zhang, X., Guo, J., Townsend, R. R., and Stanley, S. L. (2006). Comparative proteomic analysis of two *Entamoeba histolytica* strains with different virulence phenotypes identifies peroxiredoxin as an important component of amoebic virulence. *Mol. Microbiol.* 61, 1523–1532. doi: 10.1111/j.1365-2958.2006.05344.x

Denis, M., and Chadee, K. (1989). Human neutrophils activated by interferon-gamma and tumour necrosis factor-alpha kill *Entamoeba histolytica* trophozoites *in vitro*. *J. Leukocyte Biol.* 46, 270–274.

Dey, I., Keller, K., Belley, A., and Chadee, K. (2003). Identification and characterization of a cyclooxygenase-like enzyme from *Entamoeba histolytica*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13561–13566. doi: 10.1073/pnas.1835863100

Duggal, P., Guo, X., Haque, R., Peterson, K. M., Ricklefs, S., Mondal, D., et al. (2011). A mutation in the leptin receptor is associated with *Entamoeba histolytica* infection in children. *J. Clin. Invest.* 121, 1191. doi: 10.1172/JCI45294

Elnekave, K., Siman-Tov, R., and Ankri, S. (2003). Consumption of L-arginine mediated by *Entamoeba histolytica* L-arginase (EhArg) inhibits amoebicidal activity and nitric oxide production by activated macrophages. *Parasite Immunol.* 25, 597–608. doi: 10.1111/j.0141-9838.2004.00669.x

Fadok, V. A., Bratton, D. L., and Henson, P. M. (2001). Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J. Clin. Invest.* 108, 957. doi: 10.1172/JCI200114122

Ghadirian, E., and Denis, M. (1992). *Entamoeba histolytica* extract and interferon-gamma activation of macrophage-mediated amoebicidal function. *Immunobiology* 185, 1–10. doi: 10.1016/S0171-2985(11)80312-8

González-Ruiz, A., Haque, R., Aguirre, A., Castañón, G., Hall, A., Guhl, F., et al. (1994). Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *J. Clin. Pathol.* 47, 236–239. doi: 10.1136/jcp.47.3.236

Guerrant, R. L., Brush, J., Ravdin, J. I., Sullivan, J. A., and Mandell, G. L. (1981). Interaction between *Entamoeba histolytica* and human polymorphonuclear neutrophils. *J. Infect. Dis.* 143, 83–93. doi: 10.1093/infdis/143.1.83

Guo, X., Houpt, E., and Petri, W. A. (2007). Crosstalk at the initial encounter: interplay between host defense and ameba survival strategies. *Curr. Opin. Immunol.* 19, 376–384. doi: 10.1016/j.coi.2007.07.005

Guo, X., Roberts, M. R., Becker, S. M., Podd, B., Zhang, Y., Chua, S. C., et al. (2011). Leptin signaling in intestinal epithelium mediates resistance to enteric infection by *Entamoeba histolytica*. *Mucosal Immunol.* 4, 294–303. doi: 10.1038/mi.2010.76

Haque, R., Ali, I. M., Sack, R. B., Farr, B. M., Ramakrishnan, G., and Petri, W. A. (2001). Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. *J. Infect. Dis.* 183, 1787–1793. doi: 10.1086/320740

Haque, R., Mondal, D., Duggal, P., Kabir, M., Roy, S., Farr, B. M., et al. (2006). *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. *Infect. Immun.* 74, 904–909. doi: 10.1128/IAI.74.2.904-909.2006

Haque, R., Mondal, D., Shu, J., Roy, S., Kabir, M., Davis, A. N., et al. (2007). Correlation of interferon-γ production by peripheral blood mononuclear cells

with childhood malnutrition and susceptibility to amebiasis. *Am. J. Trop. Med. Hyg.* 76, 340–344.

Hellberg, A., Nickel, R., Lotter, H., Tannich, E., and Bruchhaus, I. (2001). Overexpression of cysteine proteinase 2 in *Entamoeba histolytica* or *Entamoeba dispar* increases amoeba-induced monolayer destruction *in vitro* but does not augment amoebic liver abscess formation in gerbils. *Cell. Microbiol.* 3, 13–20. doi: 10.1046/j.1462-5822.2001.00086.x

Huston, C. D., Boettner, D. R., Miller-Sims, V., and Petri, W. A. Jr. (2003). Apoptotic killing and phagocytosis of host cells by the parasite *Entamoeba histolytica*. *Infect. Immun.* 71, 964–972. doi: 10.1128/IAI.71.2.964-972.2003

Huston, C. D., Houpt, E. R., Mann, B. J., Hahn, C. S., and Petri, W. A. (2000). Caspase 3-dependent killing of host cells by the parasite *Entamoeba histolytica*. *Cell. Microbiol.* 2, 617–625. doi: 10.1046/j.1462-5822.2000.00085.x

Joly, E., and Hudrisier, D. (2003). What is trogocytosis and what is its purpose? *Nat. Immunol.* 4:815. doi: 10.1038/ni0903-815

Kammanadiminti, S. J., Mann, B. J., Dutil, L., and Chadee, K. (2004). Regulation of Toll-like receptor-2 expression by the Gal-lectin of *Entamoeba histolytica*. *FASEB J.* 18, 155–157. doi: 10.1096/fj.03-0578fje

Kaur, U., Sharma, A., Sharma, M., and Vohra, H. (2004). Distribution of *Entamoeba histolytica* Gal/GalNAc lectin—specific antibody response in an endemic area. *Scand. J. Immunol.* 60, 524–528. doi: 10.1111/j.0300-9475.2004.01512.x

Korpe, P. S., Liu, Y., Siddique, A., Kabir, M., Ralston, K., Ma, J. Z., et al. (2013). Breast milk parasite-specific antibodies and protection from amebiasis and cryptosporidiosis in Bangladeshi infants: a prospective cohort study. *Clin. Infect. Dis.* 56, 988–992. doi: 10.1093/cid/cis1044

Leippe, M., Andra, J., and Müller-Eberhard, H. J. (1994). Cytolytic and antibacterial activity of synthetic peptides derived from amoebapore, the pore-forming peptide of *Entamoeba histolytica*. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2602–2606. doi: 10.1073/pnas.91.7.2602

Leippe, M., Ebel, S., Schoenberger, O. L., Horstmann, R. D., and Müller-Eberhard, H. J. (1991). Pore-forming peptide of pathogenic *Entamoeba histolytica*. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7659–7663. doi: 10.1073/pnas.88.17.7659

Li, E., Yang, W. G., Zhang, T., and Stanley, S. L. (1995). Interaction of laminin with *Entamoeba histolytica* cysteine proteinases and its effect on amebic pathogenesis. *Infect. Immun.* 63, 4150–4153. doi: 10.1016/0169-4758(95)80056-5

Lidell, M. E., Moncada, D. M., Chadee, K., and Hansson, G. C. (2006). *Entamoeba histolytica* cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9298–9303. doi: 10.1073/pnas.0600623103

Lin, J. Y., Keller, K., and Chadee, K. (1993). *Entamoeba histolytica* proteins modulate the respiratory burst potential by murine macrophages. *Immunology* 78, 291.

Lin, J.-Y., Seguin, R., Keller, K., and Chadee, K. (1994). Tumor necrosis factor alpha augments nitric oxide-dependent macrophage cytotoxicity against *Entamoeba histolytica* by enhanced expression of the nitric oxide synthase gene. *Infect. Immun.* 62, 1534–1541.

MacFarlane, R. C., and Singh, U. (2007). Identification of an *Entamoeba histolytica* serine-, threonine-, and isoleucine-rich protein with roles in adhesion and cytotoxicity. *Eukaryotic Cell* 6, 2139–2146. doi: 10.1128/EC.00174-07

Mondal, D., Petri, W. A., Sack, R. B., Kirkpatrick, B. D., and Haque, R. (2006). *Entamoeba histolytica*-associated diarrheal illness is negatively associated with the growth of preschool children: evidence from a prospective study. *Trans. R. Soc. Trop. Med. Hyg.* 100, 1032–1038. doi: 10.1016/j.trstmh.2005.12.012

Moonah, S. N., Jiang, N. M., and Petri, W. A. Jr. (2013). Host immune response to intestinal amebiasis. *PLoS Pathog.* 9:e1003489. doi: 10.1371/journal.ppat.1003489

Mortimer, L., and Chadee, K. (2010). The immunopathogenesis of *Entamoeba histolytica*. *Exp. Parasitol.* 126, 366–380. doi: 10.1016/j.exppara.2010.03.005

Mortimer, L., Moreau, F., Cornick, S., and Chadee, K. (2014). Gal-lectin-dependent contact activates the inflammasome by invasive *Entamoeba histolytica*. *Mucosal Immunol.* 7, 829–841. doi: 10.1038/mi.2013.100

Mortimer, L., Moreau, F., Cornick, S., and Chadee, K. (2015). The NLRP3 inflammasome is a pathogen sensor for invasive *Entamoeba histolytica* via activation of α5β1 integrin at the macrophage-amebae intercellular junction. *PLoS Pathog.* 11:e1004887. doi: 10.1371/journal.ppat.1004887

Petri, W. A. (2003). Therapy of intestinal protozoa. *Trends Parasitol.* 19, 523–526. doi: 10.1016/j.pt.2003.09.003

Que, X., and Reed, S. L. (1997). The role of extracellular cysteine proteinases in pathogenesis of *Entamoeba histolytica* invasion. *Parasitol. Today* 13, 190–194. doi: 10.1016/S0169-4758(97)01043-0

Ralston, K. S. (2015). Chew on this: amoebic trogocytosis and host cell killing by *Entamoeba histolytica*. *Trends Parasitol.* 31, 442–452. doi: 10.1016/j.pt.2015.05.003

Ralston, K. S., and Petri, W. A. (2011a). Tissue destruction and invasion by *Entamoeba histolytica*. *Trends Parasitol.* 27, 254–263. doi: 10.1016/j.pt.2011.02.006

Ralston, K. S., and Petri, W. A. (2011b). The ways of a killer: how does *Entamoeba histolytica* elicit host cell death? *Essays Biochem.* 51, 193–210. doi: 10.1042/bse0510193

Ralston, K. S., Solga, M. D., Mackey-Lawrence, N. M., Bhattacharya, A., and Petri, W. A. Jr. (2014). Trogocytosis by *Entamoeba histolytica* contributes to cell killing and tissue invasion. *Nature* 508, 526–530. doi: 10.1038/nature13242

Ravdin, J. I., and Guerrant, R. L. (1981). Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*: study with mammalian tissue culture cells and human erythrocytes. *J. Clin. Invest.* 68, 1305. doi: 10.1172/JCI110377

Ravdin, J. I., Sperelakis, N., and Guerrant, R. L. (1982). Effect of ion channel inhibitors on the cytopathogenicity of *Entamoeba histolytica*. *J. Infect. Dis.* 146, 335–340. doi: 10.1093/infdis/146.3.335

Reed, S. L., Ember, J. A., Herdman, D. S., DiScipio, R. G., Hugli, T. E., and Gigli, I. (1995). The extracellular neutral cysteine proteinase of *Entamoeba histolytica* degrades anaphylatoxins C3a and C5a. *J. Immunol.* 155, 266–274.

Rico, G., Leandro, E., Rojas, S., Gimenéz, J. A., and Kretschmer, R. R. (2003). The monocyte locomotion inhibitory factor produced by *Entamoeba histolytica* inhibits induced nitric oxide production in human leukocytes. *Parasitol. Res.* 90, 264–267. doi: 10.1007/s00436-002-0780-7

Saffer, L. D., and Petri, W. A. (1991). Role of the galactose lectin of *Entamoeba histolytica* in adherence-dependent killing of mammalian cells. *Infect. Immun.* 59, 4681–4683.

Santi-Rocca, J., Weber, C., Guigon, G., Sismeiro, O., Coppee, J. Y., and Guillen, N. (2008). The lysine and glutamic acid-rich protein KERP1 plays a role in *Entamoeba histolytica* liver abscess pathogenesis. *Cell. Microbiol.* 10, 202–217. doi: 10.1111/j.1462-5822.2007.01030.x

Savill, J., and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature* 407, 784–788. doi: 10.1038/35037722

Sen, A., Chatterjee, N. S., Akbar, M. A., Nandi, N., and Das, P. (2007). The 29-kilodalton thiol-dependent peroxidase of *Entamoeba histolytica* is a factor involved in pathogenesis and survival of the parasite during oxidative stress. *Eukaryotic Cell* 6, 664–673. doi: 10.1128/EC.00308-06

Seydel, K. B., Smith, S. J., and Stanley, S. L. (2000). Innate immunity to amebic liver abscess is dependent on gamma interferon and nitric oxide in a murine model of disease. *Infect. Immun.* 68, 400–402. doi: 10.1128/IAI.68.1.400-402.2000

Seydel, K. B., and Stanley, S. L. (1998). *Entamoeba histolytica* induces host cell death in amebic liver abscess by a non-Fas-dependent, non-tumor necrosis factor alpha-dependent pathway of apoptosis. *Infect. Immun.* 66, 2980–2983.

Shrimal, S., Bhattacharya, S., and Bhattacharya, A. (2010). Serum-dependent selective expression of EhTMKB1-9, a member of *Entamoeba histolytica* B1 family of transmembrane kinases. *PLoS Pathog.* 6:e1000929. doi: 10.1371/journal.ppat.1000929

Sim, S., Yong, T.-S., Park, S.-J., Im, K.-I., Kong, Y., Ryu, J.-S., et al. (2005). NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by *Entamoeba histolytica*. *J. Immunol.* 174, 4279–4288. doi: 10.4049/jimmunol.174.7.4279

Stanley, S. L. (2003). Amoebiasis. *Lancet* 361, 1025–1034. doi: 10.1016/S0140-6736(03)12830-9

Teixeira, J. E., Heron, B. T., and Huston, C. D. (2008). C1q-and collectin-dependent phagocytosis of apoptotic host cells by the intestinal protozoan *Entamoeba histolytica*. *J. Infect. Dis.* 198, 1062–1070. doi: 10.1086/591628

Tillack, M., Biller, L., Irmer, H., Freitas, M., Gomes, M. A., Tannich, E., et al. (2007). The *Entamoeba histolytica* genome: primary structure and expression of proteolytic enzymes. *BMC Genomics* 8:170. doi: 10.1186/1471-2164-8-170

Tillack, M., Nowak, N., Lotter, H., Bracha, R., Mirelman, D., Tannich, E., et al. (2006). Increased expression of the major cysteine proteinases by stable episomal transfection underlines the important role of EhCP5 for the

pathogenicity of *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* 149, 58–64. doi: 10.1016/j.molbiopara.2006.04.009

Voigt, H., Olivo, J.-C., Sansonetti, P., and Guillén, N. (1999). Myosin IB from *Entamoeba histolytica* is involved in phagocytosis of human erythrocytes. *J. Cell Sci.* 112, 1191–1201.

Wang, W., and Chadee, K. (1995). *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatibility complex Ia molecule and IA beta mRNA expression by a prostaglandin E2-dependent mechanism. *Infect. Immun.* 63, 1089–1094.

Wang, W., Keller, K., and Chadee, K. (1994). *Entamoeba histolytica* modulates the nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxicity against amoebae and tumour cells. *Immunology* 83, 601.

Wilson, I. W., Weedall, G. D., and Hall, N. (2012). Host-parasite interactions in *Entamoeba histolytica* and *Entamoeba dispar*: what have we learned from their genomes? *Parasite Immunol.* 34, 90–99. doi: 10.1111/j.1365-3024.2011.01325.x

Yu, Y., and Chadee, K. (1997). *Entamoeba histolytica* stimulates interleukin 8 from human colonic epithelial cells without parasite-enterocyte contact. *Gastroenterology* 112, 1536–1547. doi: 10.1016/S0016-5085(97)70035-0

Zambrano-Villa, S., Rosales-Borjas, D., Carrero, J. C., and Ortiz-Ortiz, L. (2002). How protozoan parasites evade the immune response. *Trends Parasitol.* 18, 272–278. doi: 10.1016/S1471-4922(02)02289-4

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REVIEW
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Evasion of the Immune Response by *Trypanosoma cruzi* during Acute Infection

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Trypanosoma cruzi is the etiologic agent of Chagas disease, a neglected tropical disease that affects millions of people mainly in Latin America. To establish a life-long infection, *T. cruzi* must subvert the vertebrate host's immune system, using strategies that can be traced to the parasite's life cycle. Once inside the vertebrate host, metacyclic trypomastigotes rapidly invade a wide variety of nucleated host cells in a membrane-bound compartment known as the parasitophorous vacuole, which fuses to lysosomes, originating the phagolysosome. In this compartment, the parasite relies on a complex network of antioxidant enzymes to shield itself from lysosomal oxygen and nitrogen reactive species. Lysosomal acidification of the parasitophorous vacuole is an important factor that allows trypomastigote escape from the extremely oxidative environment of the phagolysosome to the cytoplasm, where it differentiates into amastigote forms. In the cytosol of infected macrophages, oxidative stress instead of being detrimental to the parasite, favors amastigote burden, which then differentiates into bloodstream trypomastigotes. Trypomastigotes released in the bloodstream upon the rupture of the host cell membrane express surface molecules, such as calreticulin and GP160 proteins, which disrupt initial and key components of the complement pathway, while others such as glycosylphosphatidylinositol-mucins stimulate immunoregulatory receptors, delaying the progression of a protective immune response. After an immunologically silent entry at the early phase of infection, *T. cruzi* elicits polyclonal B cell activation, hypergammaglobulinemia, and unspecific anti-*T. cruzi* antibodies, which are inefficient in controlling the infection. Additionally, the coexpression of several related, but not identical, epitopes derived from trypomastigote surface proteins delays the generation of *T. cruzi*-specific neutralizing antibodies. Later in the infection, the establishment of an anti-*T. cruzi* $CD8^+$ immune response focused on the parasite's immunodominant epitopes controls parasitemia and tissue infection, but fails to completely eliminate the parasite. This outcome is not detrimental to the parasite, as it reduces host mortality and maintains the parasite infectivity toward the insect vectors.

Keywords: *T. cruzi* acute infection, immune response, *T. cruzi* immune evasion, Chagas disease, immunomodulation

INTRODUCTION

Chagas disease, also known as American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*, a highly diverse taxon. This disease is endemic to Latin America, with sporadic cases mainly in the United States and Europe, and affects nearly 8 million people, accounting for the loss of 662,000 disability-adjusted life years (1–3) (WHO).[1] This parasite alternates between invertebrate hematophagous insects from the Reduviidae family and a broad range of mammalian hosts (4). Although the estimated period for *T. cruzi* speciation is still a matter of debate (5–9), recent molecular studies suggest that the ancestor of *T. cruzi* may have been introduced to South America approximately 7–10 million years ago (8, 9), and the oldest record of human infection dates from 9,000 years ago (10). Since then, this parasite has evolved fascinating strategies to evade and subvert the mammalian host immune system, leading to life-long last infections. These strategies can be traced to the parasite's life cycle.

Trypanosoma cruzi metacyclic trypomastigotes are released in the feces or urine of the triatomine vector after a blood meal. These forms are able to infect the mammalian host if they encounter mucosa or discontinuous regions in the epithelium. Once inside the host, the parasite rapidly infects a wide variety of nucleated mammalian cells (11–13). *T. cruzi* relies on an arsenal of polymorphic glycosylphosphatidylinositol (GPI)-anchored surface proteins, such as *trans*-sialidases, mucins, and others, to attach and invade host cells, leading to the formation of the parasitophorous vacuole (14, 15). After lysosomes are fused to the parasitophorous vacuole, parasite survival is mediated by a complex network of antioxidant enzymes, such as peroxidases and superoxide dismutases (SODs), that shield it from reactive oxygen and nitrogen species (16). In fact, instead of being detrimental, the lysosomal acidification is an important signal for activating key mechanisms that allow the parasite to escape from the phagosome into the cytoplasm, where it differentiates into the replicative amastigote forms. After several rounds of duplication, the amastigotes differentiate into infective bloodstream trypomastigotes, which are released upon the rupture of the host cell membrane and infect neighboring cells or enter the bloodstream. Once the trypomastigotes reach the bloodstream, the parasite circumvents complement-mediated lysis and opsonization with the aid of surface proteins, such as calreticulin and GP160 (17, 18). These proteins disrupt the initial attachment of mediators from the classical, alternative, and lectin complement pathways and dismantle the C3 convertase, a key step in all three pathways (19, 20). Thus, the parasite is allowed to disseminate through the bloodstream to many tissues during the acute phase. *T. cruzi* uses several other strategies to delay the generation of an effective immune response. During the initial phase of infection, the parasite elicits polyclonal B cell activation and hypergammaglobulinemia based on parasite-derived B cell mitogens. The antibodies produced by these cells are not parasite specific and are inefficient in controlling infection (21, 22). With the stimulation of innate immune receptors, such as the intracellular toll-like receptors (TLRs) 7 and 9, followed by proinflammatory cytokine production, a Th1-focused immune response is established (23–25). This response leads to the production of *T. cruzi*-specific $CD8^+$ cells directed to parasite immunodominant epitopes, derived from the *trans*-sialidase family, that are important for controlling parasitemia and tissue parasitism (26, 27). However, this focused immune response fails to clear parasite infection, leading to the chronic phase of Chagas disease. This control is not detrimental to *T. cruzi*, as it reduces host mortality while maintaining parasite infectivity toward its reduviid insect vectors. In this review, we will focus on the major processes behind the parasite's survival during the acute phase of Chagas disease.

[1] http://www.who.int/chagas/en/

THE INITIAL PHASE OF INFECTION: *T. cruzi* INVASION OF NON-PROFESSIONAL PHAGOCYTIC CELLS

Once a metacyclic trypomastigote penetrates the host through mucosa or lesions in the skin, it encounters host tissue cells and immune cells that populate or are recruited to that tissue. Poor parasite migration to surrounding tissues or draining lymph nodes and the evidence of parasite proliferation at the site of infection suggest that, immediately after the initial infection, the parasite invades tissues, rather than immune cells (28). In fact, in addition to being passively internalized by phagocytic cells, *T. cruzi* has the ability to invade any nucleated host cell.

Trypanosoma cruzi can actively invade a wide range of non-professional phagocytic cells through two different mechanisms. The first strategy that occurs in 20–30% of the cases is through a lysosome-dependent route, which induces Ca^{2+} signaling by inositol triphosphate (IP_3) generation, followed by the recruitment and fusion of host cell lysosomes at the parasite entry site (29–33). The second pathway, which occurs in 70–80% of invasions, is via invagination of the plasma membrane, followed by intracellular fusion with lysosomes (32, 33). Regardless of the entry route, lysosomal fusion is essential for retaining the highly mobile trypomastigotes inside the host cell; otherwise, the parasite escapes to the extracellular environment and, therefore, does not establish a productive infection (33, 34). Additionally, the lysosomal acidification of the parasitophorous vacuole contributes to trypomastigote-to-amastigote differentiation that takes place in the cytoplasm. After a transient, but crucial, association of the trypomastigotes with the lysosome-like parasitophorous vacuole, also known as a phagolysosome, this structure is disintegrated by the parasite through the action of a low pH-dependent pore-forming protein (35, 36). This process is mediated by the desialylation of the phagolysosome membrane. The lysosome-like parasitophorous vacuole internal surface is coated with two major proteins that are greatly sialylated, known as lysosome-associated membrane proteins 1 and 2 (LAMP 1 and 2) (37–39). The presence of sialic acid residues appears to protect the parasitophorous vacuole membrane from lysis. In fact, trypomastigotes escape earlier from the phagolysosome in sialic acid-deficient Lec 2 cells than from wild-type cells (38, 40). In the acidic environment of the phagolysosome, however, the parasite surface protein *trans*-sialidase is shed and becomes active

due to the low pH. Active *trans*-sialidase then transfers the sialic acid from LAMP proteins to parasite surface protein mucins, and this desialylation of the LAMP proteins renders the phagolysosomal membranes more susceptible to rupture (38, 39). Once the phagolysosome is destroyed and the trypomastigote reaches the cytoplasm, it differentiates into the replicative amastigote form and, after several rounds of replication, amastigotes differentiate into the bloodstream-infective trypomastigotes. These highly motile forms cause the rupture of the host cell membrane and can either infect neighboring cells or reach the bloodstream to disseminate the infection to distant tissues.

ROLE OF HOST-DERIVED NITROXIDATIVE STRESS IN *T. cruzi* INFECTION

Trypanosoma cruzi can also be passively internalized by phagocytic cells. Resident macrophages at the site of infection are among the first professional phagocytes to be invaded by the parasite (41, 42). To establish a productive infection in macrophages, *T. cruzi* must endure the extremely oxidative environment inside the phagolysosome (43). To this end, *T. cruzi* has a complex network of antioxidant enzymes, such as peroxidases and SODs, that protect the parasite against macrophage-released reactive oxygen and nitrogen species (44) (**Figure 1**).

During phagocytosis, the trypomastigotes trigger activation of a macrophage membrane-associated NADPH oxidase, resulting in the continuous production of superoxide radical anions $\left(O_2^{\bullet-}\right)$, which can be converted to H_2O_2 by SOD (43, 45, 46). During *T. cruzi* infection and Chagas disease progression, reactive oxygen species (ROS; e.g., $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) can be generated as a consequence of immune-mediated cytotoxic reactions, secondary damage to mitochondrion, and tissue destruction caused by the parasite. Thereafter, ROS can oxidize DNA, proteins, and lipids, killing the parasite (47).

Proinflammatory cytokines (IFN-γ and TNF) triggered by *T. cruzi* acute infection also stimulate infected macrophages to produce large amounts of nitric oxide ($^{\bullet}NO$) via the enzymatic activity of inducible nitric oxide synthase (iNOS), which oxidizes L-arginine and transfers electrons from NADPH (47–49). $^{\bullet}NO$ affects parasite survival in the macrophage by chemically modifying cysteine-containing proteins, inhibiting the catalytic activity of cruzipain, and binding to parasite metalloproteins (49, 50).

Once generated, $^{\bullet}NO$ can react with $O_2^{\bullet-}$ to produce peroxynitrite ($ONOO^-$), a potent oxidant and cytotoxic molecule that is highly effective against *T. cruzi* (46, 51). Peroxynitrite can damage cells directly by lipid peroxidation (harming membrane integrity and membrane protein function), as well as mitochondrial function and may result in apoptotic or necrotic cell death (46, 47). Moreover, secondary intermediate free radicals produced from $ONOO^-$, such as hydroxyl ($^{\bullet}OH$), nitrogen dioxide ($^{\bullet}NO_2$), and carbonate $\left(CO_3^{\bullet-}\right)$ radicals, can participate in the oxidation and nitration of proteins, lipids, and DNA, leading to mutations and transcription errors (43, 46). The oxidative stress caused by $^{\bullet}NO$ production can also be detrimental to the host, due to its high tissue-damaging potential (49). In fact, it has been shown that continuous exposure to nitroxidative-stress-induced damage can lead to Chagas disease progression and the development of myocarditis (47).

The parasite antioxidant network consists of various enzymes and non-enzymatic molecules distributed in diverse cellular compartments: the cytosol, ER, mitochondrion, and glycosome (**Figure 1A**). The final electron donor for all the enzymatic systems is the NADPH, which is derived from the pentose phosphate pathway, and their reducing equivalents are delivered to enzymatic detoxification systems via dithiol trypanothione $T(SH)_2$ and the thioredoxin homolog tryparedoxin (TXN) (43). $T(SH)_2$ is synthesized from two molecules of glutathione (GSH) and one spermidine by the enzyme trypanothione synthetase (TcTS) (52).

Trypanosoma cruzi has five peroxidases (also called peroxiredoxins) operating in its peroxide detoxification system (16). Cytosolic tryparedoxin peroxidase (TcCPX) and mitochondrial tryparedoxin peroxidase (TcMPX) have the ability to detoxify endogenous and macrophage-derived peroxynitrite, H_2O_2, and small-chain organic hydroperoxides (16, 53). Ascorbate-dependent heme-peroxidase (TcAPX), present in the ER, confers resistance against H_2O_2 (16, 54). Glutathione peroxidase-I (TcGPXI, located in the glycosome and cytosol) and glutathione peroxidase-II (TcGPXII, situated in the ER) confer resistance against lipid- and hydroperoxides (16, 55, 56).

Additionally, *T. cruzi* contains four iron SODs, which protect the parasite from the direct cytotoxic effects of $O_2^{\bullet-}$ and, hence, inhibit the formation of $ONOO^-$ by superoxide radical detoxification. TcSODs A and C neutralize the $O_2^{\bullet-}$ produced in the mitochondrion, TcSOD B1 acts in the cytosol, and TcSOD B1-2 acts in the glycosomes (43, 57).

Several studies have described the role of *T. cruzi* antioxidant enzymes as virulence factors (43). The overexpression of the peroxiredoxins TcCPX and TcMPX in *T. cruzi* results in cell lines that readily detoxify ROS generated *in vitro* or released by activated macrophages (16, 51). The protective effects of peroxidase TcCPX have also been observed *in vivo*; when compared to mice infected with wild-type parasites, mice infected with TcCPX-overexpressing *T. cruzi* showed increased parasitemia and higher inflammatory infiltrates in the skeletal muscle and heart (51). Parasites overexpressing TcAPX were more resistant to H_2O_2 but were not resistant to peroxynitrite (16). Proteomic analyses have suggested the upregulation of the *T. cruzi* antioxidant network members TXN, TcTS, TcAPX, TcMPX, and TcSOD A in the infective metacyclic trypomastigote when compared with the non-infective epimastigote stage, reinforcing the role of these enzymes in *T. cruzi* survival inside the mammalian host (58, 59). Peroxiredoxins (TcCPX and TcMPX) and a trypanothione reductase (TcTR) were upregulated during the metacyclogenesis process regardless of the *T. cruzi* strain, as observed after an analysis of 10 different isolates (44). Peroxidases were also observed in increased levels in the metacyclic forms of these virulent strains compared with attenuated isolates (44). These studies highlight the importance of the parasite antioxidant enzyme network in the successful establishment of host infection.

Reactive oxygen species are labile molecules and many of their effects are due to their rapid accumulation in different cellular compartments, such as macrophage phagolysosome. During *T. cruzi* infection, large amounts of $O_2^{\bullet-}$ are generated inside the

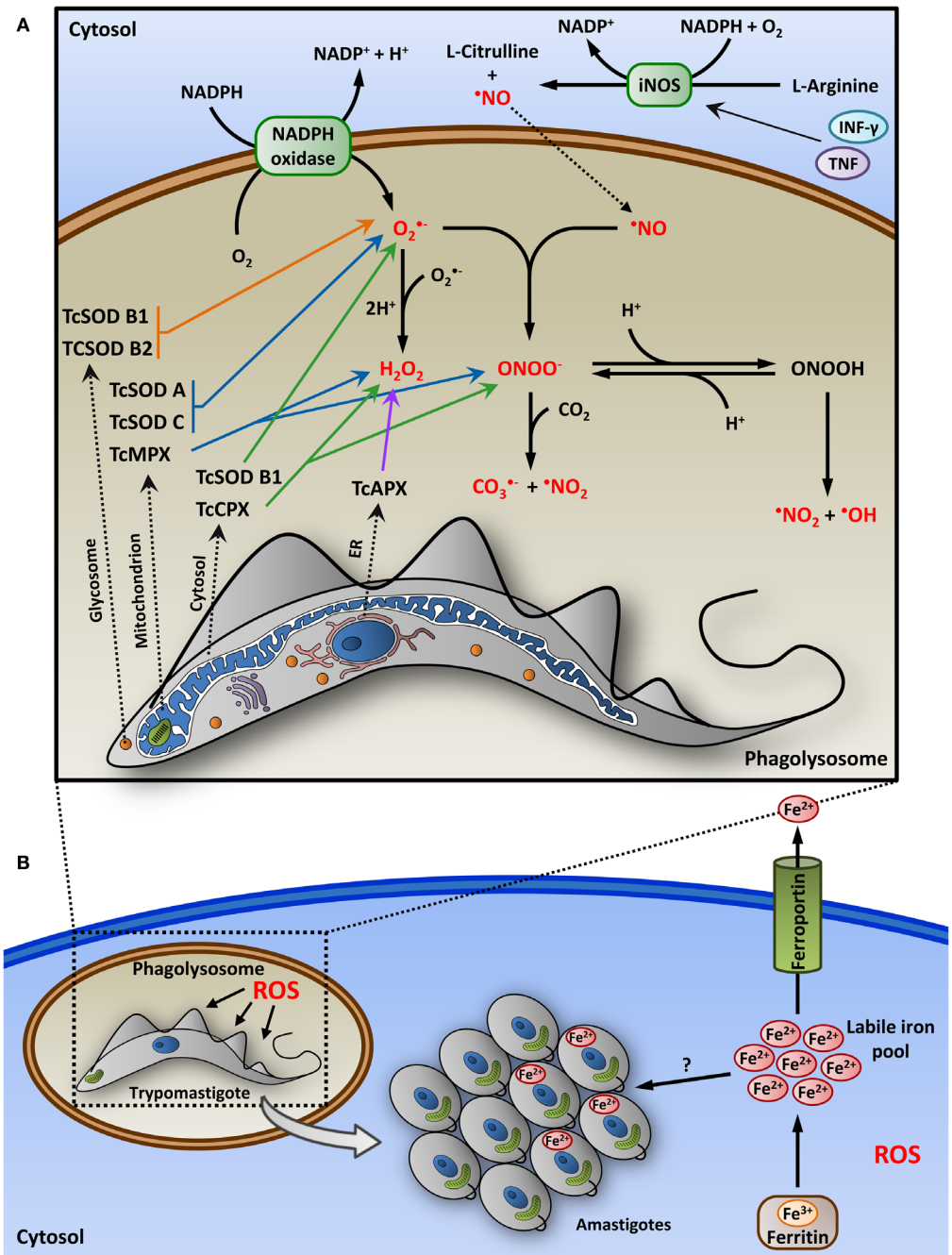

FIGURE 1 | Role of host-derived nitroxidative stress in *T. cruzi* infection. **(A)** After the phagocytosis of the parasite, macrophage membrane-associated NADPH oxidase is activated, producing the superoxide radical $\left(O_2^{\bullet-}\right)$ that can be converted into H_2O_2 inside the lumen of the phagolysosome. Macrophages stimulated with proinflammatory cytokines (IFN-γ and TNF) induce the expression of nitric oxide synthase (iNOS), generating nitric oxide (•NO) in the cytoplasm from the oxidation of L-arginine. •NO then diffuse into the phagolysosome vacuole and react with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), a potent oxidant. Secondary free radicals, such as carbonate $\left(CO_3^{\bullet-}\right)$, nitrogen dioxide ($^{\bullet}NO_2$), and hydroxyl (•OH) radicals, are produced from $ONOO^-$. These reactive oxygen species (ROS, indicated in red) can cause various cellular damages and parasite death within the phagolysosome. To survive in this highly oxidative environment, the parasite has a complex network of antioxidant enzymes, as peroxidases (TcAPX, TcCPX, and TcMPX) and superoxide dismutases (SOD), which act in the detoxification of ROS, and are distributed in various cellular compartments, such as glycosomes, mitochondrion, cytosol, and endoplasmic reticulum (ER). Enzymes derived from glycosome, mitochondrion, cytosol, and ER are indicated by orange, blue, green, and purple arrows, respectively. **(B)** To establish a productive infection, trypomastigotes should escape from the phagolysosome to the cytosol, where it differentiates into replicative amastigotes. In the cytosol of macrophages, ROS, instead of being detrimental to the parasite, can promote the intracellular growth of *T. cruzi* by a mechanism that may involve facilitating amastigote access to iron. In the cytosol, iron can be stored as ferric iron (Fe^{3+}), a redox-inert form, associated with ferritin or can be exported from the cell as ferrous iron (Fe^{2+}) through ferroportin, a macrophage-specific iron exporter. The expression of ferroportin and ferritin is upregulated by antioxidants, which can lead to reduced levels of labile iron pool in the cytosol. The mechanism of iron uptake by amastigotes is unknown, but the parasite may be dependent on the intracellular labile iron pool for growth.

phagolysosome after phagocytic stimulus (60). This radical is maintained for only 90–120 min and presents a limited diffusion capacity through the membrane due to its anionic nature (61). Although synthesized in the cytoplasm, •NO is diffused into the phagolysosome vacuole due to its hydrophobic properties (60) and has a half-life of approximately 24 h (61). In the phagolysosome, •NO reacts with $O_2^{\bullet-}$ generating $ONOO^-$, which presents a short half-life and high diffusion capacity (60). Parasite survival within the phagolysosome is broadly affected by macrophage production of $ONOO^-$ during the first hours of infection (60).

Although the parasite faces an extremely oxidative environment inside the phagolysosome (**Figure 1A**), trypomastigotes are associated with this compartment transiently and after 24 h post-infection escape to the cytosol where the parasite remains as replicative amastigotes during the majority of its intracellular life cycle. A recent study has demonstrated an unexpected role of the oxidative stress in promoting *T. cruzi* infection. Paiva et al. (62) have shown that once the parasite reaches the cytosol of macrophages, oxidative stress can also contribute to parasite burden by a mechanism that may involve facilitating amastigote access to iron, which is critical for parasite growth (**Figure 1B**). Peritoneal macrophages from mice infected with the *T. cruzi* Y strain treated with cobalt protoporphyrin (CoPP) (an activator of the transcription factor NRF2, which orchestrates antioxidant responses), as other antioxidants, lead to a notably reduced parasite burden (62). It has also been demonstrated that pro-oxidants promote *T. cruzi* growth and reverse the host-protective effects of CoPP (62). Similar results were observed *in vivo*, where CoPP reduced parasitemia and tissue parasitism in infected mice (62). The protective effect of CoPP in *T. cruzi* infection is independent of T cell-mediated immunity and does not involve apoptotic clearance of infected cells or effectors that act against the parasite, such as type I IFN, TNF, or •NO (62). These results suggest that the deleterious effects of antioxidants on parasite may occur by a mechanism different from classical innate or adaptive immune responses. Paiva et al. (62) demonstrated that the sequestration of iron, present in the host cytoplasm, is most likely involved in the parasite burden-reducing effects mediated by antioxidants, once the labile iron pool is reduced by the treatment of infected cells with antioxidants. Interestingly, these authors observed that induction of antioxidant responses reduced the parasite load in macrophages, but not in other cell types (62), suggesting that this may be a macrophage-specific mechanism. This can be explained by the role of macrophages as iron storage *in vivo*. Intracellularly, iron can be used in metabolic pathways in its ferrous form, which can also catalyze the formation of free radicals and, therefore, its concentration in the cytosol has to be tightly regulated. To this end, iron can be stored in the cytosol as ferric iron, a redox-inert form, associated with ferritin. Ferrous form can also be exported from the cell through ferroportin, a macrophage-specific iron exporter (63). The expression of ferroportin and ferritin is upregulated by the antioxidant response regulator NRF2 (64, 65), which can lead to reduced levels of labile iron pool in the cytosol. The mechanism of iron uptake by amastigotes is unknown, but the parasite may be dependent on the intracellular labile iron pool for growth (**Figure 1B**). This pathway could be the basis for the unexpected effect of antioxidants in reducing *T. cruzi* infection. Contrasting results were, however, observed in other studies, in which antioxidants had no impact in *T. cruzi* CL Brener infection (51) or increased the parasite burden in mice infected with strain Sylvio X10/4 (66). This latter study did not evaluate macrophage parasitism and, therefore, ROS production may be required to control parasitism in particular tissues (62). Additionally, strain-specific factors, such as level of expression of antioxidant enzymes, kinetics of association with the phagolysosome, and iron uptake efficiency, may contribute to differential resistance/susceptibility of distinct *T. cruzi* strains to the oxidative environment and outcome of the infection.

PATTERN-RECOGNITION RECEPTORS AND INNATE IMMUNITY AGAINST *T. cruzi*

Pattern-recognition receptors (PRRs) have been described as one of the first line of immune defense against various pathogens, including protozoans (67, 68). PPRs are expressed by cells of the innate immune system and are responsible for the recognition of molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). TLRs are among of the best-characterized PPRs and detect PAMPs that are either located on the cell surface or in the lumen of intracellular vesicles, such as endosomes or lysosomes. These receptors are more abundant in antigen-presenting cells, such as macrophages and dendritic cells, but have also been described in T cells and some somatic cells (68–71). TLR activation leads to the production of proinflammatory cytokines and chemokines that in turn lead to the recruitment of phagocytic cells to the infected tissue, which are important not only for initial infection control but also for molding the subsequent adaptive immune response (25, 68, 72). A total of 12 and 10 TLR family members have been identified in mice and humans, respectively. TLRs 1–9 are shared between mice and humans, whereas TLR11, TLR12, and TLR13 are restricted to mice, and TLR10 is expressed only in humans (73, 74). Some TLRs function as homodimers, such as TLR4 and TLR9, whereas others are heterodimers, such as TLR2/6. After stimulation, these receptors undergo required conformational changes to recruit TIR-domain-containing adaptor molecules, which, with the exception of TLR3, lead to a MyD88-dependent signaling cascade that culminates in the production of proinflammatory cytokines (25, 68, 69).

Toll-like receptors have a critical role in host resistance to *T. cruzi* infection, as evinced by a remarkable increase in the susceptibility of MyD88-deficient mice infected with *T. cruzi* compared with that of WT mice. This higher susceptibility is associated with the impaired production of IL-12 and IFN-γ proinflammatory cytokines, which are important for driving the Th1-directed protective immune response (75). *T. cruzi* has several molecules that can strongly stimulate TLRs, such as the surface molecules mucin and glycoinositolphospholipid (GIPL), as well as parasite DNA and RNA sequences (24, 25, 76–78) (**Figure 2**).

Mucins are GPI-anchored surface proteins that coat the entire surface of the parasite and are enrolled in immune evasion and host cell adhesion/infection processes (79, 80). The *T. cruzi*

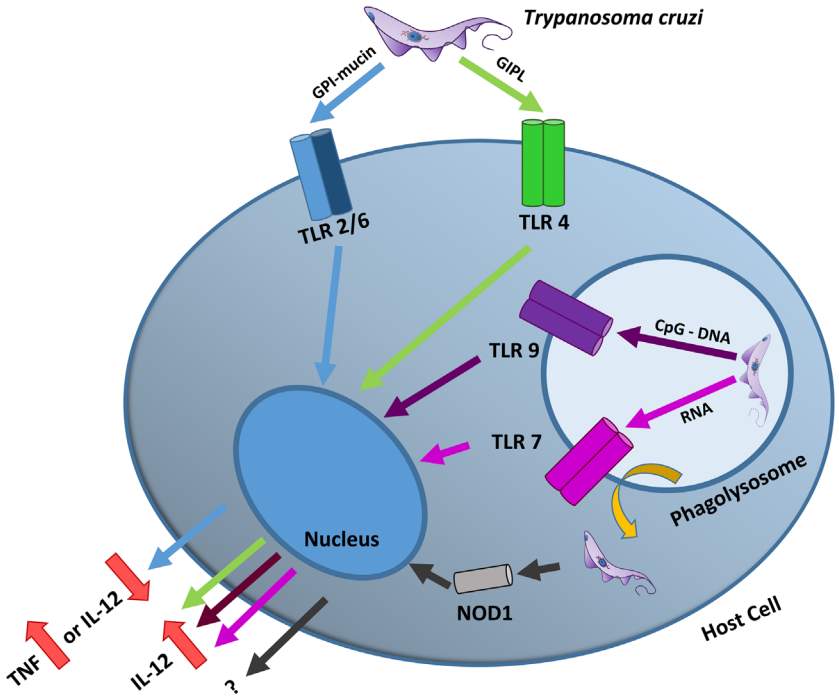

FIGURE 2 | *T. cruzi* TLR and NLR activation. *T. cruzi* possesses several molecules capable of stimulating TLRs. The activation of the heterodimer TLR2/6 by parasite GPI-mucins can lead to TNF production in macrophages or to the inhibition of IL-12 in dendritic cells (blue arrows). By contrast, the activation of TLR4 by parasite GIPLs (green arrows), TLR9 by parasite CpG DNA motifs (purple arrow) and TLR7 by parasite RNA (pink arrow) all result in the production of proinflammatory cytokines, such as IL-12. After the parasite escapes from the phagolysosome, it can activate the cytoplasmic NOD1 receptor. Although this receptor is important for controlling the infection, its mechanism of action is still unknown.

trypomastigote mucin GPI anchors, especially the unsaturated fatty acid at the sn-2 position, are potent stimulators of the extracellular heterodimer TLR2/6. *In vitro* stimulation of TLR2/6 by *T. cruzi* GPI-mucins leads to the production of proinflammatory cytokines, such as IL-12 and TNF, as well as nitric oxide, which are related to a Th1-focused immune response that is important to control parasitemia and tissue parasitism (67, 74, 81). However, in contrast to *in vitro* experiments, *in vivo* assays showed that TLR2-deficient mice infected with *T. cruzi* develop a strong proinflammatory immune response with higher IFN-γ serum levels than those of WT mice, suggesting an immunoregulatory role for TLR2 during *T. cruzi* infection (74, 75). Recently, Gravina and coworkers suggested that TLR2 assumes different functions depending on the host cell type, acting as a TNF producer in macrophages and as an immunoregulator in dendritic cells (78). *T. cruzi* covers its whole surface with as many as 2×10^6 mucin molecules (79, 80); the abundance of this molecule may be important for stimulating dendritic cells in a TLR2-dependent manner during the initial steps of infection, leading to an immunoregulatory effect *in vivo*, and may contribute to the delayed immune response and antibody production against the parasite (78, 82).

Glycoinositolphospholipids are free GPI anchors present in all *T. cruzi* life stages (72, 83–85). *T. cruzi* GIPLs share a core conserved structure [Manα(1 → 2) Manα(1 → 2) Manα(1 → 6) Manα(1 → 4) GlcNα(1 → 6) *myo*-inositol 1-PO_4-lipid], in all parasite stages and among different strains, although considerable variability exists in both the lipid and glycan portions of these molecules (84, 86). GIPLs from G, Y, and Tulahuén strains contain ceramide, whereas those from CL contain alkylacylglycerol and dihydroceramide (83, 84, 86, 87). Lipid remodeling has also been detected in GPI-anchored proteins and GIPLs in different forms of *T. cruzi* (79, 87–90). This distinct composition confers distinct biological functions, as low concentrations of GIPLs containing ceramide have been shown to induce apoptosis and regulate the activity of macrophages and dendritic cells (91, 92). This difference is also important in TLR recognition, as GIPLs containing ceramide are recognized by the homodimer TLR4, while GIPLs containing alkylacylglycerol are agonists of TLR2/6 (74, 76, 93). Although TLR2/6 stimulation by GPI-mucin appears to be 100-fold more efficient in stimulating the immune response *in vitro* (74, 93), this stimulation has also been associated with immunoregulation (74, 75, 78), whereas an anti-inflammatory outcome with respect to TLR4 stimulation has not yet been described.

In contrast to TLR2/6 and TLR4, which are localized on the cell surface, TLR7 and TLR9 are expressed in the ER and, upon *T. cruzi* cell invasion, are translocated to endolysosomes, where they recognize immunostimulatory motifs derived from parasite RNA or DNA, respectively (23–25, 94, 95). As *T. cruzi* invades the host cell and reaches the phagolysosome environment, nucleic acid molecules from lysed parasites stimulate TLR7 and 9, leading to the production of Th1 proinflammatory cytokines important

for controlling the infection (24, 77, 95, 96). The immunostimulatory CpG DNA motifs are not randomly distributed in the parasite genome; instead, they are enriched in genomic regions that encode large gene families of surface proteins, such as mucins, *trans*-sialidases, and mucin-associated surface proteins (MASPs) (24). As most of the proteins encoded by these genes are involved in parasite immune evasion mechanisms or host cell adhesion/invasion (14, 15, 80), there appears to be a trade-off between the need to invade cells and CpG immune stimulation via TLR9. One of the mechanisms that may reduce this drawback is the ability of *T. cruzi* to escape from the phagolysosome, reducing the chance of lysis and, therefore, minimizing TLR9 activation. Concomitantly, the immunoregulatory effect of TLR2 stimulation by GPI-mucin in dendritic cells may also balance TLR9 and TLR7 activation by parasite DNA and RNA (78), respectively, at least in the initial phases of infection.

In addition to TLR, other innate immune receptors important in controlling *T. cruzi* infection are the nucleotide-binding oligomerization domain (Nod)-like receptors (NLR). NLRs are localized in the cytoplasm or are associated with the plasma membrane of mammalian cells. NLRs are related to MAP kinase and NF-κB activation (NOD1 and NOD2) or with the production of a caspase 1-dependent inflammasome (NLRP3) (97). *In vitro* studies have shown that although macrophages from NOD1$^{-/-}$ and NOD2$^{-/-}$ mice infected with *T. cruzi* failed to produce nitric oxide (•NO) when stimulated with IFN-γ, only NOD1$^{-/-}$ mice failed to eliminate the intracellular parasites (98). NOD1$^{-/-}$ mice infected with *T. cruzi* showed threefold higher parasitemia than WT and NOD2$^{-/-}$ mice, and succumbed 24 days post-infection (98). Although NOD1 receptors appear to be important for *T. cruzi* infection control, the mechanisms involved are still unknown, as a deficiency in this receptor does not impair cytokine production *in vivo*, and *T. cruzi* lacks any previously described NOD1 agonists (98).

COMPLEMENT EVASION

After the first round of intracellular replication and host cell rupture, *T. cruzi* reaches the mammalian bloodstream and becomes a target of the complement pathways. The complement system consists of soluble proteins that interact with pathogen structures and activate a cascade of proteases that eliminate invading microorganisms. There are three complement pathways: classical, alternative, and lectin (**Figures 3A–C**). Although these pathways differ in the initial steps of their respective cascades, all three converge to produce a C3 convertase and then a C5 convertase, leading to the formation of the membrane attack complex (MAC) and subsequent pathogen lysis (**Figure 3D**).

Trypanosoma cruzi initially becomes a target of the host alternative and lectin complement pathways. The lectin pathway is activated by the binding of mannan-binding lectins (MBLs) or ficolins to the mannan or carbohydrates of the parasite surface,

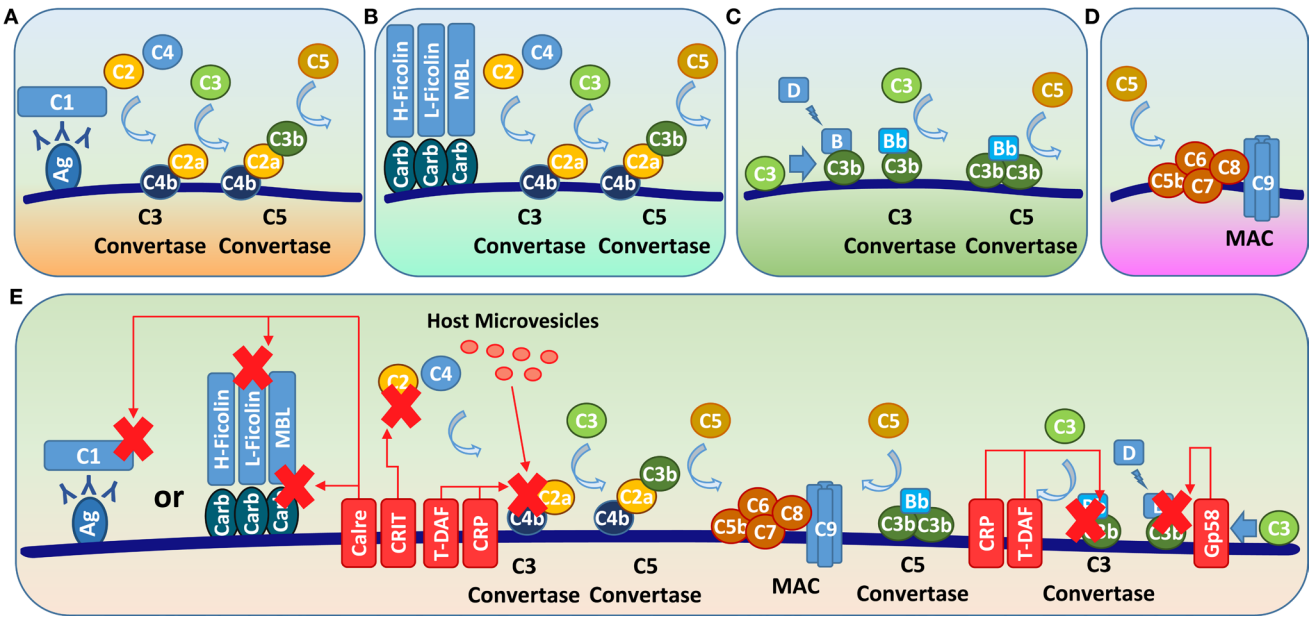

FIGURE 3 | *T. cruzi* complement evasion mechanisms. There are three complement pathways: classic, alternative, and lectin. **(A)** In the classical pathway, antibodies bound to pathogen antigens interact with the complement C1 protein, which cleaves C2 and C4 to generate C2a and C4b; these molecules bind to the pathogen surface to form the C3 convertase C4b2a. **(B)** In the lectin pathway, MBL or ficolin binds to mannan or glycosylated molecules, respectively, on the pathogen surface, and cysteine proteases bound to these molecules cleave C2 and C4, also generating the C3 convertase C4b2a. **(C)** In the alternative pathway, spontaneously hydrolyzed C3b or C3b originating from the other complement pathways interacts with factor B, which is cleaved into Bb by factor D, forming the C3 convertase C3bBb. The C3 convertases from all the complement pathways interact with newly cleaved C3b, forming a C5 convertase that cleaves C5 into C5b. **(D)** C5b interacts with C6–C9 to form the MAC, leading to the pathogen lysis. **(E)** To avoid lysis, *T. cruzi* relies on molecules, such as calreticulin and gp58/68 (Gp58), which block the initial steps of classic/lectin or alternative pathways, respectively, and CRIT, T-DAF, CRP, and host-derived microvesicles that disrupt or block C3 convertase assembly. Ag, antigen; Carb, carbohydrate; Calre, calreticulin.

respectively (99) (**Figure 3B**). This pathway is responsible for almost 70% of parasite complement-mediated lysis during infection (20, 99, 100). The alternative pathway is activated either by a low rate of spontaneous conversion of C3 to C3b or by C3b generated from the other complement pathways (100) (**Figure 3C**). As the infection progresses and anti-*T. cruzi* antibodies are produced, antibodies bound to parasite surface molecules interact with the complement C1 molecule, activating the classical pathway (**Figure 3A**).

To escape complement-mediated lysis, *T. cruzi* relies on a large set of molecules that act by blocking different steps of the complement pathways (20, 101) (**Figure 3E**). *T. cruzi* impairs the lectin pathway via calreticulin, a 45-kDa surface molecule (102) that binds to host MBL collagenous tails, preventing their interaction with parasite mannan (18), and also interacts with L-ficolin, preventing C4–C4b conversion (103). As anti-*T. cruzi* antibodies are produced, calreticulin also interacts with C1, preventing its interaction with C4 and inhibiting the classical complement pathway (18, 104, 105). Therefore, calreticulin is able to disrupt the initial steps of both the classical and lectin complement pathways, and, because it reduces the formation of C3 convertase, calreticulin also indirectly inhibits the alternative pathway.

Complement regulatory protein (CRP), also called GP160, is a trypomastigote GPI-anchored surface protein that binds to C3b and C4b, dissociating the classical and alternative complement C3 convertase (17, 106). Beucher and Norris have described several CRP paralogs within the *T. cruzi* genome that share sequence similarity with *T. cruzi trans*-sialidase superfamily members lacking TS activity (107). Nevertheless, the involvement of these sequence-related CRP paralogs in blocking the activation of the alternative and classical pathways needs experimental validation.

Complement C2 receptor inhibition trispanning (CRIT) is a *T. cruzi* transmembrane protein that blocks C2 cleavage into C2a, preventing the lectin and classical complement pathway-mediated formation of C3 convertase (99, 108). Trypomastigote decay-accelerating factor (T-DAF) is an 87- to 93-kDa protein with similarity to human decay-accelerating factor (DAF), which interferes with C3 convertase assembly efficiency, potentially affecting the three complement pathways (19, 109). *T. cruzi* gp58/68 also inhibits C3 convertase assembly, but only in the complement alternative pathway, by preventing the binding of factor B to surface-fixed C3b (110).

Finally, it has recently shown that *T. cruzi* induces the release of plasma membrane-derived vesicles from host cells (111). These vesicles are involved in diverse immune evasion processes, including binding to and inhibiting the activity of the complement C3 convertase C4b2a (111), and will be further discussed in the microvesicles section of this review.

In summary, *T. cruzi* complement evasion focuses on diverse molecules that disrupt or inhibit C3 convertase formation, a key step in all complement pathways, or neutralizes the initial steps of the complement cascade (**Figure 3**). As all complement pathways converge with C3 convertase formation, disrupting this key step is an efficient way to disturb all complement-mediated responses simultaneously. In addition to being important in the complement cascade, C3b is also an opsonin, which is recognized by macrophages and induces phagocytosis (112). Therefore, inhibiting C3b formation may also reduce macrophage-derived parasite lysis during infection.

THE ROLE OF MICROVESICLES IN *T. cruzi* INFECTION

Microvesicles (MVs) are also known as microparticles, ectosomes, exosomes, or plasma membrane-derived vesicles (111, 113–115). MVs have a complex lipid bilayer structure and carry several cell-derived molecules, such as lipids, peptides, proteins, and nucleic acids (e.g., miRNAs and mRNAs), which can be transferred to and become functional in target cells (116–119). The release of plasma membrane-derived vesicles occurs at basal levels, but may be greatly increased by extracellular stimuli, such as parasitic infection (111, 120).

Recent studies have shown that MVs play an active role in intercellular communication inside an organism or between different organisms, as occurs during pathogen infections in a host (111, 115–118). Furthermore, the involvement of MVs in various diseases, such as thrombosis, cancer, pathogen infections, autoimmune diseases, and others, has also been observed (120). Vesicles may also participate in the delivery of pathogen virulence factors, contributing to the spread of the pathogen and successful immune evasion (111, 117, 118).

As discussed above, one of the first barriers encountered by parasites is the innate immune complement system. Recently, Cestari et al. (111) observed that *T. cruzi* induces the release of host plasma membrane-derived vesicles to evade innate immunity, by inhibiting complement-mediated lysis and also facilitating host cell invasion. At the beginning of the infection, metacyclic trypomastigotes induce MV release from blood cells, such as lymphocytes, monocytes, and macrophages, in a Ca^{2+}-dependent process (111). The host-derived MVs predominantly inhibit the classical and lectin pathways of the complement system, increasing parasite survival. This inhibition is mediated by host MVs that bind to the C3 convertase C4b2a on the *T. cruzi* surface, leading to the inhibition of its catalytic activity (111).

Moreover, it has also been shown that lymphocytes- and monocytes-derived MVs carry the cytokine TGF-β, enhancing *T. cruzi* cell invasion and protecting the parasite from the complement-mediated lysis (111). This increase in cell invasion has also been demonstrated *in vivo*; mice infected with *T. cruzi* in the presence of MVs exhibited increased parasitemia (111).

In addition, parasite-shed vesicles may contain important virulence factors that contribute to the parasite–host interplay and the establishment of infection (117, 119). *T. cruzi*-derived MVs can act as messengers, preparing the cellular environment to facilitate infection, and thereby ensuring parasite survival (117, 121). This process occurs either through the interaction of parasite-derived MVs with host cell surface components or through the internalization of vesicles, which are accumulated in endocytic/phagocytic pathways (117). Proteomic analysis has revealed that the main components of the parasite-derived vesicles are TS/gp85 superfamily members, α-galactosyl-containing glycoconjugates, proteases, MASPs, and cytoskeleton proteins (117).

Previous inoculation with *T. cruzi*-derived MVs accelerates and enhances the mortality rate of infected mice, which develop more severe heart lesions with an increased number of intracellular amastigote nests (121). Furthermore, parasite vesicles induce IL-4 and IL-10 production in the heart and spleen and IL-10 and IL-12 production by resident peritoneal cells (121). Changes in host cell gene expression were also observed in HeLa cells upon the incorporation of parasite-derived extracellular vesicles containing tRNA-derived small RNAs (tsRNAs) from *T. cruzi* (119). The elicited response primarily modified the host cell extracellular matrix, cytoskeleton, and immune response pathways (119).

All together, these data indicate that both host- and parasite-derived plasma membrane MVs play an important role in the establishment and maintenance of parasite infection.

DELAYED DEVELOPMENT OF A PROTECTIVE IMMUNE RESPONSE: POLYCLONAL B CELL ACTIVATION, SMOKE SCREENS, AND IMMUNODOMINANCE

In contrast to other infectious pathogens that induce rapid changes in the gene expression of infected host cells (122), *T. cruzi* only exerts significant gene expression changes in human fibroblasts 24 h after infection (122). This delayed host transcriptional response coincides with the parasite escape from the phagolysosome to the cytoplasm and differentiation into the replicative amastigote forms. This sequence of events suggests that during the initial phase of a primary *T. cruzi* infection, the parasite does not trigger host PRRs, leading to silent entry (82, 122, 123). Besides the delayed changes in the gene expression of infected cells, *T. cruzi* immune activation coincides with the release of trypomastigotes from infected cells 4–5 days post-infection, suggesting that the parasite relies on mechanisms to avoid PAMP-derived immune stimulation during the first cycle of replication (28, 82). Three aspects may contribute to this silent entry: (i) the relatively slow kinetics of *T. cruzi* intracellular cycle, (ii) parasite escape from the phagolysosome, and (iii) immunoregulatory response meditated by TLR2/6 activation in dendritic cells. *T. cruzi* growth rate is significantly slower than virus and bacteria, taking longer to achieve a threshold necessary to mount a robust immune response, which is delayed to at least the end of the first round of intracellular replication (28). Also, parasite escape from the phagolysosome reduces its mortality, thus, reducing the amount of DNA and RNA immunostimulatory sequences available for TLR9 and TLR7 activation in this cellular compartment. Finally, the TLR2/6 immunoregulatory stimulation of dendritic cells by GPI-mucins could counteract other immune activation processes and could also delay the development of adaptive immune response (78). Another possibility for the immunologically silent entry is that *T. cruzi* PAMPs may not trigger an immunostimulatory response as effective as those of bacteria, since transgenic expression of bacterial PAMPs in *T. cruzi* enhanced the anti-parasite response leading to pathogen control and clearance (124).

After several rounds of infection/proliferation, a robust anti-*T. cruzi* immune response is developed, which is able to greatly reduce parasitemia and tissue parasitism. However, this immune response is unable to provide parasite clearance, as polymerase chain reaction (PCR) and immunocytochemistry assays have shown the presence of parasites in infected tissues in patients with cardiac (125–127) and digestive (128) manifestations. The delayed immune response and the inability to clear the parasite may be related to the large repertoire of highly polymorphic and immunogenic surface proteins that are coexpressed by the parasite (82, 123, 129, 130). This antigen arsenal may provide means of evading immune response that are distinct from the classic antigenic variation employed by parasites such as *Trypanosoma brucei* and *Giardia lamblia* (131–136).

Classic antigenic variation is achieved by the expression of identical antigenic variants on the surface of the majority of the cells in a parasite population while a small subset expresses different variants (131, 137–139). The immune response targets the parasites expressing the common variant while failing to identify those expressing rare variants (137). Long-term infection is achieved by varying the expressed antigens, leading to successive waves of parasitemia and clearance as novel antigenic determinants spread in the parasite population (133, 138, 139). There is no evidence that *T. cruzi* adopts this type of antigenic variation. Instead, the entire *T. cruzi* population simultaneously exposes a variety of antigenic surface proteins, such as mucins, *trans*-sialidase, and MASPs, encoded by highly polymorphic multigene families (22, 80, 82, 129, 130). The coexpression of this diverse antigenic repertoire drives the immune system into a series of spurious and non-neutralizing antibody responses, a mechanism known as a smoke screen, which delays the production of high-affinity anti-*T. cruzi* antibodies and the priming of effective T-CD8$^+$ cells (22, 82, 140). The presence of a broad range of antigenic motifs may also be a mechanism to drive the antibody response away from catalytic sites of key parasite surface proteins. In fact, a strong humoral response against the *trans*-sialidases C-terminal repetitive motif shed acute phase antigen (SAPA) has been observed, followed by a weak antibody response against several epitopes at the N-terminal catalytic region in a later stage that was unable to inhibit the enzyme activity (141).

In addition to the high variability of parasite surface antigens, the presence of parasite-derived B cell mitogens also causes polyclonal B cell activation and hypergammaglobulinemia, resulting in a delayed parasite-specific antibody response (21, 22, 142, 143). This unfocused response is important for parasite survival, as most of the antibodies produced by splenic cells during the initial acute phase do not target the parasite, and specific anti-*T. cruzi* antibodies are only produced later (22). Interestingly, although the humoral response in the chronic stage shows a preferential IgG2a pattern, the acute infection comprises a broader range of immunoglobulin isotypes: IgM, IgG1, IgG2a, IgG2b, and IgG3 (22, 144). In addition to B cell mitogens, another driving factor of this polyclonal activation may be the coexpression and shedding of a large repertoire of immunogenic surface proteins, delaying the immune response to immunodominant epitopes. In fact, *trans*-sialidases and their terminal long tandem repeats have been shown to be T-independent polyclonal activators of mouse B cells (143, 145, 146). Even though polyclonal B-cell activation is transient and its role as a parasite escape mechanism needs further *in vivo* experimental validation,

this may be a strategy that could contribute for parasite survival during the initial stage of infection, when parasitemia is low and the parasite has not yet reached the sites where it persists, such as muscle, adipose tissue, and nervous system.

In contrast to previous studies, Bryan and coworkers have shown that C57BL/6 mice infected with *T. cruzi* Y strain presented lower polyclonal B cell activation than BALB/c mice, suggesting that polyclonal activation is not a generalized response in *T. cruzi* infection and is highly dependent on the host strain (147). The authors associated this difference with the protective Th1-focused C57BL/6 immune response, in contrast to the susceptible Th2-focused response developed by BALB/c mice (147). Distinct parasite strains also show different degrees of B-cell polyclonal activation. Parasites from TcVI DTU such as CL (144) and Tulahuén (22) strains and the clone CL Brener (145) induced polyclonal B-cell activation in BALB/c and C3H/Hej mice, while polyclonal activation induced by Y strain was restricted to BALB/c (147). The TcVI DTU was originated by a hybridization event between TcII and TcIII strains (148), which result in an increased repertoire of multigene families encoding surface proteins when compared to Sylvio X10 (TcI) (149). As these surface proteins are highly immunogenic, this larger repertoire of antigens could contribute to B-cell polyclonal activation observed in infections by TcVI strains.

Trypanosoma cruzi antigens released in the intracellular host cell environment, either from live parasites shedding or parasite lysis, become available for presentation by the class I major compatibility complex (MHC) through the endogenous pathway (25, 26). This presentation promotes the priming of a strong but delayed $CD8^+$ T immune response that is highly effective for controlling parasite levels, but only becomes evident 5–6 days post-infection, coinciding with the first round of intracellular replication (82, 150–153). The delayed anti-*T. cruzi* immune response may be due to the need for a sufficient number of antigen-producing amastigotes accumulating in the cytosol, and/or by the large number of different polymorphic antigens that are simultaneously expressed by the parasite. These antigens may compete for presentation through host cell MHC class I molecules, delaying a fast and focused immune response (82, 123, 152, 153). This immunologically silent initial phase of infection may allow the parasite to reach a critical level before activating the host immune system (82, 123, 154). As the infection advances, pathogen-specific T cells appear to preferentially recognize a small number of epitopes in a hierarchical manner, a process called immunodominance (26, 27, 152, 155). Immunodominant antigens can be selected based on several factors, such as the abundance of the parasite epitope and its affinity to MHC and T-cell receptors (26, 156–158). *Trans*-sialidases are among the major known $CD8^+$ T immunodominant targets in *T. cruzi* infection, due to high expression in the infective forms and repetitive/antigenic content; as such, these enzymes have also been proposed as vaccine targets (27, 74, 152, 159–162). As previously stated, *trans*-sialidase is a highly polymorphic, multicopy gene family in *T. cruzi*, with several potential immunogenic candidates that can generate an unfocused immune response. To overcome this, the anti-*trans*-sialidase immune response is focused on a relatively small number of epitopes encoded by multiple genes (82, 152). *Trans*-sialidase immunodominant antigens can account for more than 30% of the entire $CD8^+$ response in mice (152) and a significant proportion in humans (153). The presence of subdominant/cryptic antigens was demonstrated after the tolerization of the major immunodominant epitopes of *T. cruzi* during infection in BALB/c and C57BL/6 mice (27). These mice exhibited an immune response against novel antigens and a transient increase in parasite load but were ultimately able to control the acute infection, suggesting that a focused immune response *per se*, but not the presence of these immunodominant antigens, is required to control the infection (27, 82). This result is not surprising, as the *trans*-sialidase gene family varies in sequence and expression among *T. cruzi* strains (148, 149, 163), and immunodominance also depends on interaction between the antigen and host receptors, which vary among host species. Although immunodominance is a well-described phenomenon in *T. cruzi*, its direct implications for parasite clearance are still under debate. While some authors state that specific T cells for a single epitope can hinder the development of immunity to several other epitopes, allowing a small set of variant parasites to escape from the immune system (26, 164), others argue that immunodominance is probably not the major factor governing *T. cruzi* escape from sterile immunity (27). The second group argued that vaccination to boost specific immunodominant epitopes enhanced mice protection, instead of being deleterious to the hosts by strongly focusing the immune response on the immunodominant epitope (161, 165), and the tolerization of immunodominant epitopes did not lead to higher parasite clearance (27).

CONCLUSION

Trypanosoma cruzi has been interacting and coevolving with humans for 6,000–9,000 years (5, 10, 166), and infecting wild mammals even longer (6–8, 167). Because of this extensive interaction with mammalian hosts and its obligatory parasitic lifestyle, this protozoan has developed several mechanisms to evade the host immune system (**Figure 4**), and simultaneously reduce host damage while maintaining its transmissibility to insect vectors (168). It is not surprising that as the disease reaches its chronic stage, only 30% of the patients progress to cardiac or digestive manifestations, whereas 70% show no clinical symptoms but are still able to infect triatomine insect vectors (168). However, when this equilibrium is lost and symptoms do occur, the disease causes great morbidity, resulting in a loss of 662,000 disability-adjusted life years (1–3) (WHO) (see text footnote 1). Among the trypanosomatids whose genomes have already been sequenced, *T. cruzi* exhibits the largest expansion of the multigene families that encode surface proteins, many of which are antigenic (130, 148, 163). A driving force for the expansion of these polymorphic surface proteins may be their involvement in the parasite's ability to invade any mammalian nucleated cell, which is a critical strategy that allows the parasite to spread in different host tissues during the initial infection. In addition, this impressive surface protein polymorphism also contributes to antigenic variability, leading to the coexpression of several polymorphic antigens that delay the development of an effective immune response. The delayed immune activation in host cell newly infected with *T. cruzi*, the polyclonal B cell activation, and *T. cruzi* intra- and inter-strain

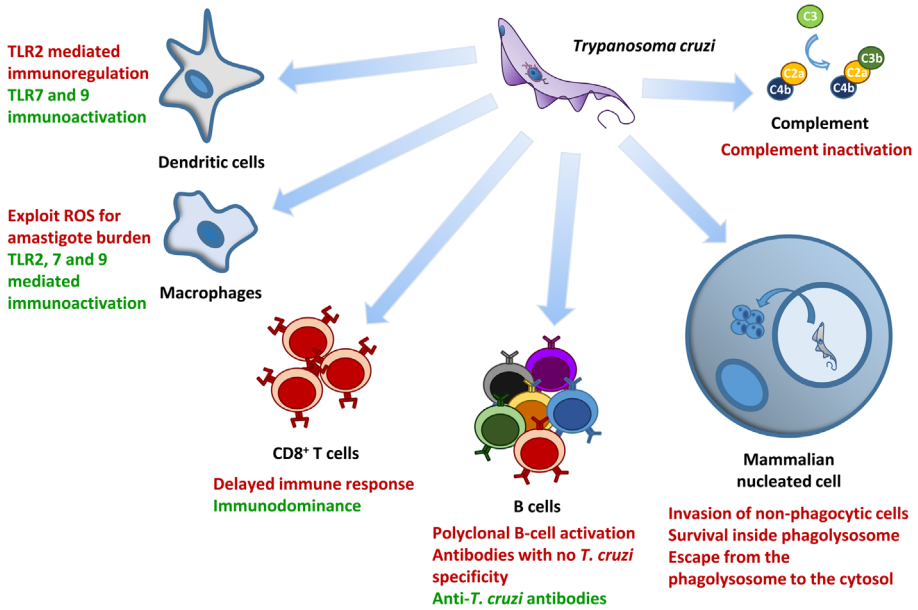

FIGURE 4 | Major mechanisms involved in *T. cruzi* survival and control during the initial phase of infection. This figure summarizes the major interaction mechanisms between *T. cruzi* and several host components addressed in this review. Mechanisms associated with the control of parasite load are highlighted in green, whereas those involved with parasite modulation of the host immune system and/or with increased parasite load are highlighted in red.

surface antigenic variability makes prophylactic vaccine target identification nearly impossible (82). An effective pan-*T. cruzi* vaccine would have to include immunodominant and cryptic antigens from a broad variety of parasite isolates.

AUTHOR CONTRIBUTIONS

MC: participated in design and manuscript writing. JR-C: participated in design and manuscript writing. DB: participated in design, coordination, and manuscript writing.

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REFERENCES

1. Hotez PJ, Bottazzi ME, Franco-Paredes C, Ault SK, Periago MR. The neglected tropical diseases of Latin America and the Caribbean: a review of disease burden and distribution and a roadmap for control and elimination. *PLoS Negl Trop Dis* (2008) **2**(9):e300. doi:10.1371/journal.pntd.0000300
2. Coura JR, Dias JC. Epidemiology, control and surveillance of Chagas disease: 100 years after its discovery. *Mem Inst Oswaldo Cruz* (2009) **104**(Suppl 1):31–40. doi:10.1590/S0074-02762009000900006
3. Martins-Melo FR, Ramos AN Jr, Alencar CH, Heukelbach J. Mortality due to Chagas disease in Brazil from 1979 to 2009: trends and regional differences. *J Infect Dev Ctries* (2012) **6**(11):817–24. doi:10.3855/jidc.2459
4. Brener Z. Biology of *Trypanosoma cruzi*. *Annu Rev Microbiol* (1973) **27**:347–82. doi:10.1146/annurev.mi.27.100173.002023
5. Stevens JR, Noyes HA, Dover GA, Gibson WC. The ancient and divergent origins of the human pathogenic trypanosomes, *Trypanosoma brucei* and *T. cruzi*. *Parasitology* (1999) **118**(Pt 1):107–16. doi:10.1017/S0031182098003473
6. Flores-Lopez CA, Machado CA. Analyses of 32 loci clarify phylogenetic relationships among *Trypanosoma cruzi* lineages and support a single hybridization prior to human contact. *PLoS Negl Trop Dis* (2011) **5**(8):e1272. doi:10.1371/journal.pntd.0001272
7. Zumaya-Estrada FA, Messenger LA, Lopez-Ordonez T, Lewis MD, Flores-Lopez CA, Martinez-Ibarra AJ, et al. North American import? Charting the origins of an enigmatic *Trypanosoma cruzi* domestic genotype. *Parasit Vectors* (2012) **5**:226. doi:10.1186/1756-3305-5-226
8. Hamilton PB, Teixeira MM, Stevens JR. The evolution of *Trypanosoma cruzi*: the 'bat seeding' hypothesis. *Trends Parasitol* (2012) **28**(4):136–41. doi:10.1016/j.pt.2012.01.006
9. Steverding D. The history of Chagas disease. *Parasit Vectors* (2014) **7**:317. doi:10.1186/1756-3305-7-317
10. Aufderheide AC, Salo W, Madden M, Streitz J, Buikstra J, Guhl F, et al. A 9,000-year record of Chagas' disease. *Proc Natl Acad Sci U S A* (2004) **101**(7):2034–9. doi:10.1073/pnas.0307312101
11. Marioni JC, Thorne NP, Valsesia A, Fitzgerald T, Redon R, Fiegler H, et al. Breaking the waves: improved detection of copy number variation from microarray-based comparative genomic hybridization. *Genome Biol* (2007) **8**(10):R228. doi:10.1186/gb-2007-8-10-r228
12. Epting CL, Coates BM, Engman DM. Molecular mechanisms of host cell invasion by *Trypanosoma cruzi*. *Exp Parasitol* (2010) **126**(3):283–91. doi:10.1016/j.exppara.2010.06.023
13. Andrews NW. Living dangerously: how *Trypanosoma cruzi* uses lysosomes to get inside host cells, and then escapes into the cytoplasm. *Biol Res* (1993) **26**(1–2):65–7.
14. De Pablos LM, Osuna A. Multigene families in *Trypanosoma cruzi* and their role in infectivity. *Infect Immun* (2012) **80**(7):2258–64. doi:10.1128/IAI.06225-11

15. Bartholomeu DC, de Paiva RM, Mendes TA, DaRocha WD, Teixeira SM. Unveiling the intracellular survival gene kit of trypanosomatid parasites. *PLoS Pathog* (2014) **10**(12):e1004399. doi:10.1371/journal.ppat.1004399
16. Piacenza L, Peluffo G, Alvarez MN, Kelly JM, Wilkinson SR, Radi R. Peroxiredoxins play a major role in protecting *Trypanosoma cruzi* against macrophage- and endogenously-derived peroxynitrite. *Biochem J* (2008) **410**(2):359–68. doi:10.1042/BJ20071138
17. Norris KA, Bradt B, Cooper NR, So M. Characterization of a *Trypanosoma cruzi* C3 binding protein with functional and genetic similarities to the human complement regulatory protein, decay-accelerating factor. *J Immunol* (1991) **147**(7):2240–7.
18. Ferreira V, Valck C, Sanchez G, Gingras A, Tzima S, Molina MC, et al. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*. *J Immunol* (2004) **172**(5):3042–50. doi:10.4049/jimmunol.172.5.3042
19. Tambourgi DV, Kipnis TL, da Silva WD, Joiner KA, Sher A, Heath S, et al. A partial cDNA clone of trypomastigote decay-accelerating factor (T-DAF), a developmentally regulated complement inhibitor of *Trypanosoma cruzi*, has genetic and functional similarities to the human complement inhibitor DAF. *Infect Immun* (1993) **61**(9):3656–63.
20. Cestari I, Evans-Osses I, Schlapbach LJ, de Messias-Reason I, Ramirez MI. Mechanisms of complement lectin pathway activation and resistance by trypanosomatid parasites. *Mol Immunol* (2013) **53**(4):328–34. doi:10.1016/j.molimm.2012.08.015
21. Minoprio P. Parasite polyclonal activators: new targets for vaccination approaches? *Int J Parasitol* (2001) **31**(5–6):588–91. doi:10.1016/S0020-7519(01)00171-0
22. Bermejo DA, Amezcua Vesely MC, Khan M, Acosta Rodriguez EV, Montes CL, Merino MC, et al. *Trypanosoma cruzi* infection induces a massive extrafollicular and follicular splenic B-cell response which is a high source of non-parasite-specific antibodies. *Immunology* (2011) **132**(1):123–33. doi:10.1111/j.1365-2567.2010.03347.x
23. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* (2004) **5**(2):190–8. doi:10.1038/ni1028
24. Bartholomeu DC, Ropert C, Melo MB, Parroche P, Junqueira CF, Teixeira SM, et al. Recruitment and endo-lysosomal activation of TLR9 in dendritic cells infected with *Trypanosoma cruzi*. *J Immunol* (2008) **181**(2):1333–44. doi:10.4049/jimmunol.181.2.1333
25. Junqueira C, Caetano B, Bartholomeu DC, Melo MB, Ropert C, Rodrigues MM, et al. The endless race between *Trypanosoma cruzi* and host immunity: lessons for and beyond Chagas disease. *Expert Rev Mol Med* (2010) **12**:e29. doi:10.1017/S1462399410001560
26. Rodrigues MM, Alencar BC, Claser C, Tzelepis F. Immunodominance: a new hypothesis to explain parasite escape and host/parasite equilibrium leading to the chronic phase of Chagas' disease? *Braz J Med Biol Res* (2009) **42**(3):220–3.
27. Rosenberg CS, Martin DL, Tarleton RL. CD8+ T cells specific for immunodominant trans-sialidase epitopes contribute to control of *Trypanosoma cruzi* infection but are not required for resistance. *J Immunol* (2010) **185**(1):560–8. doi:10.4049/jimmunol.1000432
28. Padilla AM, Simpson LJ, Tarleton RL. Insufficient TLR activation contributes to the slow development of CD8+ T cell responses in *Trypanosoma cruzi* infection. *J Immunol* (2009) **183**(2):1245–52. doi:10.4049/jimmunol.0901178
29. Tardieux I, Nathanson MH, Andrews NW. Role in host cell invasion of *Trypanosoma cruzi*-induced cytosolic-free Ca2+ transients. *J Exp Med* (1994) **179**(3):1017–22. doi:10.1084/jem.179.3.1017
30. Rodriguez A, Rioult MG, Ora A, Andrews NW. A trypanosome-soluble factor induces IP3 formation, intracellular Ca2+ mobilization and microfilament rearrangement in host cells. *J Cell Biol* (1995) **129**(5):1263–73. doi:10.1083/jcb.129.5.1263
31. Rodriguez A, Samoff E, Rioult MG, Chung A, Andrews NW. Host cell invasion by trypanosomes requires lysosomes and microtubule/kinesin-mediated transport. *J Cell Biol* (1996) **134**(2):349–62. doi:10.1083/jcb.134.2.349
32. Woolsey AM, Sunwoo L, Petersen CA, Brachmann SM, Cantley LC, Burleigh BA. Novel PI 3-kinase-dependent mechanisms of trypanosome invasion and vacuole maturation. *J Cell Sci* (2003) **116**(Pt 17):3611–22. doi:10.1242/jcs.00666
33. Andrade LO, Andrews NW. Lysosomal fusion is essential for the retention of *Trypanosoma cruzi* inside host cells. *J Exp Med* (2004) **200**(9):1135–43. doi:10.1084/jem.20041408
34. Andrade LO, Andrews NW. The *Trypanosoma cruzi*-host-cell interplay: location, invasion, retention. *Nat Rev Microbiol* (2005) **3**(10):819–23. doi:10.1038/nrmicro1249
35. Andrews NW, Abrams CK, Slatin SL, Griffiths G. A *T. cruzi*-secreted protein immunologically related to the complement component C9: evidence for membrane pore-forming activity at low pH. *Cell* (1990) **61**(7):1277–87. doi:10.1016/0092-8674(90)90692-8
36. Manning-Cela R, Cortes A, Gonzalez-Rey E, Van Voorhis WC, Swindle J, Gonzalez A. LYT1 protein is required for efficient in vitro infection by *Trypanosoma cruzi*. *Infect Immun* (2001) **69**(6):3916–23. doi:10.1128/IAI.69.6.3916-3923.2001
37. Kornfeld S, Mellman I. The biogenesis of lysosomes. *Annu Rev Cell Biol* (1989) **5**:483–525. doi:10.1146/annurev.cb.05.110189.002411
38. Hall BF, Webster P, Ma AK, Joiner KA, Andrews NW. Desialylation of lysosomal membrane glycoproteins by *Trypanosoma cruzi*: a role for the surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. *J Exp Med* (1992) **176**(2):313–25. doi:10.1084/jem.176.2.313
39. Albertti LA, Macedo AM, Chiari E, Andrews NW, Andrade LO. Role of host lysosomal associated membrane protein (LAMP) in *Trypanosoma cruzi* invasion and intracellular development. *Microbes Infect* (2010) **12**(10):784–9. doi:10.1016/j.micinf.2010.05.015
40. Rubin-de-Celis SS, Uemura H, Yoshida N, Schenkman S. Expression of trypomastigote trans-sialidase in metacyclic forms of *Trypanosoma cruzi* increases parasite escape from its parasitophorous vacuole. *Cell Microbiol* (2006) **8**(12):1888–98. doi:10.1111/j.1462-5822.2006.00755.x
41. Burleigh BA, Andrews NW. The mechanisms of *Trypanosoma cruzi* invasion of mammalian cells. *Annu Rev Microbiol* (1995) **49**:175–200. doi:10.1146/annurev.mi.49.100195.001135
42. Kierszenbaum F, Knecht E, Budzko DB, Pizzimenti MC. Phagocytosis: a defense mechanism against infection with *Trypanosoma cruzi*. *J Immunol* (1974) **112**(5):1839–44.
43. Piacenza L, Peluffo G, Alvarez MN, Martinez A, Radi R. *Trypanosoma cruzi* antioxidant enzymes as virulence factors in Chagas disease. *Antioxid Redox Signal* (2013) **19**(7):723–34. doi:10.1089/ars.2012.4618
44. Piacenza L, Zago MP, Peluffo G, Alvarez MN, Basombrio MA, Radi R. Enzymes of the antioxidant network as novel determiners of *Trypanosoma cruzi* virulence. *Int J Parasitol* (2009) **39**(13):1455–64. doi:10.1016/j.ijpara.2009.05.010
45. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* (1995) **64**:97–112. doi:10.1146/annurev.bi.64.070195.000525
46. Radi R. Peroxynitrite, a stealthy biological oxidant. *J Biol Chem* (2013) **288**(37):26464–72. doi:10.1074/jbc.R113.472936
47. Gupta S, Wen JJ, Garg NJ. Oxidative stress in Chagas disease. *Interdiscip Perspect Infect Dis* (2009) **2009**:190354. doi:10.1155/2009/190354
48. Munoz-Fernandez MA, Fernandez MA, Fresno M. Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-alpha and IFN-gamma through a nitric oxide-dependent mechanism. *Immunol Lett* (1992) **33**(1):35–40. doi:10.1016/0165-2478(92)90090-B
49. Gutierrez FR, Mineo TW, Pavanelli WR, Guedes PM, Silva JS. The effects of nitric oxide on the immune system during *Trypanosoma cruzi* infection. *Mem Inst Oswaldo Cruz* (2009) **104**(Suppl 1):236–45. doi:10.1590/S0074-02762009000900030
50. Venturini G, Salvati L, Muolo M, Colasanti M, Gradoni L, Ascenzi P. Nitric oxide inhibits cruzipain, the major papain-like cysteine proteinase from *Trypanosoma cruzi*. *Biochem Biophys Res Commun* (2000) **270**(2):437–41. doi:10.1006/bbrc.2000.2447
51. Alvarez MN, Peluffo G, Piacenza L, Radi R. Intraphagosomal peroxynitrite as a macrophage-derived cytotoxin against internalized *Trypanosoma cruzi*: consequences for oxidative killing and role of microbial peroxiredoxins in infectivity. *J Biol Chem* (2011) **286**(8):6627–40. doi:10.1074/jbc.M110.167247
52. Oza SL, Tetaud E, Ariyanayagam MR, Warnon SS, Fairlamb AH. A single enzyme catalyses formation of trypanothione from glutathione and spermidine in *Trypanosoma cruzi*. *J Biol Chem* (2002) **277**(39):35853–61. doi:10.1074/jbc.M204403200
53. Wilkinson SR, Temperton NJ, Mondragon A, Kelly JM. Distinct mitochondrial and cytosolic enzymes mediate trypanothione-dependent peroxide

metabolism in *Trypanosoma cruzi*. *J Biol Chem* (2000) **275**(11):8220–5. doi:10.1074/jbc.275.11.8220
54. Wilkinson SR, Obado SO, Mauricio IL, Kelly JM. *Trypanosoma cruzi* expresses a plant-like ascorbate-dependent hemoperoxidase localized to the endoplasmic reticulum. *Proc Natl Acad Sci U S A* (2002) **99**(21):13453–8. doi:10.1073/pnas.202422899
55. Wilkinson SR, Taylor MC, Touitha S, Mauricio IL, Meyer DJ, Kelly JM. TcGPXII, a glutathione-dependent *Trypanosoma cruzi* peroxidase with substrate specificity restricted to fatty acid and phospholipid hydroperoxides, is localized to the endoplasmic reticulum. *Biochem J* (2002) **364**(Pt 3):787–94. doi:10.1042/bj20020038
56. Wilkinson SR, Meyer DJ, Taylor MC, Bromley EV, Miles MA, Kelly JM. The *Trypanosoma cruzi* enzyme TcGPXI is a glycosomal peroxidase and can be linked to trypanothione reduction by glutathione or tryparedoxin. *J Biol Chem* (2002) **277**(19):17062–71. doi:10.1074/jbc.M111126200
57. Mateo H, Marin C, Perez-Cordon G, Sanchez-Moreno M. Purification and biochemical characterization of four iron superoxide dismutases in *Trypanosoma cruzi*. *Mem Inst Oswaldo Cruz* (2008) **103**(3):271–6. doi:10.1590/S0074-02762008000300008
58. Atwood JA III, Weatherly DB, Minning TA, Bundy B, Cavola C, Opperdoes FR, et al. The *Trypanosoma cruzi* proteome. *Science* (2005) **309**(5733):473–6. doi:10.1126/science.1110289
59. Parodi-Talice A, Monteiro-Goes V, Arrambide N, Avila AR, Duran R, Correa A, et al. Proteomic analysis of metacyclic trypomastigotes undergoing *Trypanosoma cruzi* metacyclogenesis. *J Mass Spectrom* (2007) **42**(11):1422–32. doi:10.1002/jms.1267
60. Piacenza L, Alvarez MN, Peluffo G, Radi R. Fighting the oxidative assault: the *Trypanosoma cruzi* journey to infection. *Curr Opin Microbiol* (2009) **12**(4):415–21. doi:10.1016/j.mib.2009.06.011
61. Alvarez MN, Piacenza L, Irigoin F, Peluffo G, Radi R. Macrophage-derived peroxynitrite diffusion and toxicity to *Trypanosoma cruzi*. *Arch Biochem Biophys* (2004) **432**(2):222–32. doi:10.1016/j.abb.2004.09.015
62. Paiva CN, Feijo DF, Dutra FF, Carneiro VC, Freitas GB, Alves LS, et al. Oxidative stress fuels *Trypanosoma cruzi* infection in mice. *J Clin Invest* (2012) **122**(7):2531–42. doi:10.1172/JCI58525
63. Kaplan J. Mechanisms of cellular iron acquisition: another iron in the fire. *Cell* (2002) **111**(5):603–6. doi:10.1016/S0092-8674(02)01164-9
64. Marro S, Chiabrando D, Messana E, Stolte J, Turco E, Tolosano E, et al. Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. *Haematologica* (2010) **95**(8):1261–8. doi:10.3324/haematol.2009.020123
65. Hintze KJ, Theil EC. DNA and mRNA elements with complementary responses to hemin, antioxidant inducers, and iron control ferritin-L expression. *Proc Natl Acad Sci U S A* (2005) **102**(42):15048–52. doi:10.1073/pnas.0505148102
66. Dhiman M, Garg NJ. NADPH oxidase inhibition ameliorates *Trypanosoma cruzi*-induced myocarditis during Chagas disease. *J Pathol* (2011) **225**(4):583–96. doi:10.1002/path.2975
67. Gazzinelli RT, Denkers EY. Protozoan encounters with toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol* (2006) **6**(12):895–906. doi:10.1038/nri1978
68. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* (2010) **140**(6):805–20. doi:10.1016/j.cell.2010.01.022
69. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* (2006) **124**(4):783–801. doi:10.1016/j.cell.2006.02.015
70. Kulkarni R, Behboudi S, Sharif S. Insights into the role of toll-like receptors in modulation of T cell responses. *Cell Tissue Res* (2011) **343**(1):141–52. doi:10.1007/s00441-010-1017-1
71. Stahl P, Ruppert V, Meyer T, Schmidt J, Campos MA, Gazzinelli RT, et al. Trypomastigotes and amastigotes of *Trypanosoma cruzi* induce apoptosis and STAT3 activation in cardiomyocytes in vitro. *Apoptosis* (2013) **18**(6):653–63. doi:10.1007/s10495-013-0822-x
72. Gazzinelli RT, Ropert C, Campos MA. Role of the toll/interleukin-1 receptor signaling pathway in host resistance and pathogenesis during infection with protozoan parasites. *Immunol Rev* (2004) **201**:9–25. doi:10.1111/j.0105-2896.2004.00174.x
73. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol* (2010) **11**(5):373–84. doi:10.1038/ni.1863
74. Rodrigues MM, Oliveira AC, Bellio M. The immune response to *Trypanosoma cruzi*: role of toll-like receptors and perspectives for vaccine development. *J Parasitol Res* (2012) **2012**:507874. doi:10.1155/2012/507874
75. Campos MA, Closel M, Valente EP, Cardoso JE, Akira S, Alvarez-Leite JI, et al. Impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeloid differentiation factor 88. *J Immunol* (2004) **172**(3):1711–8. doi:10.4049/jimmunol.172.3.1711
76. Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, Golenbock DT, et al. Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with *T. cruzi*. *J Immunol* (2004) **173**(9):5688–96. doi:10.4049/jimmunol.173.9.5688
77. Caetano BC, Carmo BB, Melo MB, Cerny A, dos Santos SL, Bartholomeu DC, et al. Requirement of UNC93B1 reveals a critical role for TLR7 in host resistance to primary infection with *Trypanosoma cruzi*. *J Immunol* (2011) **187**(4):1903–11. doi:10.4049/jimmunol.1003911
78. Gravina HD, Antonelli L, Gazzinelli RT, Ropert C. Differential use of TLR2 and TLR9 in the regulation of immune responses during the infection with *Trypanosoma cruzi*. *PLoS One* (2013) **8**(5):e63100. doi:10.1371/journal.pone.0063100
79. Serrano AA, Schenkman S, Yoshida N, Mehlert A, Richardson JM, Ferguson MA. The lipid structure of the glycosylphosphatidylinositol-anchored mucin-like sialic acid acceptors of *Trypanosoma cruzi* changes during parasite differentiation from epimastigotes to infective metacyclic trypomastigote forms. *J Biol Chem* (1995) **270**(45):27244–53. doi:10.1074/jbc.270.45.27244
80. Buscaglia CA, Campo VA, Frasch ACC, Di Noia JM. *Trypanosoma cruzi* surface mucins: host-dependent coat diversity. *Nat Rev Microbiol* (2006) **4**(3):229–36. doi:10.1038/nrmicro1351
81. Coelho PS, Klein A, Talvani A, Coutinho SF, Takeuchi O, Akira S, et al. Glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from *Trypanosoma cruzi* trypomastigotes induce in vivo leukocyte recruitment dependent on MCP-1 production by IFN-gamma-primed-macrophages. *J Leukoc Biol* (2002) **71**(5):837–44.
82. Tarleton RL. CD8+ T cells in *Trypanosoma cruzi* infection. *Semin Immunopathol* (2015) **37**(3):233–8. doi:10.1007/s00281-015-0481-9
83. Lederkremer RM, Bertello LE. Glycoinositolphospholipids, free and as anchors of proteins, in *Trypanosoma cruzi*. *Curr Pharm Des* (2001) **7**(12):1165–79. doi:10.2174/1381612013397519
84. Previato JO, Wait R, Jones C, DosReis GA, Todeschini AR, Heise N, et al. Glycoinositolphospholipid from *Trypanosoma cruzi*: structure, biosynthesis and immunobiology. *Adv Parasitol* (2004) **56**:1–41. doi:10.1016/S0065-308X(03)56001-8
85. Almeida IC, Gazzinelli RT. Proinflammatory activity of glycosylphosphatidylinositol anchors derived from *Trypanosoma cruzi*: structural and functional analyses. *J Leukoc Biol* (2001) **70**(4):467–77.
86. Carreira JC, Jones C, Wait R, Previato JO, Mendonca-Previato L. Structural variation in the glycoinositolphospholipids of different strains of *Trypanosoma cruzi*. *Glycoconj J* (1996) **13**(6):955–66. doi:10.1007/BF01053191
87. de Lederkremer RM, Agusti R. Glycobiology of *Trypanosoma cruzi*. *Adv Carbohydr Chem Biochem* (2009) **62**:311–66. doi:10.1016/S0065-2318(09)00007-9
88. de Lederkremer RM, Lima CE, Ramirez MI, Goncalvez MF, Colli W. Hexadecylpalmitoylglycerol or ceramide is linked to similar glycophosphoinositol anchor-like structures in *Trypanosoma cruzi*. *Eur J Biochem* (1993) **218**(3):929–36. doi:10.1111/j.1432-1033.1993.tb18449.x
89. Agusti R, Couto AS, Campetella OE, Frasch AC, de Lederkremer RM. The trans-sialidase of *Trypanosoma cruzi* is anchored by two different lipids. *Glycobiology* (1997) **7**(6):731–5. doi:10.1093/glycob/7.6.731
90. Salto ML, Bertello LE, Vieira M, Docampo R, Moreno SN, de Lederkremer RM. Formation and remodeling of inositolphosphoceramide during differentiation of *Trypanosoma cruzi* from trypomastigote to amastigote. *Eukaryot Cell* (2003) **2**(4):756–68. doi:10.1128/EC.2.4.756-768.2003
91. Freire-de-Lima CG, Nunes MP, Corte-Real S, Soares MP, Previato JO, Mendonca-Previato L, et al. Proapoptotic activity of a *Trypanosoma cruzi* ceramide-containing glycolipid turned on in host macrophages by IFN-gamma. *J Immunol* (1998) **161**(9):4909–16.
92. Brodskyn C, Patricio J, Oliveira R, Lobo L, Arnholdt A, Mendonca-Previato L, et al. Glycoinositolphospholipids from *Trypanosoma cruzi* interfere with

macrophages and dendritic cell responses. *Infect Immun* (2002) **70**(7):3736–43. doi:10.1128/IAI.70.7.3736-3743.2002
93. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procopio DO, et al. Activation of toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J Immunol* (2001) **167**(1):416–23. doi:10.4049/jimmunol.167.1.416
94. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A toll-like receptor recognizes bacterial DNA. *Nature* (2000) **408**(6813):740–5. doi:10.1038/35047123
95. Latz E, Visintin A, Espevik T, Golenbock DT. Mechanisms of TLR9 activation. *J Endotoxin Res* (2004) **10**(6):406–12. doi:10.1179/096805104225006525
96. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. *J Immunol* (2006) **177**(6):3515–9. doi:10.4049/jimmunol.177.6.3515
97. Geddes K, Magalhaes JG, Girardin SE. Unleashing the therapeutic potential of NOD-like receptors. *Nat Rev Drug Discov* (2009) **8**(6):465–79. doi:10.1038/nrd2783
98. Silva GK, Gutierrez FR, Guedes PM, Horta CV, Cunha LD, Mineo TW, et al. Cutting edge: nucleotide-binding oligomerization domain 1-dependent responses account for murine resistance against *Trypanosoma cruzi* infection. *J Immunol* (2010) **184**(3):1148–52. doi:10.4049/jimmunol.0902254
99. Cestari Idos S, Krarup A, Sim RB, Inal JM, Ramirez MI. Role of early lectin pathway activation in the complement-mediated killing of *Trypanosoma cruzi*. *Mol Immunol* (2009) **47**(2–3):426–37. doi:10.1016/j.molimm.2009.08.030
100. Cestari I, Ramirez MI. Inefficient complement system clearance of *Trypanosoma cruzi* metacyclic trypomastigotes enables resistant strains to invade eukaryotic cells. *PLoS One* (2010) **5**(3):e9721. doi:10.1371/journal.pone.0009721
101. Osorio L, Rios I, Gutierrez B, Gonzalez J. Virulence factors of *Trypanosoma cruzi*: who is who? *Microbes Infect* (2012) **14**(15):1390–402. doi:10.1016/j.micinf.2012.09.003
102. Aguillon JC, Ferreira L, Perez C, Colombo A, Molina MC, Wallace A, et al. Tc45, a dimorphic *Trypanosoma cruzi* immunogen with variable chromosomal localization, is calreticulin. *Am J Trop Med Hyg* (2000) **63**(5–6):306–12.
103. Sosoniuk E, Vallejos G, Kenawy H, Gaboriaud C, Thielens N, Fujita T, et al. *Trypanosoma cruzi* calreticulin inhibits the complement lectin pathway activation by direct interaction with L-ficolin. *Mol Immunol* (2014) **60**(1):80–5. doi:10.1016/j.molimm.2014.03.014
104. Valck C, Ramirez G, Lopez N, Ribeiro CH, Maldonado I, Sanchez G, et al. Molecular mechanisms involved in the inactivation of the first component of human complement by *Trypanosoma cruzi* calreticulin. *Mol Immunol* (2010) **47**(7–8):1516–21. doi:10.1016/j.molimm.2010.01.019
105. Ramirez G, Valck C, Molina MC, Ribeiro CH, Lopez N, Sanchez G, et al. *Trypanosoma cruzi* calreticulin: a novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity. *Immunobiology* (2011) **216**(1–2):265–73. doi:10.1016/j.imbio.2010.04.001
106. Norris KA. Stable transfection of *Trypanosoma cruzi* epimastigotes with the trypomastigote-specific complement regulatory protein cDNA confers complement resistance. *Infect Immun* (1998) **66**(6):2460–5.
107. Beucher M, Norris KA. Sequence diversity of the *Trypanosoma cruzi* complement regulatory protein family. *Infect Immun* (2008) **76**(2):750–8. doi:10.1128/IAI.01104-07
108. Cestari Idos S, Evans-Osses I, Freitas JC, Inal JM, Ramirez MI. Complement C2 receptor inhibitor trispanning confers an increased ability to resist complement-mediated lysis in *Trypanosoma cruzi*. *J Infect Dis* (2008) **198**(9):1276–83. doi:10.1086/592167
109. Joiner KA, daSilva WD, Rimoldi MT, Hammer CH, Sher A, Kipnis TL. Biochemical characterization of a factor produced by trypomastigotes of *Trypanosoma cruzi* that accelerates the decay of complement C3 convertases. *J Biol Chem* (1988) **263**(23):11327–35.
110. Fischer E, Ouaissi MA, Velge P, Cornette J, Kazatchkine MD. gp 58/68, a parasite component that contributes to the escape of the trypomastigote form of *T. cruzi* from damage by the human alternative complement pathway. *Immunology* (1988) **65**(2):299–303.
111. Cestari I, Ansa-Addo E, Deolindo P, Inal JM, Ramirez MI. *Trypanosoma cruzi* immune evasion mediated by host cell-derived microvesicles. *J Immunol* (2012) **188**(4):1942–52. doi:10.4049/jimmunol.1102053
112. van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. *Cell Microbiol* (2007) **9**(9):2095–102. doi:10.1111/j.1462-5822.2007.00981.x
113. Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, et al. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med* (2000) **6**(7):769–75. doi:10.1038/77498
114. Gasser O, Hess C, Miot S, Deon C, Sanchez JC, Schifferli JA. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res* (2003) **285**(2):243–57. doi:10.1016/S0014-4827(03)00055-7
115. Thery C. Exosomes: secreted vesicles and intercellular communications. *F1000 Biology Rep* (2011) **3**:15. doi:10.3410/B3-15
116. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* (2009) **9**(8):581–93. doi:10.1038/nri2567
117. Torrecilhas AC, Schumacher RI, Alves MJ, Colli W. Vesicles as carriers of virulence factors in parasitic protozoan diseases. *Microbes Infect* (2012) **14**(15):1465–74. doi:10.1016/j.micinf.2012.07.008
118. Barteneva NS, Maltsev N, Vorobjev IA. Microvesicles and intercellular communication in the context of parasitism. *Front Cell Infect Microbiol* (2013) **3**:49. doi:10.3389/fcimb.2013.00049
119. Garcia-Silva MR, Cabrera-Cabrera F, das Neves RF, Souto-Padron T, de Souza W, Cayota A. Gene expression changes induced by *Trypanosoma cruzi* shed microvesicles in mammalian host cells: relevance of tRNA-derived halves. *Biomed Res Int* (2014) **2014**:305239. doi:10.1155/2014/305239
120. Barteneva NS, Fasler-Kan E, Bernimoulin M, Stern JN, Ponomarev ED, Duckett L, et al. Circulating microparticles: square the circle. *BMC Cell Biol* (2013) **14**:23. doi:10.1186/1471-2121-14-23
121. Trocoli Torrecilhas AC, Tonelli RR, Pavanelli WR, da Silva JS, Schumacher RI, de Souza W, et al. *Trypanosoma cruzi*: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. *Microbes Infect* (2009) **11**(1):29–39. doi:10.1016/j.micinf.2008.10.003
122. Vaena de Avalos S, Blader IJ, Fisher M, Boothroyd JC, Burleigh BA. Immediate/early response to *Trypanosoma cruzi* infection involves minimal modulation of host cell transcription. *J Biol Chem* (2002) **277**(1):639–44. doi:10.1074/jbc.M109037200
123. Tarleton RL. Immune system recognition of *Trypanosoma cruzi*. *Curr Opin Immunol* (2007) **19**(4):430–4. doi:10.1016/j.coi.2007.06.003
124. Kurup SP, Tarleton RL. Perpetual expression of PAMPs necessary for optimal immune control and clearance of a persistent pathogen. *Nat Commun* (2013) **4**:2616. doi:10.1038/ncomms3616
125. Higuchi Mde L, De Brito T, Martins Reis M, Barbosa A, Bellotti G, Pereira-Barreto AC, et al. Correlation between *Trypanosoma cruzi* parasitism and myocardial inflammatory infiltrate in human chronic chagasic myocarditis: light microscopy and immunohistochemical findings. *Cardiovasc Pathol* (1993) **2**(2):101–6. doi:10.1016/1054-8807(93)90021-S
126. Jones EM, Colley DG, Tostes S, Lopes ER, Vnencak-Jones CL, McCurley TL. Amplification of a *Trypanosoma cruzi* DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. *Am J Trop Med Hyg* (1993) **48**(3):348–57.
127. Gutierrez FR, Guedes PM, Gazzinelli RT, Silva JS. The role of parasite persistence in pathogenesis of Chagas heart disease. *Parasite Immunol* (2009) **31**(11):673–85. doi:10.1111/j.1365-3024.2009.01108.x
128. Vago AR, Macedo AM, Adad SJ, Reis DD, Correa-Oliveira R. PCR detection of *Trypanosoma cruzi* DNA in oesophageal tissues of patients with chronic digestive Chagas' disease. *Lancet* (1996) **348**(9031):891–2. doi:10.1016/S0140-6736(05)64761-7
129. Bartholomeu DC, Cerqueira GC, Leao AC, daRocha WD, Pais FS, Macedo C, et al. Genomic organization and expression profile of the mucin-associated surface protein (MASP) family of the human pathogen *Trypanosoma cruzi*. *Nucleic Acids Res* (2009) **37**(10):3407–17. doi:10.1093/nar/gkp172
130. dos Santos SL, Freitas LM, Lobo FP, Rodrigues-Luiz GF, Mendes TA, Oliveira AC, et al. The MASP family of *Trypanosoma cruzi*: changes in gene expression and antigenic profile during the acute phase of experimental infection. *PLoS Negl Trop Dis* (2012) **6**(8):e1779. doi:10.1371/journal.pntd.0001779
131. Borst P. Antigenic variation and allelic exclusion. *Cell* (2002) **109**(1):5–8. doi:10.1016/S0092-8674(02)00711-0
132. Prucca CG, Slavin I, Quiroga R, Elias EV, Rivero FD, Saura A, et al. Antigenic variation in *Giardia lamblia* is regulated by RNA interference. *Nature* (2008) **456**(7223):750–4. doi:10.1038/nature07585

133. McCulloch R, Horn D. What has DNA sequencing revealed about the VSG expression sites of African trypanosomes? *Trends Parasitol* (2009) **25**(8):359–63. doi:10.1016/j.pt.2009.05.007
134. Prucca CG, Lujan HD. Antigenic variation in *Giardia lamblia*. *Cell Microbiol* (2009) **11**(12):1706–15. doi:10.1111/j.1462-5822.2009.01367.x
135. Prucca CG, Rivero FD, Lujan HD. Regulation of antigenic variation in *Giardia lamblia*. *Annu Rev Microbiol* (2011) **65**:611–30. doi:10.1146/annurev-micro-090110-102940
136. Horn D, McCulloch R. Molecular mechanisms underlying the control of antigenic variation in African trypanosomes. *Curr Opin Microbiol* (2010) **13**(6):700–5. doi:10.1016/j.mib.2010.08.009
137. Borst P, Ulbert S. Control of VSG gene expression sites. *Mol Biochem Parasitol* (2001) **114**(1):17–27. doi:10.1016/S0166-6851(01)00243-2
138. Pays E, Vanhamme L, Perez-Morga D. Antigenic variation in *Trypanosoma brucei*: facts, challenges and mysteries. *Curr Opin Microbiol* (2004) **7**(4):369–74. doi:10.1016/j.mib.2004.05.001
139. Guizetti J, Scherf A. Silence, activate, poise and switch! Mechanisms of antigenic variation in *Plasmodium falciparum*. *Cell Microbiol* (2013) **15**(5):718–26. doi:10.1111/cmi.12115
140. Pitcovsky TA, Buscaglia CA, Mucci J, Campetella O. A functional network of intramolecular cross-reacting epitopes delays the elicitation of neutralizing antibodies to *Trypanosoma cruzi* trans-sialidase. *J Infect Dis* (2002) **186**(3):397–404. doi:10.1086/341463
141. Pitcovsky TA, Mucci J, Alvarez P, Leguizamon MS, Burrone O, Alzari PM, et al. Epitope mapping of trans-sialidase from *Trypanosoma cruzi* reveals the presence of several cross-reactive determinants. *Infect Immun* (2001) **69**(3):1869–75. doi:10.1128/IAI.69.3.1869-1875.2001
142. Minoprio P, Burlen O, Pereira P, Guilbert B, Andrade L, Hontebeyrie-Joskowicz M, et al. Most B cells in acute *Trypanosoma cruzi* infection lack parasite specificity. *Scand J Immunol* (1988) **28**(5):553–61. doi:10.1111/j.1365-3083.1988.tb01487.x
143. Reina-San-Martin B, Cosson A, Minoprio P. Lymphocyte polyclonal activation: a pitfall for vaccine design against infectious agents. *Parasitol Today* (2000) **16**(2):62–7. doi:10.1016/S0169-4758(99)01591-4
144. Spinella S, Liegeard P, Hontebeyrie-Joskowicz M. *Trypanosoma cruzi*: predominance of IgG2a in nonspecific humoral response during experimental Chagas' disease. *Exp Parasitol* (1992) **74**(1):46–56. doi:10.1016/0014-4894(92)90138-Z
145. Reina-San-Martin B, Degrave W, Rougeot C, Cosson A, Chamond N, Cordeiro-Da-Silva A, et al. A B-cell mitogen from a pathogenic trypanosome is a eukaryotic proline racemase. *Nat Med* (2000) **6**(8):890–7. doi:10.1038/78651
146. Gao W, Wortis HH, Pereira MA. The *Trypanosoma cruzi* trans-sialidase is a T cell-independent B cell mitogen and an inducer of non-specific Ig secretion. *Int Immunol* (2002) **14**(3):299–308. doi:10.1093/intimm/14.3.299
147. Bryan MA, Guyach SE, Norris KA. Specific humoral immunity versus polyclonal B cell activation in *Trypanosoma cruzi* infection of susceptible and resistant mice. *PLoS Negl Trop Dis* (2010) **4**(7):e733. doi:10.1371/journal.pntd.0000733
148. El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* (2005) **309**(5733):409–15. doi:10.1126/science.1112631
149. Franzen O, Ochaya S, Sherwood E, Lewis MD, Llewellyn MS, Miles MA, et al. Shotgun sequencing analysis of *Trypanosoma cruzi* I Sylvio X10/1 and comparison with *T. cruzi* VI CL Brener. *PLoS Negl Trop Dis* (2011) **5**(3):e984. doi:10.1371/journal.pntd.0000984
150. Tarleton RL. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. *J Immunol* (1990) **144**(2):717–24.
151. Tarleton RL, Koller BH, Latour A, Postan M. Susceptibility of beta 2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. *Nature* (1992) **356**(6367):338–40. doi:10.1038/356338a0
152. Martin DL, Weatherly DB, Laucella SA, Cabinian MA, Crim MT, Sullivan S, et al. CD8+ T-Cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. *PLoS Pathog* (2006) **2**(8):e77. doi:10.1371/journal.ppat.0020077
153. Alvarez MG, Postan M, Weatherly DB, Albareda MC, Sidney J, Sette A, et al. HLA class I-T cell epitopes from trans-sialidase proteins reveal functionally distinct subsets of CD8+ T cells in chronic Chagas disease. *PLoS Negl Trop Dis* (2008) **2**(9):e288. doi:10.1371/journal.pntd.0000288
154. Tarleton RL. New approaches in vaccine development for parasitic infections. *Cell Microbiol* (2005) **7**(10):1379–86. doi:10.1111/j.1462-5822.2005.00589.x
155. Thomas MC, Fernandez-Villegas A, Carrilero B, Maranon C, Saura D, Noya O, et al. Characterization of an immunodominant antigenic epitope from *Trypanosoma cruzi* as a biomarker of chronic Chagas' disease pathology. *Clin Vaccine Immunol* (2012) **19**(2):167–73. doi:10.1128/CVI.05566-11
156. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol* (1999) **17**:51–88. doi:10.1146/annurev.immunol.17.1.51
157. Chen W, Anton LC, Bennink JR, Yewdell JW. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. *Immunity* (2000) **12**(1):83–93. doi:10.1016/S1074-7613(00)80161-2
158. Goto Y, Carter D, Reed SG. Immunological dominance of *Trypanosoma cruzi* tandem repeat proteins. *Infect Immun* (2008) **76**(9):3967–74. doi:10.1128/IAI.00604-08
159. Wizel B, Garg N, Tarleton RL. Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* infection. *Infect Immun* (1998) **66**(11):5073–81.
160. Wizel B, Nunes M, Tarleton RL. Identification of *Trypanosoma cruzi* trans-sialidase family members as targets of protective CD8+ TC1 responses. *J Immunol* (1997) **159**(12):6120–30.
161. Hoft DF, Eickhoff CS, Giddings OK, Vasconcelos JR, Rodrigues MM. Trans-sialidase recombinant protein mixed with CpG motif-containing oligodeoxynucleotide induces protective mucosal and systemic *Trypanosoma cruzi* immunity involving CD8+ CTL and B cell-mediated cross-priming. *J Immunol* (2007) **179**(10):6889–900. doi:10.4049/jimmunol.179.10.6889
162. Dominguez MR, Silveira EL, de Vasconcelos JR, de Alencar BC, Machado AV, Bruna-Romero O, et al. Subdominant/cryptic CD8 T cell epitopes contribute to resistance against experimental infection with a human protozoan parasite. *PLoS One* (2011) **6**(7):e22011. doi:10.1371/journal.pone.0022011
163. Freitas LM, dos Santos SL, Rodrigues-Luiz GF, Mendes TA, Rodrigues TS, Gazzinelli RT, et al. Genomic analyses, gene expression and antigenic profile of the trans-sialidase superfamily of *Trypanosoma cruzi* reveal an undetected level of complexity. *PLoS One* (2011) **6**(10):e25914. doi:10.1371/journal.pone.0025914
164. Tzelepis F, de Alencar BC, Penido ML, Claser C, Machado AV, Bruna-Romero O, et al. Infection with *Trypanosoma cruzi* restricts the repertoire of parasite-specific CD8+ T cells leading to immunodominance. *J Immunol* (2008) **180**(3):1737–48. doi:10.4049/jimmunol.180.3.1737
165. Miyahira Y. *Trypanosoma cruzi* infection from the view of CD8+ T cell immunity – an infection model for developing T cell vaccine. *Parasitol Int* (2008) **57**(1):38–48. doi:10.1016/j.parint.2007.07.005
166. Guhl F, Jaramillo C, Vallejo GA, Yockteng R, Cardenas-Arroyo F, Fornaciari G, et al. Isolation of *Trypanosoma cruzi* DNA in 4,000-year-old mummified human tissue from northern Chile. *Am J Phys Anthropol* (1999) **108**(4):401–7. doi:10.1002/(SICI)1096-8644(199904)108:4<401::AID-AJPA2>3.3.CO;2-G
167. Kawashita SY, Sanson GF, Fernandes O, Zingales B, Briones MR. Maximum-likelihood divergence date estimates based on rRNA gene sequences suggest two scenarios of *Trypanosoma cruzi* intraspecific evolution. *Mol Biol Evol* (2001) **18**(12):2250–9. doi:10.1093/oxfordjournals.molbev.a003771
168. Perlowagora-Szumlewicz A, Muller CA, Moreira CJ. Studies in search of a suitable experimental insect model for xenodiagnosis of hosts with Chagas' disease. 4 – the reflection of parasite stock in the responsiveness of different vector species to chronic infection with different *Trypanosoma cruzi* stocks. *Rev Saude Publica* (1990) **24**(3):165–77. doi:10.1590/S0034-89101990000300002

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REVIEW
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Major Histocompatibility Complex and Malaria: Focus on *Plasmodium vivax* Infection

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The importance of host and parasite genetic factors in malaria resistance or susceptibility has been investigated since the middle of the last century. Nowadays, of all diseases that affect man, malaria still plays one of the highest levels of selective pressure on human genome. Susceptibility to malaria depends on exposure profile, epidemiological characteristics, and several components of the innate and adaptive immune system that influences the quality of the immune response generated during the *Plasmodium* lifecycle in the vertebrate host. But it is well known that the parasite's enormous capacity of genetic variation in conjunction with the host genetics polymorphism is also associated with a wide spectrum of susceptibility degrees to complicated or severe forms of the disease. In this scenario, variations in genes of the major histocompatibility complex (MHC) associated with host resistance or susceptibility to malaria have been identified and used as markers in host–pathogen interaction studies, mainly those evaluating the impact on the immune response, acquisition of resistance, or increased susceptibility to infection or vulnerability to disease. However, due to the intense selective pressure, number of cases, and mortality rates, the majority of the reported associations reported concerned *Plasmodium falciparum* malaria. Studies on the MHC polymorphism and its association with *Plasmodium vivax*, which is the most widespread *Plasmodium* and the most prevalent species outside the African continent, are less frequent but equally important. Despite punctual contributions, there are accumulated evidences of human genetic control in *P. vivax* infection and disease. Herein, we review the current knowledge in the field of MHC and derived molecules (HLA Class I, Class II, TNF-α, LTA, BAT1, and CTL4) regarding *P. vivax* malaria. We discuss particularly the results of *P. vivax* studies on HLA class I and II polymorphisms in relation to host susceptibility, naturally acquired immune response against specific antigens and the implication of this knowledge to overcome the parasite immune evasion. Finally, the potential impact of such polymorphisms on the development of vaccine candidate antigens against *P. vivax* will be studied.

Keywords: malaria, *P.vivax*, MHC, HLA, vaccine

INTRODUCTION

Caused by blood-borne apicomplexan parasites of the genus *Plasmodium*, malaria remains a major public health problem. Malaria transmission occurs in 96 countries and territories, and according to the latest estimates, 3.3 billion people are at risk of infection. Approximately 214 million cases and 438,000 deaths due to malaria occur worldwide, mainly of children under 5 years. The great majority of cases (88%) and deaths (90%) occurs in Africa, followed by Southeast Asia (10%) and Eastern Mediterranean region (2%) (1). Of the five *Plasmodium* species that affect humans, *Plasmodium vivax* is responsible for about 6% of the world estimated cases. However, outside sub-Saharan Africa, *P. vivax* accounts for 51% of all malaria cases, being the most widespread *Plasmodium* species (1).

Plasmodium parasites have a complex lifecycle, which includes the development of a sexual cycle in the invertebrate vector, the female of the *Anopheles* mosquitos, and an asexual cycle in the vertebrate hosts. Infection with *Plasmodium* parasites presents an asymptomatic stage, pre-erythrocytic, which occurs in the liver followed by a symptomatic erythrocytic stage, when merozoites arisen during pre-erythrocytic stage invade red blood cells. The rupture of the erythrocytic schizont is typically accompanied by clinical symptoms, because of the release of parasite derived toxins, such as phospholipids, that can activate immune cells leading to the production of inflammatory cytokines that can, directly or indirectly, contribute to the elimination of the parasite and complications associated with infection.

At first, humans are susceptible to malaria, even those who have already contracted the disease several times. However, young children, pregnant women, and adults from non-endemic areas are particularly susceptible to develop severe malaria. In high endemic malaria areas, with repeated exposure, older children and adults develop considerable degree of protection from death and severe malaria, and thus, the clinical manifestations are milder, or even absent, although sterile immunity is probably never achieved and the infected immune individuals continue to present parasites in the blood for long periods, probably in the presence of very mild symptomatology (2, 3). It has been proposed that these changes reflect the parasitological and clinical immunity collectively referred to as naturally acquired immunity, which generally determines not only the age-specific incidence and prevalence of infections but also the expression of pathological processes that underlie the clinical manifestations of infection.

The spectrum of malaria clinical manifestations generally differs between adults and children and from person to person, ranging from asymptomatic infection to clinical symptoms as fever, nausea, headache, and muscle pain, chills and vomiting and, in 1–2% of the cases, to severe malaria, leading to multiorgan system involvement, severe anemia, and death (4–6). *Plasmodium falciparum* is the most virulent agent and responsible for the majority of severe malaria deaths (1). Severe malaria due to *P. falciparum* may present as confusion, drowsiness, excitement, convulsions, delirium, and coma. The differences observed in the clinical forms of the disease as well as the underlying pathophysiological processes are still under investigation, but it is now clear that the genetic factors influence the spectrum of clinical manifestations and the evolution and severity of the disease (7, 8).

The classical framework of the influence of genetic factors in malaria evolution and severity is the protective effect of certain hemoglobinopathies. The first observations were postulated in the late 40s by Haldane, known as one of the three founders of population genetics and acknowledged as the first person to suggest that disease could be an important evolutionary force in humans (9). Based on the distribution of thalassemia in the Mediterranean, Haldane proposed that certain hemoglobinopathies are highly prevalent in regions where malaria is endemic due to the protection against the severe disease (10). According to the Haldane's malaria hypothesis, this could result in a "balanced polymorphism" where the homozygote disadvantage for inherited erythrocyte disease is compensated through the resistance of the heterozygote where malaria is endemic (11–14). Thus, it has been proposed that malaria is associated to gene selective pressure in the human genome, and it has been associated with some genetic diseases. After that, several reports have shown that genetic disorders, such as thalassemias, sickle-cell trait, glucose-6-phosphate dehydrogenase (G6PD) deficiency, ovalocytosis, Hemoglobin (Hb) S, HbC, HbE, and complement receptor-1 (CR1) deficiency, are associated with malaria susceptibility or resistance. Case–control studies have shown that these polymorphisms reduce the risk of severe and complicated malaria. Among the mechanisms involved in the protection against *P. falciparum* severe malaria are reduced invasion of erythrocyte by the parasite, decreased intracellular parasite growth, increased phagocytosis, and enhanced immune response against parasite-infected erythrocyte (14–17). Besides these genetic disorders, other polymorphisms in genes encoding the immune system molecules may also be involved in malaria outcome (**Table 1**).

Considering the intense selective pressure, the number of cases and the mortality rates associated with *P. falciparum* infection, specific studies of association between genetic factors and *P. vivax* are less frequent, even though this species is the most widespread *Plasmodium*, the most prevalent species outside the African continent, and with increasing evidences of associated death (57, 58). The observation that *P. vivax* malaria is rare in West Africa and that most sub-Saharan Africans are negative to blood group Duffy was the first evidence regarding *P. vivax* natural resistance. It led to the discovery that *P. vivax* uses the Duffy blood group antigen as a receptor to invade erythrocytes (59). Populations with the null phenotype of Duffy, although susceptible to the hepatic malaria stage, are less susceptible to *P. vivax* merozoite invasion. Moreover, there are accumulated evidences of the relationship between immune response to *P. vivax* antigens and major histocompatibility complex (MHC) genes. Therefore, in the present study, we review the current knowledge in the field of MHC molecules regarding *P. vivax* malaria.

P. vivax MALARIA AND THE IMMUNE SYSTEM

Like in other species of the *Plasmodium* genus, *P. vivax* lifecycle is a complex process and requires an invertebrate and

TABLE 1 | Genetic polymorphisms of the vertebrate host and associations with the natural resistance to malaria.

Genetically based resistance mechanisms		Gene/locus	Function	Phenotype	Reference
Hemoglobinopathies	α-Thalassemia	HBA (16p13.3)	Hemoglobin composition	Protection against severe malaria	(18–20)
	β-Thalassemia	HBB (11p15.5)	Hemoglobin composition	Protection against severe malaria	(11, 21)
	Sickle cell disease	HBB (11p15.5)	Hemoglobin composition	Protection against severe malaria	(19, 22, 23)
	Hemoglobin C (HbC)	HBB (11p15.5)	Hemoglobin composition	Reduced risk of severe and non-severe *P. falciparum* infections	(24, 25)
	Hemoglobin E (HbE)	HBB (11p15.5)	Hemoglobin composition	Protection against severe malaria and high parasitemia	(26, 27)
Enzymes	Glucose-6-phosphate dehydrogenase (G6PD)	G6PD (Xq28)	Protection of erythrocyte against oxidative stress	Resistance against *P. falciparum* infection and severe malaria	(16, 28–30)
	Pyruvate kinase (PK)	PKLR (1q21)	Erythrocyte metabolism	Protection against *P. falciparum* infection	(31)
Erythrocyte	Ovalocytosis	SLC4A1 (17q21-22)	Anion exchanger	Protection against severe malaria by *P. falciparum* and reduced risk of *P. vivax* infection	(32–34)
	Duffy antigen	ACKR1/FY (1q21-q22)	Chemokine receptor	Decreased risk/resistance of *P. vivax* infection	(35–38)
Immunogenetic variants	Human leukocyte antigens (HLA)	HLA (6p21.3)	Component of the immune system	Protection against severe malaria and antiplasmodial immune response	(39–44)
	Complement component (3b/4b) receptor 1 (CR1)	CR1 (1q32)	Removing immune complexes/cytoadherence	Protection against severe malaria	(45)
	Nitric oxide synthase 2 r	NOS2A (17q11.2)	Nitric oxide production	Protection against severe malaria	(46, 47)
	Tumor necrosis factor (TNF)	TNF (6p21.3)	Proinflamatory activities	Severe malaria	(48–51)
	Interferon gamma (IFN)	IFNG (12q14)	Proinflamatory activities	Reduced risk to develop severe malaria	(52)
	Interleukin 4 (IL4)	IL4 (5q31.1)	Anti-inflammatory activities	Antimalarial antibody levels and reduced risk to develop severe malaria	(53, 54)
	Interleukin 10 (IL10)	IL10 (1q31-q32)	Regulation of the immune response	Reduced risk to develop severe malaria and anemia	(55, 56)

a vertebrate host for survival and perpetuation (**Figure 1**). Therefore, during its entire life cycle in humans, *P. vivax* undergoes multiple morphological and antigenically distinct stages and can be attacked by different immune mechanisms, depending on the stage and whether the parasite is within or outside the host cell. During the migration through the bloodstream to the liver, antibodies can block sporozoite migration and/or invasion of hepatocytes, repressing lifecycle progression (60–63). In the liver stage, infected hepatocytes are potential targets of $CD4^+$ and $CD8^+$ T cells, although the immune response mediated by NK cells and T gamma-delta T cells also participates in the immune response against pre-erythrocytic forms stimulating other cell populations secreting cytokines or acting directly on the infected hepatocyte (64–66). After being released from merosomes, free merozoites are susceptible to host immune responses. Merozoites can be the target of opsonizing antibodies, triggering cell-mediated merozoite killing or blocking merozoite proteins responsible for the initial interaction with the molecules on the surface of erythrocytes, preventing invasion (67, 68). Considering that the MHCs Class I and II antigens are absent on the surface of the erythrocytes, the immune response against blood stage forms involves mainly antibodies. During the intraerythrocytic stage, antibodies may coalesce merozoites at or just before the rupture of erythrocytes, preventing their release and spread into the bloodstream, essential for the clearance of parasitemia in the later stages of the infection (2, 67, 68). Although antibodies have a critical role in the development of immunity against erythrocytic forms, studies indicate that the development of the immune response also involves monocytes, neutrophils, $CD4^+$ T cells, NK cells, and NKT cells (68).

Overall, one may say that the cellular immune response is more important in the control of the hepatic forms of the parasite, whereas the humoral immune response seems to be more important to the control of its erythrocytic stage. Since *P. vivax* stimulates various components of the immune system, the balance of this activation can represent a fine line between inhibition of the parasite growth and immunopathology. Thus, it is acceptable to consider that polymorphisms in genes encoding immune system molecules, especially those located at MHC locus, could be involved in *P. vivax* malaria outcome.

HLA GENETIC REGION

The MHC, referred as human leukocyte antigen (HLA) system in humans, is an extremely polymorphic region encoding for the major molecules in charge of antigen presentation on the cell surface, and it has been one of the most intensively studied areas in the human genome (69, 70). Located in the short arm of the chromosome 6, HLA complex consists of more than 200 genes categorized into three basic groups: class I, class II, and class III (**Figure 2**). Class I molecule is a heterodimer consisting of a heavy chain and a light chain, the beta-2 microglobulin. HLA Class I genetic region encodes the heavy chain of the classics HLA-A, -B, and -C molecules, besides HLA-E, -F, -G, and the MHC class I polypeptide-related sequence A (MICA) and MICB. Class

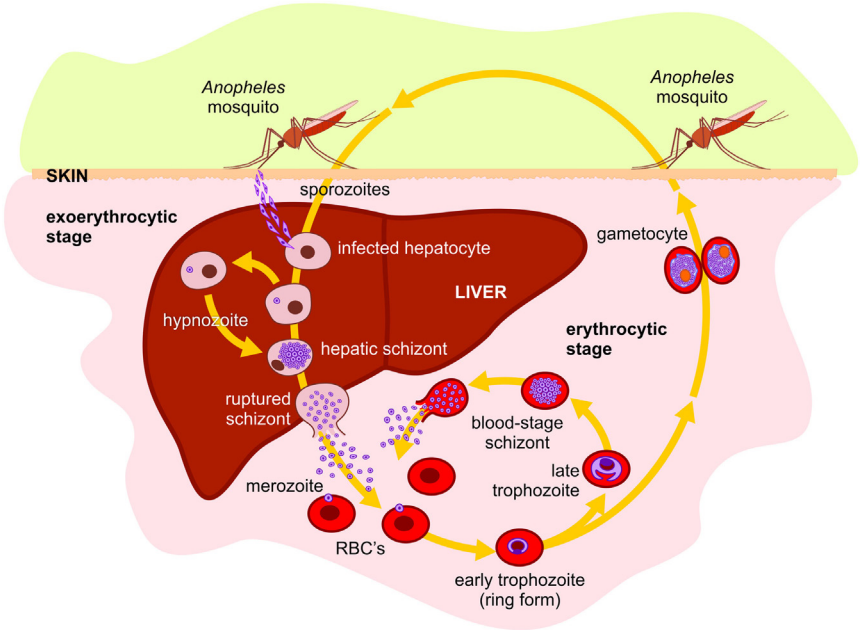

FIGURE 1 | *Plasmodium vivax* lifecycle in human host: *P. vivax* is transmitted to humans by the bite of an infected female of the *Anopheles* mosquito, releasing the salivary fluid carrying sporozoites into the tissues or directly into the bloodstream. From the tissues, the motile sporozoites can penetrate small blood vessels. In hepatic sinusoids, they penetrate through Kupffer cells into Space of Disse and invade hepatocytes to begin the exo-erythrocytic or liver-stage cycle. The sporozoite differentiates into mature liver-stage schizont with thousands of uninucleated merozoites surrounded by a parasitophorous membrane. The hepatocyte containing mature liver schizonts ruptures releasing merosomes. These merosomes are transported into the general blood circulation and break, releasing merozoites which invade young red blood cells (reticulocytes), beginning the erythrocytic or blood-stage cycle. *P. vivax* has dormant liver hypnozoite stages, which can reactivate and lead to blood-stage relapses. Within the erythrocyte, the merozoite differentiates in erythrocytic trophozoite. When fully mature, the infected erythrocyte ruptures, releasing the merozoites, which then invade new erythrocytes, initiating the entire intraerythrocytic-stage cycle, rupture, and reinvasion. Alternatively, some merozoites can develop gametocytes. During blood feeding, female mosquito of a susceptible *Anopheles* species can ingest the gametocytes, beginning the sexual stage of the life cycle. In the midgut of the mosquito, gametocytes escape from erythrocytes and become sexually stimulated. The male gamete fuses with the female, forming a diploid zygote. Therefore, the zygote is transformed into an invasive parasite stage ookinete. The ookinete traverses the midgut wall by passing through epithelial cells and comes to rest adjacent to the basal lamina where it transforms into an oocyst that undergoes multiple nuclear divisions producing several thousand sporozoites. At maturity, the oocyst breaks open and the sporozoites are released into the hemocele of the mosquito, migrating and penetrating the salivary glands. In the salivary glands, the sporozoites become infectious to humans, completing the life cycle.

I molecules are expressed in nearly all cells and play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. Class II molecules are heterodimers formed by α and β chains. HLA class II genetic region, initially called Immune response (Ir) genes due to its role in controlling the immune response, encodes the α and β chains of the HLA-DR, -DQ, -DP, -DM, and -DO molecules and peptide transporter proteins (TAP) 1 and (TAP) 2 (69–71). Class II molecules are predominantly expressed on antigen-presenting cells (APC), such as macrophages, dendritic cells, B cells, Langerhans cells, and Kupffer cells, although some cells may express class II molecules during inflammatory process (70, 72, 73). The proteins produced from HLA class III genes have somewhat different functions, some of which involve participation in inflammation processes and other immune system activities. HLA-Class III genetic region encodes C2 and C4 complement components and tumor necrosis factor (TNF) superfamily (70). The functions of some HLA genes are unknown.

The polymorphism of HLA has been useful in the search of donors with compatible grafts in tracing population migration as well as in its potential relationship to pathogen-mediated selection. Further, assessing and comparing the polymorphism of HLA allows to better define the extent of the genetic variability in humans as well as the reasons of this diversity. The HLA region is associated with more diseases (mainly autoimmune and infectious diseases) than any other region of the genome (74–78).

HLA CLASS I AND II GENES AND *P. vivax* ANTIGENS

The importance of HLA genes influencing malaria outcome has been demonstrated since studies conducted by Hill and colleagues who elegantly illustrated the influence of HLA genes in the protection against an intracellular pathogen and how the polymorphism of HLA genes may have evolved through selection of molecules induced by the pathogen. In a study of over 2000 children in West Africa, Hill et al. showed that carriers of HLA Class I Bw53 and HLA class II DRB1*1302-DQB1*0501, frequently occurring in sub-Saharan Africa, were protected against severe malaria (39).

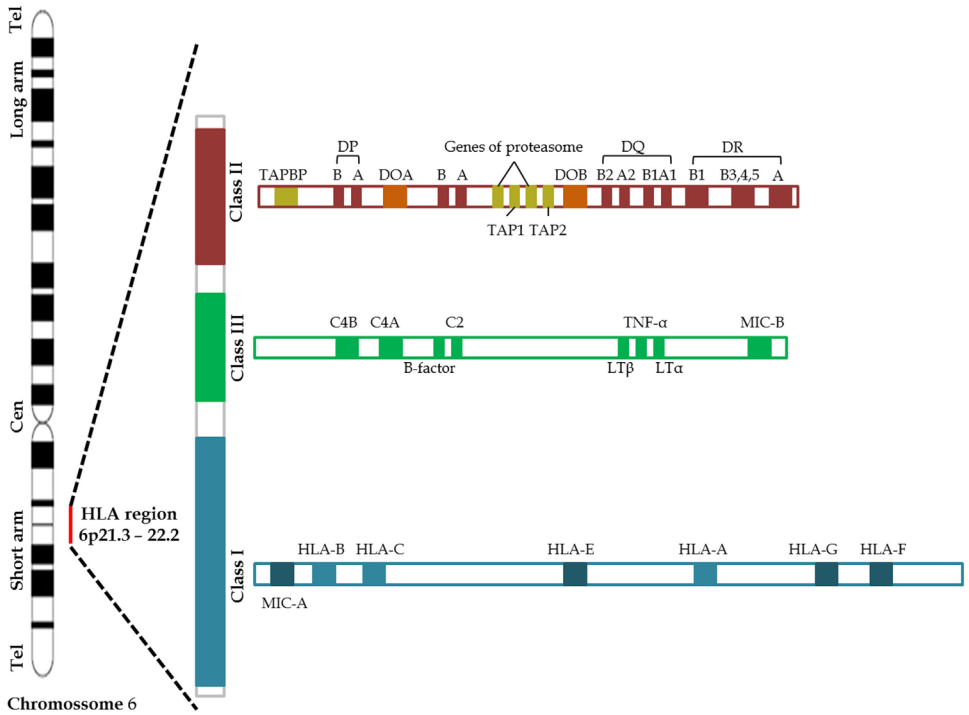

FIGURE 2 | Schematic representation of the human Chromosome 6 including the main MHC genes (69).

Later studies showed that HLA-B53 restricted cytotoxic T cells recognize peptides corresponding to regions of *P. falciparum* Liver Stage Antigen-1 (79). Thus, HLA molecules have been used as genetic markers in an attempt to determine the presence of genetic modulation of the immune response during malaria infection. Considering the increasing focus on the development of subunit malaria vaccines, studies on the influence of HLA molecules in the immune response in ethnically diverse populations are important before the implementation of vaccine trials. This is particularly relevant for *P. vivax*, which affects populations with high diversity of genetic backgrounds.

In this scenario, the circumsporozoite surface protein (CSP) is the most abundant polypeptide present in the sporozoite covering. This protein is involved in the motility and invasion of the sporozoite during its entrance in the hepatocyte (80). The *csp* gene encodes a protein, which has in its central portion, a highly immunogenic repetitive region. Based on the *csp* gene, two variants, VK247 and *P. vivax*-like, have been described. They differ from the classical form (VK210) by sequence variations in the central region of the gene (81, 82). A study performed by Oliveira-Ferreira and others with 108 individuals living in Rondonia State, in the Southwestern part of the Brazilian Amazon, observed a significant association between the antibody response to the CSP repeats of VK247and the presence of HLA-DRB1*16 and between the presence of HLA-DRB1*07 and the absence of antibody responses to the CSP repeats of VK210 (83). More recently, Storti-Mello and co-workers described a significant association between the absence of antibody response to the CSP amino-terminal region and the presence of HLA-DRB1*03 and DR5 in a study with 55 individuals from different regions of the Brazilian Amazon (84) (**Table 2**).

TABLE 2 | Associations between HLA-DRB1 and HLA-DQB1 allelic groups and antibody response to *P. vivax* antigens.

Antigen/protein	HLA	Association	Reference
CSP VK247 variant	DRB1*16	+	(83)
CSP VK210 variant	DRB1*07	–	(83)
CSP peptide N	DRB1*03	–	(84)
CSP peptide N	DRB1*11, *12 (DR5)	–	(84)
MSP3-NT	DRB1*04	+	(85)
MSP3-CT	DRB1*04	+	(85)
MSP3-CT	DQB1*03	+	(85)
MSP3-CT	DQB1*06	–	(85)
MSP3-FL	DRB1*16	–	(85)
MSP9-RIRII	DRB1*01	–	(85)
MSP9-RIRII	DRB1*04	+	(85)
MSP9-RII	DRB1*01	–	(85)
MSP9-RII	DRB1*04	+	(85)
MSP9-CT	DRB1*04	+	(85)
AMA-1	DRB1*03	+	(84)
RBP-1	DRB1 and DQB1 alleles	Not found	(86)
MSP1-19	DRB1 and DQB1 alleles	Not found	(84, 85)
DBP	DRB1 alleles	Not found	(87)

Merozoite surface proteins (MSPs) have been reported as abundantly expressed on the surface of merozoites and can contribute to the initial recognition of erythrocytes. MSP-1, MSP-3, and MSP-9 have been considered important vaccine candidates

based on their location, on their recognition by antibodies from individuals naturally exposed to *P. vivax*, their immunogenic properties in animal models, and evidence of the induction of antibodies able to inhibit parasite-growth (88–95). Therefore, considering the importance of the antibodies against MSPs in the development of anti-parasite immunity, studies have also focused the evaluation of the genetic restriction of the anti-MSP humoral response. Storti-Melo et al. analyzed the influence of the HLA-DRB1 alleles on antibody levels against the amino-terminal region of the MSP-1 in individuals from the Brazilian Amazon and observed significant association between high levels of antibodies for MSP-1 and the presence of HLA-DRB1*03 (84). In contrast, no evidence of a specific HLA-DR or HLA-DQ restriction for the antibody response to MSP-1 was observed in a study carried out by Lima-Junior et al. in 276 individuals living in Rondonia State in the Brazilian Amazon (85). However, in that paper, the authors showed HLA associations with IgG antibody response against different regions of MSP-3 and MSP-9 proteins. A high frequency of responders to carboxy-terminal (CT) and amino-terminal (NT) regions of MSP-3 were defined in HLA-DRB1*04 carriers and to MSP-3CT also defined in HLA-DQB1*03 carriers. Additionally, a high frequency of non-responders to MSP-3CT and the presence of HLA-DQB1*06 and to a recombinant protein representing the full length (FL) of MSP-3 with the presence of the HLA-DRB1*16 allele were observed. Regarding MSP-9, the presence of HLA-DRB1*04 was positively associated with the IgG immune response against all constructions used in the study, the amino-terminal domain (NT) and the C-terminal blocks of tandem repeats (RII and RIRII), while the presence of the HLA-DRB1*01 was associated with the high frequency of non-responders only to the repeated region (**Table 2**).

However, other studies did not find associations between HLA-DR or HLA-DQ alleles and antibody response to *P. vivax* antigens. In a study performed by Ferreira and co-workers, no genetic restriction mediated by HLA-DRB1* and HLA-DQB1* against two constructions of *P. vivax* Reticulocyte Binding Protein-1 (PvRBP1) was verified in more than 500 HLA alleles from different individuals from communities in the Amazon region of Brazil (86). Moreover, regarding the cellular response, Arevalo-Herrera et al. also did not observe association between HLA and cellular immune response of healthy volunteers vaccinated with CSP derived long synthetic peptides (96) and Lima-junior et al. describe five promiscuous peptides from MSP-9 which also presented no association between HLA-DRB1 alleles and the cellular immune response (97).

HLA CLASS III GENES AND *P. vivax*

Several genes of the immune system have proved to be important in relation to the susceptibility or resistance to malaria, especially those associated with severe malaria. Therefore, a common strategy is to identify the mutations in such genes and observe their possible association with the disease outcome. Since Kwiatkowski et al. showed that the TNF was associated with the susceptibility to cerebral malaria (98), numerous mutations have already been identified in the promoter of this gene, which can influence on TNF production rate. In one of those vanguard studies, McGuire and colleagues showed that mutation at position -308 of the TNF promoter region is associated with increased risk of death from cerebral malaria in Africa (51). Analysis of other clinical complications experienced in African children with severe malaria also showed that severe anemia due to malaria is associated with the mutation at position -238 suggesting that the clinical manifestations could also be influenced by genetic determinants located near the TNF gene. In fact, the guanine-to-adenine transition at position -308 in the TNF promoter, which defines the rare allele TNF2 is strongly associated with the MHC haplotypes HLA-A1, B8, DR3 and was also reported to influence the TNF promoter activity, enhancing TNF-α production (99). In patients with cerebral, severe malaria and mucocutaneous leishmaniasis, the TNF-α -308G/A polymorphism has been shown to be associated with the outcome and clinical course of the disease (100). However, only in the last years, the influence of these polymorphisms on *P. vivax* infection began to be investigated. On the one hand, in patients with *P. vivax* malaria from India, two single nucleotide polymorphisms (SNP) in the TNF promoter (–308G > A and –1031C > T) were associated with cytokine levels and temperature, but no association related to susceptibility were reported (101). On the other hand, there was neither association between six different TNF SNP polymorphisms and *P. vivax* malaria in Thailand nor differences in allelic distribution among the three distinct ethnic groups assessed by the study: Thai, Burmese, and Karen (102). In Brazil, even TNF-308 GA genotype or A allele carriers presented higher levels of TNF than those with the GG genotype or G allele, no association related to susceptibility was observed in *P. vivax* infected individuals (103, 104). In fact, we tend to reinforce the idea that a SNP is often not sufficient for predicting the susceptibility or resistance of individuals to *P. vivax* malaria. Therefore, the usual approach when investigating the differences in response to malaria infection should be the haplotype analysis. For example, Sortica et al. reported the association of TNF haplotype with a lower susceptibility to *P. vivax* infections, since an uninfected group presented a significantly higher frequency of a specific haplotype (T1031/A863/C857/G308/G238) when compared to *P. vivax* infected individuals (104). However, despite these several evidences of polymorphism in TNF gene in relation to malaria susceptibility in the studies, a larger number of samples and different clinical and epidemiological scenarios are necessary to confirm the associations.

Aside the TNF association studies, the associations between malaria and polymorphisms in other genes located at HLA locus were also the focus of investigations. For example, the nuclear protein HLA-B-associated transcript 1 (BAT1), which is an RNA helicase encoded by the DDX39B gene, has been described as a negative regulator of inflammation by modulating expression of proinflammatory cytokines (such as TNF) (105). Therefore, using mutations in two MHC genes located approximately at 150 kb from each other (TNF and DDX39B) Mendonça et al. reported associations between DDX39B haplotypes and complicated *P. vivax* malaria. Participants with *DDX39B*-22/*DDX39B*-348/*TNF*-308/*IL6*-176 genotype combinations GC/CC/GG/GG and GG/CT/GG/GG had reduced and increased risk, respectively, of developing malaria symptoms (103).

Lastly, other HLA-Class III host candidate gene polymorphisms were also associated with susceptibility/resistance to *Plasmodium* infection. However, the absence of studies using only *P. vivax* infected/exposed individuals makes the definition of genetic polymorphism of HLA-class III genes associated specifically to this species particularly difficult. In *P. falciparum* studies conducted in Africa, a trend of association between LTA polymorphism with antimalarial IgG subclass levels was found but not confirmed by statistical tests (106). Moreover, no LTA polymorphisms were associated with severe malaria in cohorts in Kenya and Malawi in a large study involving >10,000 individuals from three African populations. In Brazilian endemic areas, recently, a study with a large number of candidate gene polymorphisms was performed and the association with susceptibility/resistance to *Plasmodium* infection with clinical (mild) malaria in a population infected with *P. falciparum* or *P. vivax* was investigated. Although no differences between species were found, the results showed, for the first time, an association between alleles of CTL4 gene with malaria, which displayed a significant association with reduced risk for clinical malaria. In addition, two other associations with cytokines were identified, both within MHC class III region, that included TNF and the lymphotoxin alpha (LT-α/LTA) and beta (LT-β/LTB) genes, which are closely related (107).

HLA AND BIOINFORMATICS APPLIED TO *P. vivax* ANTIGEN DISCOVERY

The ultimate goal of MHC binding antigenic peptide prediction is to identify epitopes that activate T-cells and mediate cell-mediated immunity without HLA genotype/haplotype restriction. Recognition of peptide bound to an MHC molecule by a T-cell receptor is a critical step and for T-cell activation binding of peptide to the MHC molecule is a necessary requirement (108). The association of immunogenic fragments (epitopes) to the HLA molecules of class I or II determines what type of cell is to be stimulated and, consequently, what kind of response will be generated. Conventional vaccinology approaches accumulate successes and failures aiming at experimental screening methods to evaluate the presence of HLA restriction in immune response to vaccine candidates. But this conventional process is still laborious, expensive, and time-consuming. Computational prediction methods complement experimental studies, minimize the number of validation experiments, and significantly speed up the epitope mapping process (109). The bioinformatics tools have already e helped identifying promiscuous epitopes within *Leishmania* (110), *Mycobacterium tuberculosis* (111) and HIV (112) antigens. In malaria, epitope identification is particularly challenging, as more than 5000 proteins are encoded by the genome (113, 114), which could generate hundreds of thousands of possible $CD4^+$ T cell epitopes. On the other hand, the identification of $CD4^+$ and $CD8^+$ epitopes from malaria is urgently required to track various vaccine approaches, mainly to evaluate candidates for compositions of subunit vaccines. For example, in *P. falciparum* vaccine research, Doolan et al. first used proteomic approaches to identify 27 highly expressed candidate antigens, and then used HLA-DR binding predictions to identify 723 predicted HLA-DR binders. Of these, 39 peptides binding tightly to HLA-DR variants derived from four newly identified antigenic targets were identified (115). Beyond antigen identification, this application of proteomics and bioinformatics was confirmed as particularly powerful and is likely to prove useful in other applications, particularly as consensus motif prediction approaches.

Despite several T-cell epitopes from pre-erythrocytic (116–118), asexual blood stage (119–121), and gametocyte (122) antigens have been predicted and/or experimentally confirmed for *P. falciparum*, the use of bioinformatics strategies to identify potentially important epitopes in *P. vivax* is still restricted. The majority of the studies focusing on the detection of B or T-cell epitopes have used conventional screening methods (94, 123–125). Only few studies have already used prediction servers to trial the most promising epitopes to be used in validation assays (**Table 3**). One of the first prediction studies on *P. vivax* reported the results of *in silico* analysis of PvMSP-1 vaccine candidates in relation to potential HLA restricted or promiscuous CD4 and CD8 epitopes (126). More recently, Kumar et al. using several computational screening methods analyzed 10 protein sequences of *P. vivax* proteins, including vaccine candidates, such as MSP-1, MSP-9, Pvs25, and PvS28 in relation to potential antigenicity, promiscuity and binding to several HLA class I and II alleles. The best scored T-CD4 and T-CD8 epitopes for each antigen were also identified (127). Even with promising results, these bioinformatics approach reported is still dependent on experimental validation. In this scenario, our previous studies reported that along all PvMSP-9 N-terminal 11 peptides were highly predicted by the ProPred algorithm to be promiscuous, of which only five of them were recognized at high frequency by PBMCs from individuals living in malaria endemic areas presenting a large variety of HLA class II allelic groups (97). If the conventional screening methods had been applied, at least 40 overlapping peptides should have been synthetized and tested individually in order to select these promiscuous epitopes; on the other hand, if we had used only prediction approaches, five non-immunogenic peptides could have been selected. Although bioinformatics approach has lately accumulated more successes than failures, the confidence level (approximately 50%) for predicting epitopes to MHC class II molecules is far from perfection and in some cases can cause mismatches between predicted versus experimental results. This can happen mainly because these molecules accept a wider range of peptides in size and binding registers (16). For example, two universal epitopes were described in PvDBP sequence using conventional vaccinology experiments; however, the SYFPEITHI-binding prediction for the HLA-DRB1*0101 molecule was not in accordance with the experimental results (128). This comparison between experimental and theoretical data sets suggests that class II binding prediction tools are useful, but they have to be used with caution. Therefore, by different ways, both PvMSP-9 and PvDBP studies highlighted the combination of *in silico* analysis and the experimental confirmation as the ideal method. Therefore, actually there are accumulated evidences of successful use of bioinformatics on *P. vivax* vaccine research. For example, peptide sequences of PvRBP1 promiscuous for binding to HLA class II molecules were selected by ProPred algorithm and the IEDB server (http://www.iedb.org/) for allele binding

TABLE 3 | Bioinformatics approaches applied to epitope selection in relation to MHC alleles.

Program/database	Approach	HLA	*P. vivax* research aplication	Reference
SYFPEITHI	Database comprising more than 7000 endogenous peptide sequences known to bind class I and class II MHC molecules (131)	Class I and Class II	Comparative analysis of epitopes of *P. vivax* and *P. falciparum* lactate dehydrogenase (LDH) protein, based on LDH sequences Comparison of immunologically identified universal epitopes in *Plasmodium vivax* Duffy-binding protein and *in silico* prediction results	(128, 130)
TEPITOPE/ProPred	Promiscuity evaluation based on virtual IC50 assay of single amino acid variants of peptide sequences (132, 133)	Class II	T-cell epitope mapping on the 33-kDa region of *P. vivax* MSP1 vaccine candidate Identification and confirmation of promiscuous epitopes in PvMSP-9 Selection of promiscuous epitopes in PvRBP-1 for inclusion in a chimeric recombinant protein	(86, 97, 134)
RANKPEP	Position-specific scoring matrices of known T cell epitopes (135)	Class I and Class II	Not found	–
MULTIPRED	Evaluation of potential promiscuous T cell epitopes using neural network and hidden Markov model algorithms (136)	Class I and Class II	Not found	–
NetMHC	Neural network approach to associate binding preferences and MHC sequences known T cell epitopes, MHC structures, and sequences (137)	Class I and Class II	*In silico* analysis of PvMSP-1 sequence to find potential promiscuous T CD4 and T CD8 epitopes Identification, localization, and confirmation of MHC-restricted $CD8^+$ T cell epitopes within the *Pf*AMA1 protein and *Pv*AMA1 domain III	(126, 138)
EpiDOCK	Converts the input sequence into a collection of overlapping non-amers and predicts binding to the 23 most frequent human MHC class II and a score is assigned (139)	Class II	Not found	–
IEDB	Immune Epitope Database Consensus method consisting of NN-align, SMM-align, MetMHCPan, and/or Combinatory Library available for the sequence (140, 141)	Class I and Class II	Identification of allele binding score of predicted promiscuous epitopes and evaluation of population coverage selected PvRBP-1 antigens Identification and selection of potential multi-specie (*P. vivax* and *P. falciparum*) antigens by protein structure, binding predictions and protein motifs	(86, 129)

score and population coverage. The most promising peptide sequences were included in a PvRBP-1 chimeric antigen containing the predicted promiscuous T-cell epitopes and known B-cell epitopes and presented no HLA restriction in naturally acquired immune response of exposed individuals (86). Moreover, Cespedes et al. also used the identification and selection of novel antigens by structure, binding predictions and protein motifs. A total of 50 *P. vivax* antigens were selected based on proteome and transcriptome data of *P. falciparum* orthologs. After immunological confirmation, four peptides were experimentally confirmed as truly immunogenic peptides and were preselected for further preclinical trials (129). Lastly, beyond the vaccine field, the prediction tools have also been used in a comparative analysis of epitopes from lactate dehydrogenase (LDH) protein (130). Based on *P. vivax* and *P. falciparum* LDH sequences, T-cell epitope prediction indicated that 28 HLA alleles could recognize pLDH antigen epitopes. Interestingly, despite a large number of potentially common or similar epitopes, specific Pv-LDH and Pf-LDH epitopes were also predicted and, if experimentally confirmed, could be involved in future specific diagnostic rapid tests.

In summary, with the concomitant advent of whole-genome sequencing and advances in bioinformatics, the vaccinology field changed in the last few decades, providing the opportunity of describing novel antigens and improving the already known. Consequently, the focus in vaccine design shifted to explore antigens susceptible to antibody recognition and T-cell induction through comparative pan genome reverse vaccinology. Even though, in most of cases, experimental confirmation is necessary, high-accuracy predictions are available for any HLA known, non-human primates, mouse strains, and other mammals. Therefore, those "reverse immunology" systems have become highly accessible, and they can be a fast and efficient alternative when some conventional vaccinology strategies are difficult, especially when dealing with non-culturable microorganisms, as *P. vivax*.

CONCLUDING REMARKS

The number of studies involving MHC polymorphism and *P. vivax* specific immune response and clinical outcome are

still increasing, and there are several similarities and disparities among these association studies. Despite the variation of MHC genes, alleles and/or haplotypes in different clinical and epidemiological scenarios, the association between MHC genes and *P. vivax* has been demonstrated in the majority of studies presented. We believe that the inconsistency of some data may derive from the fact that a large number of potential risk factors, such as nutritional status, coinfections, and relapses, which could influence the specific immune response, are almost impossible to be controlled in malaria endemic areas. Other issues are the small sample size, the heterogeneity of human populations in different endemic areas, and of course, the complexity of MHC genes. Therefore, since population-based cohorts with a single *P. vivax* infection represent a valuable but uncommon resource for genetic studies, more sophisticated analytical approaches are needed to study the expression of MHC genes in such different exposure conditions to determine the precise role of such polymorphisms as determinant for *P. vivax* susceptibility, immune response, and its outcome in disease progression.

AUTHOR CONTRIBUTIONS

JL-J and LP-R wrote the review.

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REFERENCES

1. WHO. *Global Malaria Report*. Geneva (2015).
2. Langhorne J, Ndungu FM, Sponaas AM, Marsh K. Immunity to malaria: more questions than answers. *Nat Immunol* (2008) **9**:725–32. doi:10.1038/ni.f.205
3. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. *Lancet* (2014) **383**:723–35. doi:10.1016/S0140-6736(13)60024-0
4. Schumacher RF, Spinelli E. Malaria in children. *Mediterr J Hematol Infect Dis* (2012) **4**:e2012073. doi:10.4084/MJHID.2012.073
5. Maitland K, Marsh K. Pathophysiology of severe malaria in children. *Acta Trop* (2004) **90**:131–40. doi:10.1016/j.actatropica.2003.11.010
6. Menendez C, Fleming AF, Alonso PL. Malaria-related anaemia. *Parasitol Today* (2000) **16**:469–76. doi:10.1016/S0169-4758(00)01774-9
7. Mangano VD, Modiano D. Host genetics and parasitic infections. *Clin Microbiol Infect* (2014) **20**:1265–75. doi:10.1111/1469-0691.12793
8. Mangano VD, Modiano D. An evolutionary perspective of how infection drives human genome diversity: the case of malaria. *Curr Opin Immunol* (2014) **30**:39–47. doi:10.1016/j.coi.2014.06.004
9. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Hum Genet* (2005) **77**:171–92. doi:10.1086/432519
10. Haldane J. *Disease and Evolution. La Ricerca Scientifica Suppl. A*. Roma, Italy (1949). p. 68–76.
11. Yuthavong Y, Wilairat P. Protection against malaria by thalassaemia and haemoglobin variants. *Parasitol Today* (1993) **9**:241–5. doi:10.1016/0169-4758(93)90065-N
12. Cooke BM, Mohandas N, Coppel RL. Malaria and the red blood cell membrane. *Semin Hematol* (2004) **41**:173–88. doi:10.1053/j.seminhematol.2004.01.004
13. Richer J, Chudley AE. The hemoglobinopathies and malaria. *Clin Genet* (2005) **68**:332–6. doi:10.1111/j.1399-0004.2005.00503.x
14. Duffy PE, Fried M. Red blood cells that do and red blood cells that don't: how to resist a persistent parasite. *Trends Parasitol* (2006) **22**:99–101. doi:10.1016/j.pt.2006.01.009
15. Pasvol G, Weatherall DJ, Wilson RJ. Cellular mechanism for the protective effect of haemoglobin S against *P. falciparum* malaria. *Nature* (1978) **274**:701–3. doi:10.1038/274701a0
16. Cappadoro M, Giribaldi G, O'Brien E, Turrini F, Mannu F, Ulliers D, et al. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency. *Blood* (1998) **92**:2527–34.
17. Ayi K, Turrini F, Piga A, Arese P. Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against *falciparum* malaria in sickle trait and beta-thalassemia trait. *Blood* (2004) **104**:3364–71. doi:10.1182/blood-2003-11-3820
18. Allen SJ, O'Donnell A, Alexander ND, Alpers MP, Peto TE, Clegg JB, et al. alpha+-Thalassemia protects children against disease caused by other infections as well as malaria. *Proc Natl Acad Sci U S A* (1997) **94**:14736–41. doi:10.1073/pnas.94.26.14736
19. Williams TN, Mwangi TW, Wambua S, Alexander ND, Kortok M, Snow RW, et al. Sickle cell trait and the risk of *Plasmodium falciparum* malaria and other childhood diseases. *J Infect Dis* (2005) **192**:178–86. doi:10.1086/430744
20. Wambua S, Mwangi TW, Kortok M, Uyoga SM, Macharia AW, Mwacharo JK, et al. The effect of alpha+-thalassaemia on the incidence of malaria and other diseases in children living on the coast of Kenya. *PLoS Med* (2006) **3**:e158. doi:10.1371/journal.pmed.0030158
21. Willcox M, Bjorkman A, Brohult J. *Falciparum* malaria and beta-thalassaemia trait in northern Liberia. *Ann Trop Med Parasitol* (1983) **77**:335–47.
22. Friedman MJ. Erythrocytic mechanism of sickle cell resistance to malaria. *Proc Natl Acad Sci U S A* (1978) **75**:1994–7. doi:10.1073/pnas.75.4.1994
23. Ackerman H, Usen S, Jallow M, Sisay-Joof F, Pinder M, Kwiatkowski DP. A comparison of case-control and family-based association methods: the example of sickle-cell and malaria. *Ann Hum Genet* (2005) **69**:559–65. doi:10.1111/j.1529-8817.2005.00180.x
24. Friedman MJ, Roth EF, Nagel RL, Trager W. The role of hemoglobins C, S, and Nbalt in the inhibition of malaria parasite development in vitro. *Am J Trop Med Hyg* (1979) **28**:777–80.
25. Rihet P, Flori L, Tall F, Traore AS, Fumoux F. Hemoglobin C is associated with reduced *Plasmodium falciparum* parasitemia and low risk of mild malaria attack. *Hum Mol Genet* (2004) **13**:1–6. doi:10.1093/hmg/ddh002
26. Bunyaratvej A, Butthep P, Sae-Ung N, Fucharoen S, Yuthavong Y. Reduced deformability of thalassemic erythrocytes and erythrocytes with abnormal hemoglobins and relation with susceptibility to *Plasmodium falciparum* invasion. *Blood* (1992) **79**:2460–3.
27. Chotivanich K, Udomsangpetch R, Pattanapanyasat K, Chierakul W, Simpson J, Looareesuwan S, et al. Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe *P falciparum* malaria. *Blood* (2002) **100**:1172–6.
28. Roth EF Jr, Raventos-Suarez C, Rinaldi A, Nagel RL. Glucose-6-phosphate dehydrogenase deficiency inhibits in vitro growth of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* (1983) **80**:298–9. doi:10.1073/pnas.80.1.298
29. Guindo A, Fairhurst RM, Doumbo OK, Wellems TE, Diallo DA. X-linked G6PD deficiency protects hemizygous males but not heterozygous females

against severe malaria. *PLoS Med* (2007) **4**:e66. doi:10.1371/journal.pmed.0040066
30. Ouattara AK, Bisseye C, Bazie BV, Diarra B, Compaore TR, Djigma F, et al. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is associated with asymptomatic malaria in a rural community in Burkina Faso. *Asian Pac J Trop Biomed* (2014) **4**:655–8. doi:10.12980/APJTB.4.2014APJTB-2014-0100
31. Ayi K, Min-Oo G, Serghides L, Crockett M, Kirby-Allen M, Quirt I, et al. Pyruvate kinase deficiency and malaria. *N Engl J Med* (2008) **358**:1805–10. doi:10.1056/NEJMoa072464
32. Allen SJ, O'Donnell A, Alexander ND, Mgone CS, Peto TE, Clegg JB, et al. Prevention of cerebral malaria in children in Papua New Guinea by southeast Asian ovalocytosis band 3. *Am J Trop Med Hyg* (1999) **60**:1056–60.
33. Genton B, al-Yaman F, Mgone CS, Alexander N, Paniu MM, Alpers MP, et al. Ovalocytosis and cerebral malaria. *Nature* (1995) **378**:564–5. doi:10.1038/378564a0
34. Rosanas-Urgell A, Lin E, Manning L, Rarau P, Laman M, Senn N, et al. Reduced risk of *Plasmodium vivax* malaria in Papua New Guinean children with Southeast Asian ovalocytosis in two cohorts and a case-control study. *PLoS Med* (2012) **9**:e1001305. doi:10.1371/journal.pmed.1001305
35. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med* (1976) **295**:302–4. doi:10.1056/NEJM197608052950602
36. Spencer HC, Miller LH, Collins WE, Knud-Hansen C, McGinnis MH, Shiroishi T, et al. The Duffy blood group and resistance to *Plasmodium vivax* in Honduras. *Am J Trop Med Hyg* (1978) **27**:664–70.
37. Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, et al. Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis* (2011) **5**:e1192. doi:10.1371/journal.pntd.0001192
38. Woldearegai TG, Kremsner PG, Kun JF, Mordmuller B. *Plasmodium vivax* malaria in Duffy-negative individuals from Ethiopia. *Trans R Soc Trop Med Hyg* (2013) **107**:328–31. doi:10.1093/trstmh/trt016
39. Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, et al. Common west African HLA antigens are associated with protection from severe malaria. *Nature* (1991) **352**:595–600. doi:10.1038/352595a0
40. Hill AV, Bennett S, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, et al. HLA, malaria and dominant protective associations. *Parasitol Today* (1992) **8**:57. doi:10.1016/0169-4758(92)90089-K
41. Wilkinson RJ, Pasvol G. Host resistance to malaria runs into swampy water. *Trends Microbiol* (1997) **5**:213–5. doi:10.1016/S0966-842X(97)01053-6
42. Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, et al. Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* (1998) **279**:1173–7. doi:10.1126/science.279.5354.1173
43. Banic DM, Goldberg AC, Pratt-Riccio LR, De Oliveira-Ferreira J, Santos F, Gras-Masse H, et al. Human leukocyte antigen class II control of the immune response to p126-derived amino terminal peptide from *Plasmodium falciparum*. *Am J Trop Med Hyg* (2002) **66**:509–15.
44. Pratt-Riccio LR, Lima-Junior JC, Carvalho LJ, Theisen M, Espindola-Mendes EC, Santos F, et al. Antibody response profiles induced by *Plasmodium falciparum* glutamate-rich protein in naturally exposed individuals from a Brazilian area endemic for malaria. *Am J Trop Med Hyg* (2005) **73**:1096–103.
45. Thathy V, Moulds JM, Guyah B, Otieno W, Stoute JA. Complement receptor 1 polymorphisms associated with resistance to severe malaria in Kenya. *Malar J* (2005) **4**:54. doi:10.1186/1475-2875-4-54
46. Hobbs MR, Udhayakumar V, Levesque MC, Booth J, Roberts JM, Tkachuk AN, et al. A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children. *Lancet* (2002) **360**:1468–75. doi:10.1016/S0140-6736(02)11474-7
47. Planche T, Macallan DC, Sobande T, Borrmann S, Kun JF, Krishna S, et al. Nitric oxide generation in children with malaria and the NOS2G-954C promoter polymorphism. *Am J Physiol Regul Integr Comp Physiol* (2010) **299**:R1248–53. doi:10.1152/ajpregu.00390.2010
48. Clark TG, Diakite M, Auburn S, Campino S, Fry AE, Green A, et al. Tumor necrosis factor and lymphotoxin-alpha polymorphisms and severe malaria in African populations. *J Infect Dis* (2009) **199**:569–75. doi:10.1086/596320
49. Hananantachai H, Patarapotikul J, Ohashi J, Naka I, Krudsood S, Looareesuwan S, et al. Significant association between TNF-alpha (TNF) promoter allele (-1031C, -863C, and -857C) and cerebral malaria in Thailand. *Tissue Antigens* (2007) **69**:277–80. doi:10.1111/j.1399-0039.2006.00756.x
50. Wattavidanage J, Carter R, Perera KL, Munasingha A, Bandara S, McGuinness D, et al. TNFalpha*2 marks high risk of severe disease during *Plasmodium falciparum* malaria and other infections in Sri Lankans. *Clin Exp Immunol* (1999) **115**:350–5. doi:10.1046/j.1365-2249.1999.00804.x
51. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature* (1994) **371**:508–10. doi:10.1038/371508a0
52. Kanchan K, Jha P, Pati SS, Mohanty S, Mishra SK, Sharma SK, et al. Interferon-gamma (IFNG) microsatellite repeat and single nucleotide polymorphism haplotypes of IFN-alpha receptor (IFNAR1) associated with enhanced malaria susceptibility in Indian populations. *Infect Genet Evol* (2015) **29**:6–14. doi:10.1016/j.meegid.2014.10.030
53. Cabantous S, Ranque S, Poudiougou B, Traore A, Berbache S, Vitte J, et al. Genotype combinations of two IL4 polymorphisms influencing IL-4 plasma levels are associated with different risks of severe malaria in the Malian population. *Immunogenetics* (2015) **67**:283–8. doi:10.1007/s00251-015-0836-3
54. Luoni G, Verra F, Arca B, Sirima BS, Troye-Blomberg M, Coluzzi M, et al. Antimalarial antibody levels and IL4 polymorphism in the Fulani of West Africa. *Genes Immun* (2001) **2**:411–4. doi:10.1038/sj.gene.6363797
55. Wilson JN, Rockett K, Jallow M, Pinder M, Sisay-Joof F, Newport M, et al. Analysis of IL10 haplotypic associations with severe malaria. *Genes Immun* (2005) **6**:462–6. doi:10.1038/sj.gene.6364227
56. Ouma C, Davenport GC, Were T, Otieno MF, Hittner JB, Vulule JM, et al. Haplotypes of IL-10 promoter variants are associated with susceptibility to severe malarial anemia and functional changes in IL-10 production. *Hum Genet* (2008) **124**:515–24. doi:10.1007/s00439-008-0578-5
57. Baird JK. Evidence and implications of mortality associated with acute *Plasmodium vivax* malaria. *Clin Microbiol Rev* (2013) **26**:36–57. doi:10.1128/CMR.00074-12
58. Galinski MR, Barnwell JW. *Plasmodium vivax*: who cares? *Malar J* (2008) **7**(Suppl 1):S9. doi:10.1186/1475-2875-7-S1-S9
59. Welch SG, McGregor IA, Williams K. The Duffy blood group and malaria prevalence in Gambian West Africans. *Trans R Soc Trop Med Hyg* (1977) **71**:295–6. doi:10.1016/0035-9203(77)90102-X
60. Nardin E, Zavala F, Nussenzweig V, Nussenzweig RS. Pre-erythrocytic malaria vaccine: mechanisms of protective immunity and human vaccine trials. *Parassitologia* (1999) **41**:397–402.
61. Luo S, Liu D, Ye B, Shu H, Fu R. Effect of monoclonal antibodies on the entry and development of *Plasmodium vivax* sporozoite in cultured cells. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* (1995) **13**:284–9.
62. Millet P, Chizzolini C, Wirtz RA, Bathurst I, Broderson JR, Campbell GH, et al. Inhibitory activity against sporozoites induced by antibodies directed against nonrepetitive regions of the circumsporozoite protein of *Plasmodium vivax*. *Eur J Immunol* (1992) **22**:519–24. doi:10.1002/eji.1830220234
63. Hollingdale MR, Nardin EH, Tharavanij S, Schwartz AL, Nussenzweig RS. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells; an in vitro assay of protective antibodies. *J Immunol* (1984) **132**:909–13.
64. Doolan DL, Hoffman SL. The complexity of protective immunity against liver-stage malaria. *J Immunol* (2000) **165**:1453–62. doi:10.4049/jimmunol.165.3.1453
65. Cockburn IA, Amino R, Kelemen RK, Kuo SC, Tse SW, Radtke A, et al. In vivo imaging of CD8+ T cell-mediated elimination of malaria liver stages. *Proc Natl Acad Sci U S A* (2013) **110**:9090–5. doi:10.1073/pnas.1303858110
66. Chia WN, Goh YS, Renia L. Novel approaches to identify protective malaria vaccine candidates. *Front Microbiol* (2014) **5**:586. doi:10.3389/fmicb.2014.00586
67. Herrera S, Corradin G, Arevalo-Herrera M. An update on the search for a *Plasmodium vivax* vaccine. *Trends Parasitol* (2007) **23**:122–8. doi:10.1016/j.pt.2007.01.008
68. Mueller I, Galinski MR, Tsuboi T, Arevalo-Herrera M, Collins WE, King CL. Natural acquisition of immunity to *Plasmodium vivax*: epidemiological observations and potential targets. *Adv Parasitol* (2013) **81**:77–131. doi:10.1016/B978-0-12-407826-0.00003-5

69. Beck S, Geraghty D, Inoko H, Rowen L, Aguado B, Bahram S, et al. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature* (1999) **401**:921–3.
70. Goldberg AC, Rizzo LV. MHC structure and function – antigen presentation. Part 1. *Einstein (Sao Paulo)* (2015) **13**:153–6. doi:10.1590/S1679-45082015RB3122
71. Trowsdale J, Campbell RD. Human MHC genes and products. *Curr Protoc immunol* (2001) **1**:1K.
72. Hershberg RM, Cho DH, Youakim A, Bradley MB, Lee JS, Framson PE, et al. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *J Clin Invest* (1998) **102**:792–803. doi:10.1172/JCI3201
73. Wu Z, Biro PA, Mirakian R, Hammond L, Curcio F, Ambesi-Impiombato FS, et al. HLA-DMB expression by thyrocytes: indication of the antigen-processing and possible presenting capability of thyroid cells. *Clin Exp Immunol* (1999) **116**:62–9. doi:10.1046/j.1365-2249.1999.00831.x
74. Price P, Calder DM, Witt CS, Allcock RJ, Christiansen FT, Davies GR, et al. Periodontal attachment loss in HIV-infected patients is associated with the major histocompatibility complex 8.1 haplotype (HLA-A1,B8,DR3). *Tissue Antigens* (1999) **54**:391–9. doi:10.1034/j.1399-0039.1999.540409.x
75. Trowsdale J, Knight JC. Major histocompatibility complex genomics and human disease. *Annu Rev Genomics Hum Genet* (2013) **14**:301–23. doi:10.1146/annurev-genom-091212-153455
76. Li CP, Zhou Y, Xiang X, Zhou Y, He M. Relationship of HLA-DRB1 gene polymorphism with susceptibility to pulmonary tuberculosis: updated meta-analysis. *Int J Tuberc Lung Dis* (2015) **19**:841–9. doi:10.5588/ijtld.14.0521
77. Magira EE, Papasteriades C, Kanterakis S, Toubis M, Roussos C, Monos DS. HLA-A and HLA-DRB1 amino acid polymorphisms are associated with susceptibility and protection to pulmonary tuberculosis in a Greek population. *Hum Immunol* (2012) **73**:641–6. doi:10.1016/j.humimm.2012.03.008
78. Haag S, Tuncel J, Thordardottir S, Mason DE, Yau AC, Dobritzsch D, et al. Positional identification of RT1-B (HLA-DQ) as susceptibility locus for autoimmune arthritis. *J Immunol* (2015) **194**:2539–50. doi:10.4049/jimmunol.1402238
79. Hill AV, Elvin J, Willis AC, Aidoo M, Allsopp CE, Gotch FM, et al. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature* (1992) **360**:434–9. doi:10.1038/360434a0
80. Zavala F, Cochrane AH, Nardin EH, Nussenzweig RS, Nussenzweig V. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. *J Exp Med* (1983) **157**:1947–57. doi:10.1084/jem.157.6.1947
81. Qari SH, Shi YP, Goldman IF, Udhayakumar V, Alpers MP, Collins WE, et al. Identification of *Plasmodium vivax*-like human malaria parasite. *Lancet* (1993) **341**:780–3. doi:10.1016/0140-6736(93)90559-Y
82. Qari SH, Goldman IF, Povoa MM, Oliveira S, Alpers MP, Lal AA. Wide distribution of the variant form of the human malaria parasite *Plasmodium vivax*. *J Biol Chem* (1991) **266**:16297–300.
83. Oliveira-Ferreira J, Pratt-Riccio LR, Arruda M, Santos F, Ribeiro CT, Goldberg AC, et al. HLA class II and antibody responses to circumsporozoite protein repeats of *P. vivax* (VK210, VK247 and *P. vivax*-like) in individuals naturally exposed to malaria. *Acta Trop* (2004) **92**:63–9. doi:10.1016/j.actatropica.2004.02.011
84. Storti-Melo LM, da Costa DR, Souza-Neiras WC, Cassiano GC, Couto VS, Povoa MM, et al. Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with *Plasmodium vivax*. *Acta Trop* (2012) **121**:152–5. doi:10.1016/j.actatropica.2011.10.009
85. Lima-Junior JC, Rodrigues-da-Silva RN, Banic DM, Jiang J, Singh B, Fabricio-Silva GM, et al. Influence of HLA-DRB1 and HLA-DQB1 alleles on IgG antibody response to the *P. vivax* MSP-1, MSP-3alpha and MSP-9 in individuals from Brazilian endemic area. *PLoS One* (2012) **7**:e36419. doi:10.1371/journal.pone.0036419
86. Ferreira AR, Singh B, Cabrera-Mora M, Magri De Souza AC, Queiroz Marques MT, Porto LC, et al. Evaluation of naturally acquired IgG antibodies to a chimeric and non-chimeric recombinant species of *Plasmodium vivax* reticulocyte binding protein-1: lack of association with HLA-DRB1*/DQB1* in malaria exposed individuals from the Brazilian Amazon. *PLoS One* (2014) **9**:e105828. doi:10.1371/journal.pone.0105828
87. Martinez P, Lopez C, Saravia C, Vanegas M, Patarroyo MA. Evaluation of the antigenicity of universal epitopes from PvDBPII in individuals exposed to *Plasmodium vivax* malaria. *Microbes Infect* (2010) **12**:1188–97. doi:10.1016/j.micinf.2010.08.007
88. Soares IS, Levitus G, Souza JM, Del Portillo HA, Rodrigues MM. Acquired immune responses to the N- and C-terminal regions of *Plasmodium vivax* merozoite surface protein 1 in individuals exposed to malaria. *Infect Immun* (1997) **65**:1606–14.
89. Galinski MR, Corredor-Medina C, Povoa M, Crosby J, Ingravallo P, Barnwell JW. *Plasmodium vivax* merozoite surface protein-3 contains coiled-coil motifs in an alanine-rich central domain. *Mol Biochem Parasitol* (1999) **101**:131–47. doi:10.1016/S0166-6851(99)00063-8
90. de Oliveira CI, Wunderlich G, Levitus G, Soares IS, Rodrigues MM, Tsuji M, et al. Antigenic properties of the merozoite surface protein 1 gene of *Plasmodium vivax*. *Vaccine* (1999) **17**:2959–68. doi:10.1016/S0264-410X(99)00176-0
91. Barnwell JW, Galinski MR, DeSimone SG, Perler F, Ingravallo P. *Plasmodium vivax*, P. cynomolgi, and P. knowlesi: identification of homologue proteins associated with the surface of merozoites. *Exp Parasitol* (1999) **91**:238–49. doi:10.1006/expr.1998.4372
92. Oliveira-Ferreira J, Vargas-Serrato E, Barnwell JW, Moreno A, Galinski MR. Immunogenicity of *Plasmodium vivax* merozoite surface protein-9 recombinant proteins expressed in *E. coli*. *Vaccine* (2004) **22**:2023–30. doi:10.1016/j.vaccine.2003.07.021
93. Soares IS, Rodrigues MM. Immunogenic properties of the *Plasmodium vivax* vaccine candidate MSP1(19) expressed as a secreted non-glycosylated polypeptide from *Pichia pastoris*. *Parasitology* (2002) **124**:237–46. doi:10.1017/S003118200100110X
94. Lima-Junior JC, Jiang J, Rodrigues-da-Silva RN, Banic DM, Tran TM, Ribeiro RY, et al. B cell epitope mapping and characterization of naturally acquired antibodies to the *Plasmodium vivax* merozoite surface protein-3alpha (PvMSP-3alpha) in malaria exposed individuals from Brazilian Amazon. *Vaccine* (2011) **29**:1801–11. doi:10.1016/j.vaccine.2010.12.099
95. Lima-Junior JC, Tran TM, Meyer EV, Singh B, De-Simone SG, Santos F, et al. Naturally acquired humoral and cellular immune responses to *Plasmodium vivax* merozoite surface protein 9 in Northwestern Amazon individuals. *Vaccine* (2008) **26**:6645–54. doi:10.1016/j.vaccine.2008.09.029
96. Arevalo-Herrera M, Soto L, Perlaza BL, Cespedes N, Vera O, Lenis AM, et al. Antibody-mediated and cellular immune responses induced in naive volunteers by vaccination with long synthetic peptides derived from the *Plasmodium vivax* circumsporozoite protein. *Am J Trop Med Hyg* (2011) **84**:35–42. doi:10.4269/ajtmh.2011.09-0507
97. Lima-Junior JC, Banic DM, Tran TM, Meyer VS, De-Simone SG, Santos F, et al. Promiscuous T-cell epitopes of *Plasmodium* merozoite surface protein 9 (PvMSP9) induces IFN-gamma and IL-4 responses in individuals naturally exposed to malaria in the Brazilian Amazon. *Vaccine* (2010) **28**:3185–91. doi:10.1016/j.vaccine.2010.02.046
98. Kwiatkowski D, Hill AV, Sambou I, Twumasi P, Castracane J, Manogue KR, et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet* (1990) **336**:1201–4. doi:10.1016/0140-6736(90)92827-5
99. Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci U S A* (1997) **94**:3195–9. doi:10.1073/pnas.94.7.3195
100. Cabrera M, Shaw MA, Sharples C, Williams H, Castes M, Convit J, et al. Polymorphism in tumor necrosis factor genes associated with mucocutaneous leishmaniasis. *J Exp Med* (1995) **182**:1259–64. doi:10.1084/jem.182.5.1259
101. Sohail M, Kaul A, Bali P, Raziuddin M, Singh MP, Singh OP, et al. Alleles -308A and -1031C in the TNF-alpha gene promoter do not increase the risk but associated with circulating levels of TNF-alpha and clinical features of *vivax* malaria in Indian patients. *Mol Immunol* (2008) **45**:1682–92. doi:10.1016/j.molimm.2007.10.002
102. Kuesap J, Hirayama K, Kikuchi M, Ruangweerayut R, Na-Bangchang K. Study on association between genetic polymorphisms of haem oxygenase-1,

tumour necrosis factor, cadmium exposure and malaria pathogenicity and severity. *Malar J* (2010) **9**:260. doi:10.1186/1475-2875-9-260
103. Mendonca VR, Souza LC, Garcia GC, Magalhaes BM, Lacerda MV, Andrade BB, et al. DDX39B (BAT1), TNF and IL6 gene polymorphisms and association with clinical outcomes of patients with *Plasmodium vivax* malaria. *Malar J* (2014) **13**:278. doi:10.1186/1475-2875-13-278
104. Sortica VA, Cunha MG, Ohnishi MD, Souza JM, Ribeiro-Dos-Santos AK, Santos NP, et al. IL1B, IL4R, IL12RB1 and TNF gene polymorphisms are associated with *Plasmodium vivax* malaria in Brazil. *Malar J* (2012) **11**:409. doi:10.1186/1475-2875-11-409
105. Allcock RJ, Williams JH, Price P. The central MHC gene, BAT1, may encode a protein that down-regulates cytokine production. *Genes Cells* (2001) **6**:487–94. doi:10.1046/j.1365-2443.2001.00435.x
106. Afridi S, Atkinson A, Garnier S, Fumoux F, Rihet P. Malaria resistance genes are associated with the levels of IgG subclasses directed against *Plasmodium falciparum* blood-stage antigens in Burkina Faso. *Malar J* (2012) **11**:308. doi:10.1186/1475-2875-11-308
107. da Silva Santos S, Clark TG, Campino S, Suarez-Mutis MC, Rockett KA, Kwiatkowski DP, et al. Investigation of host candidate malaria-associated risk/protective SNPs in a Brazilian Amazonian population. *PLoS One* (2012) **7**:e36692. doi:10.1371/journal.pone.0036692
108. van der Merwe PA, Davis SJ. Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol* (2003) **21**:659–84. doi:10.1146/annurev.immunol.21.120601.141036
109. Lin HH, Zhang GL, Tongchusak S, Reinherz EL, Brusic V. Evaluation of MHC-II peptide binding prediction servers: applications for vaccine research. *BMC Bioinformatics* (2008) **9**(Suppl 12):S22. doi:10.1186/1471-2105-9-S12-S22
110. Alves CR, Pontes de Carvalho LC, Souza AL, De Simone SG. A strategy for the identification of T-cell epitopes on *Leishmania* cysteine proteinases. *Cytobios* (2001) **104**:33–41.
111. Mustafa AS. Th1 cell reactivity and HLA-DR binding prediction for promiscuous recognition of MPT63 (Rv1926c), a major secreted protein of *Mycobacterium tuberculosis*. *Scand J Immunol* (2009) **69**:213–22. doi:10.1111/j.1365-3083.2008.02221.x
112. Fonseca SG, Coutinho-Silva A, Fonseca LA, Segurado AC, Moraes SL, Rodrigues H, et al. Identification of novel consensus CD4 T-cell epitopes from clade B HIV-1 whole genome that are frequently recognized by HIV-1 infected patients. *AIDS* (2006) **20**:2263–73. doi:10.1097/01.aids.0000253353.48331.5f
113. Carlton JM, Escalante AA, Neafsey D, Volkman SK. Comparative evolutionary genomics of human malaria parasites. *Trends Parasitol* (2008) **24**:545–50. doi:10.1016/j.pt.2008.09.003
114. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* (2008) **455**:757–63. doi:10.1038/nature07327
115. Doolan DL, Southwood S, Freilich DA, Sidney J, Graber NL, Shatney L, et al. Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. *Proc Natl Acad Sci U S A* (2003) **100**:9952–7. doi:10.1073/pnas.1633254100
116. Doolan DL, Southwood S, Chesnut R, Appella E, Gomez E, Richards A, et al. HLA-DR-promiscuous T cell epitopes from *Plasmodium falciparum* pre-erythrocytic-stage antigens restricted by multiple HLA class II alleles. *J Immunol* (2000) **165**:1123–37. doi:10.4049/jimmunol.165.2.1123
117. Moreno A, Clavijo P, Edelman R, Davis J, Sztein M, Sinigaglia F, et al. CD4+ T cell clones obtained from *Plasmodium falciparum* sporozoite-immunized volunteers recognize polymorphic sequences of the circumsporozoite protein. *J Immunol* (1993) **151**:489–99.
118. Parra-Lopez C, Calvo-Calle JM, Cameron TO, Vargas LE, Salazar LM, Patarroyo ME, et al. Major histocompatibility complex and T cell interactions of a universal T cell epitope from *Plasmodium falciparum* circumsporozoite protein. *J Biol Chem* (2006) **281**:14907–17. doi:10.1074/jbc.M511571200
119. Daubenberger CA, Nickel B, Ciatto C, Grutter MG, Poltl-Frank F, Rossi L, et al. Amino acid dimorphism and parasite immune evasion: cellular immune responses to a promiscuous epitope of *Plasmodium falciparum* merozoite surface protein 1 displaying dimorphic amino acid polymorphism are highly constrained. *Eur J Immunol* (2002) **32**:3667–77. doi:10.1002/1521-4141(200212)32:12<3667::AID-IMMU3667>3.0.CO;2-C
120. Ohta N, Iwaki K, Itoh M, Fu J, Nakashima S, Hato M, et al. Epitope analysis of human T-cell response to MSP-1 of *Plasmodium falciparum* in malaria-nonexposed individuals. *Int Arch Allergy Immunol* (1997) **114**:15–22. doi:10.1159/000237637
121. Vargas LE, Parra CA, Salazar LM, Guzman F, Pinto M, Patarroyo ME. MHC allele-specific binding of a malaria peptide makes it become promiscuous on fitting a glycine residue into pocket 6. *Biochem Biophys Res Commun* (2003) **307**:148–56. doi:10.1016/S0006-291X(03)01129-X
122. Contreras CE, Ploton IN, Siliciano RF, Karp CL, Viscidi R, Kumar N. Mapping of specific and promiscuous HLA-DR-restricted T-cell epitopes on the *Plasmodium falciparum* 27-kilodalton sexual stage-specific antigen. *Infect Immun* (1998) **66**:3579–90.
123. Caro-Aguilar I, Rodriguez A, Calvo-Calle JM, Guzman F, De la Vega P, Patarroyo ME, et al. *Plasmodium vivax* promiscuous T-helper epitopes defined and evaluated as linear peptide chimera immunogens. *Infect Immun* (2002) **70**:3479–92. doi:10.1128/IAI.70.7.3479-3492.2002
124. Herrera MA, de Plata C, Gonzalez JM, Corradin G, Herrera S. Immunogenicity of multiple antigen peptides containing *Plasmodium vivax* CS epitopes in BALB/c mice. *Mem Inst Oswaldo Cruz* (1994) **89**(Suppl 2):71–6. doi:10.1590/S0074-02761994000600017
125. Fern J, Good MF. Promiscuous malaria peptide epitope stimulates CD45Ra T cells from peripheral blood of nonexposed donors. *J Immunol* (1992) **148**:907–13.
126. Wiwanitkit V. Predicted epitopes of malarial merozoite surface protein 1 by bioinformatics method: a clue for further vaccine development. *J Microbiol Immunol Infect* (2009) **42**:19–21.
127. Nanda Kumar Y, Jeyakodi G, Gunasekaran K, Jambulingam P. Computational screening and characterization of putative vaccine candidates of *Plasmodium vivax*. *J Biomol Struct Dyn* (2015) **29**:1–59. doi:10.1080/07391102.2015.1090344
128. Saravia C, Martinez P, Granados DS, Lopez C, Reyes C, Patarroyo MA. Identification and evaluation of universal epitopes in *Plasmodium vivax* Duffy binding protein. *Biochem Biophys Res Commun* (2008) **377**:1279–83. doi:10.1016/j.bbrc.2008.10.153
129. Cespedes N, Habel C, Lopez-Perez M, Castellanos A, Kajava AV, Servis C, et al. *Plasmodium vivax* antigen discovery based on alpha-helical coiled coil protein motif. *PLoS One* (2014) **9**:e100440. doi:10.1371/journal.pone.0100440
130. Jiang L, Wang ZY, Ma XJ, Zhang XP, Cai L. [Comparative analysis of nucleotide sequences of lactate dehydrogenase (LDH) gene and LDH epitopes of *Plasmodium vivax* and *Plasmodium falciparum*]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* (2010) **28**:103–7.
131. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* (1999) **50**:213–9. doi:10.1007/s002510050595
132. Sturniolo T, Bono E, Ding J, Raddrizzani L, Tuereci O, Sahin U, et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol* (1999) **17**:555–61. doi:10.1038/9858
133. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. *Bioinformatics* (2001) **17**:1236–7. doi:10.1093/bioinformatics/17.12.1236
134. Rosa DS, Iwai LK, Tzelepis F, Bargieri DY, Medeiros MA, Soares IS, et al. Immunogenicity of a recombinant protein containing the *Plasmodium vivax* vaccine candidate MSP1(19) and two human CD4+ T-cell epitopes administered to non-human primates (*Callithrix jacchus jacchus*). *Microbes Infect* (2006) **8**:2130–7. doi:10.1016/j.micinf.2006.03.012
135. Reche PA, Glutting JP, Reinherz EL. Prediction of MHC class I binding peptides using profile motifs. *Hum Immunol* (2002) **63**:701–9. doi:10.1016/S0198-8859(02)00432-9
136. Zhang GL, Khan AM, Srinivasan KN, August JT, Brusic V. MULTIPRED: a computational system for prediction of promiscuous HLA binding peptides. *Nucleic Acids Res* (2005) **33**:W172–9. doi:10.1093/nar/gki452
137. Nielsen M, Lundegaard C, Blicher T, Peters B, Sette A, Justesen S, et al. Quantitative predictions of peptide binding to any HLA-DR molecule of known sequence: NetMHCIIpan. *PLoS Comput Biol* (2008) **4**:e1000107. doi:10.1371/journal.pcbi.1000107
138. Sedegah M, Kim Y, Peters B, McGrath S, Ganeshan H, Lejano J, et al. Identification and localization of minimal MHC-restricted CD8+ T cell

epitopes within the *Plasmodium falciparum* AMA1 protein. *Malar J* (2010) **9**:241. doi:10.1186/1475-2875-9-241

139. Atanasova M, Patronov A, Dimitrov I, Flower DR, Doytchinova I. EpiDOCK: a molecular docking-based tool for MHC class II binding prediction. *Protein Eng Des Sel* (2013) **26**:631–4. doi:10.1093/protein/gzt018
140. Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, et al. Immune epitope database analysis resource. *Nucleic Acids Res* (2012) **40**:W525–30. doi:10.1093/nar/gks438
141. Kim Y, Sette A, Peters B. Applications for T-cell epitope queries and tools in the immune epitope database and analysis resource. *J Immunol Methods* (2011) **374**:62–9. doi:10.1016/j.jim.2010.10.010

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Plasmodium falciparum Secretome in Erythrocyte and Beyond

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Plasmodium falciparum is the causative agent of deadly malaria disease. It is an intracellular eukaryote and completes its multi-stage life cycle spanning the two hosts viz, mosquito and human. In order to habituate within host environment, parasite conform several strategies to evade host immune responses such as surface antigen polymorphism or modulation of host immune system and it is mediated by secretion of proteins from parasite to the host erythrocyte and beyond, collectively known as, malaria secretome. In this review, we will discuss about the deployment of parasitic secretory protein in mechanism implicated for immune evasion, protein trafficking, providing virulence, changing permeability and cyto-adherence of infected erythrocyte. We will be covering the possibilities of developing malaria secretome as a drug/vaccine target. This gathered information will be worthwhile in depicting a well-organized picture for host-pathogen interplay during the malaria infection and may also provide some clues for the development of novel anti-malarial therapies.

Keywords: plasmodium, secretome, cytoadherence, immune modulation, anti-malarial therapy

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INTRODUCTION

World Health Organization report summarized that about 198 million cases and 0.58 million deaths occurred in year 2013 (World Health Organization [WHO], 2014). Amongst different species of *Plasmodium*, *P. falciparum* is the most dangerous and responsible for severe complexities during infection like multi-organ failure, cerebral malaria, coma, and death (Miller et al., 1994; Mendis and Carter, 1995). *P. falciparum* completes its life cycle spanning two alternate host, human, and mosquito. Within the human host, parasite undergoes series of developmental stages in the liver and erythrocytes (RBCs). The intra-erythrocytic cycle is found to be important as it is responsible for patho-physiology of the disease (Miller et al., 2002). Within the erythrocyte, the parasite proceeds through the different morphological stages such as ring, trophozoite, and schizont (Bannister et al., 2000; Florens et al., 2002). After completion of infection cycle, erythrocyte gets ruptured, and merozoites are released into the host bloodstream. The released merozoites initiate next round of erythrocytic cycle by infecting fresh erythrocytes (Gilson and Crabb, 2009). The survival of parasite inside the host cell is difficult, ascribed to which *Plasmodium* adapts various strategies to avoid the host immune response (Miller et al., 1994; Hisaeda et al., 2005). The strategies encompass the secretion of hitherto of proteins against infected RBC (iRBC) surface and beyond it into the host plasma (Singh et al., 2009). Infected erythrocyte surface remodeling is an example of such phenomena, involving the insertion of secreted proteins into the iRBC membrane (Deitsch and Wellems, 1996; Parker et al., 2004). Remodeling assists in cyto-adherence of iRBCs to the endothelial lining of blood vessels and thus averting them from splenic clearance (Newbold et al., 1999; Cooke et al., 2001; Rowe et al., 2009). Host immune modulation

is another phenomenon involving the release of secretory proteins before or along with rupture of iRBCs. The released proteins interact with the components of the host immune system to provide conducive environment for merozoites before they invade new erythrocytes (Singh et al., 2009). The entire set of secreted proteins is known as 'Secretome.' These proteins are implicated in the processes essential for parasite survival such as host–parasite interactions and immune modulation (Ranganathan and Garg, 2009). Hence, in this review we will be focusing on parasite secretome and its significance in the malaria biology.

IDENTIFICATION OF MALARIA PARASITE'S SECRETOME

The export of secretory proteins from various species including *P. falciparum* has been thoroughly studied (van Ooij et al., 2008). Transport of secretory proteins follows a complicated route due to the presence of three membranes of parasite and vacuole inside the host cell (Martin et al., 2009). There must be a defined mechanism or recognizable sequence motifs behind such complex transport. Advent of bioinformatics tools facilitates the prediction of such conserved signal sequences responsible for the export and localization of the secretory proteins (Hiller et al., 2004; Marti et al., 2004, 2005).

The identification of sequence motifs necessary for export of parasite proteins is required for unearthing the complete secretome of the parasite. The first report proposed the presence of host targeting signal (HT motif) (Hiller et al., 2004) or *Plasmodium* export element (PEXEL motif) (Marti et al., 2004) in the sequence that is a requisite for the export of secretory proteins from parasitophorous vacuole (PV). The HT/PEXEL motif, present in more than 400 parasitic proteins, comprises short amino-terminal sequence, 'R/KxLxE/Q'. The role of motif in the export of both soluble and surface-associated protein is determined by green fluorescent protein (GFP) and yellow fluorescent protein (YFP) assays involving the fusion of secretory proteins such as Knob associated histidine rich protein (KAHRP), *Pf*EMP-1 (with PEXEL like motif), Glycophorin binding protein (GBP130), and members of repetitive interspersed family (rifin). Mutation or truncation of the PEXEL motif interrupted the transport of proteins and rendered their accumulation in PV itself (Marti et al., 2004; van Ooij et al., 2008). Prior to the secretion of PEXEL containing proteins to destination, N-terminus is processed in endoplasmic reticulum (ER) by protease enzyme plasmepsin V (PMV), followed by N-acetylation of the cleaved product (Chang et al., 2008; Boddey et al., 2010; Russo et al., 2010). The decisive role of PMV in export of proteins is also demonstrated by identification of transition state (TS) inhibitor, WEHI-916 (Walter and Eliza Hall Institute of Medical Research). The WEHI-916 inhibitor is found to compete with the PEXEL containing substrate resulting in blocking of activity of PMV and ultimately lead to the cessation of parasite growth at the trophozoite stage. Knockdown studies of PMV further supported the inhibitory role on PMV. Indirect hindrance of export of PfEMP-1 and the loss of virulence and cytoadherence of iRBC has been observed due to inhibitory activity of WEHI-916 (Sleebs et al., 2014a,b). Recently, another inhibitor of PMV, WEHI-842 has been identified. The inhibitory role of WEHI-842 is assessed through the immunoblotting of GFP tagged PEXEL containing PfEMP-3. It is found to be more effective in comparison to WEHI-916 (Hodder et al., 2015). The presence of PEXEL motif leads to identification of first set of parasite secretome. However, HT/PEXEL is found missing in various secretory proteins (Lingelbach and Przyborski, 2006). Analysis of such sequences showed the presence of a hydrophobic stretch in the internal region of trans-membrane proteins which helps in crossing the Parasitophorous Vacuolar Membrane (PVM). Immune localization experiments and GFP-tagged approach investigated that first 20 amino acids of N-terminus along with hydrophobic residues in trans-membrane domain are common features of all PEXEL negative export proteins (PNEPs). Thus PNEPs have further expanded the secretome repertoire of the parasite (Spielmann et al., 2006; Spielmann and Gilberger, 2010; Heiber et al., 2013).

Apart from sequence-motifs based approach, other approaches are used to predict secretory proteins of *Plasmodium*. A Position-Specific Scoring Matrix (PSSM) profile based method is adapted that employs phylogenetic relationship derived through PSI–Blast against the non-redundant database. Based on these data, web server called '*Plasmodium* Secretory and Infected erythrocyte Associated Protein prediction' (PSEApred) is developed to predict the secretory nature of *plasmodium* proteins (Verma et al., 2008). Similarly, the presence of N-myristoylation site, a cysteine S-palmitoylation site and some basic residues at N-terminus of parasite proteins are found to be responsible for the targeting to PVM and beyond (Gunaratne et al., 2000; Ma et al., 2012; Thavayogarajah et al., 2015; Wetzel et al., 2015). Together, both classical and non-classical path of secretion of proteins from parasite to the host cell have enhanced the secretome of the parasite. However, there is a possibility of appending more proteins to the growing secretome of parasite by identifying new signatures and patterns of secretion.

TRAFFICKING PATHWAY OF SECRETOME

The protein containing PEXEL motif moves from ER after cleavage by PMV to the PV either in the form of vesicular cargo (Barnwell, 1989) or through the secretory apparatus (Hinterberg et al., 1994; Taraschi et al., 2003). Proteins that are not cleaved by PMV have been shown to bind with phosphatidylinositol 3-phosphate (PI3-P) in the ER and proceed for further trafficking pathway (Bhattacharjee et al., 2012). The PNEP proteins require transmembrane domain for their transport (Heiber et al., 2013). All the proteins in the PV are found to be exported through *Plasmodium* Translocon of Exported protein (PTEX) complex (de Koning-Ward et al., 2009; Beck et al., 2014; Elsworth et al., 2014). These proteins undergo unfolding for their translocation across PV (Charpian and Przyborski, 2008; Gehde et al., 2009; Gruring et al., 2012). *P. falciparum* has developed a membranous

structure in the cytoplasm of the host cell called 'Maurer's cleft'. It is a secondary organelle and required for the export of the proteins involved in virulence, modification of host cell environment (Trager et al., 1966; Rudzinska and Trager, 1968) and for trafficking of membrane localized proteins (Przyborski et al., 2003; Lanzer et al., 2006; Mundwiler-Pachlatko and Beck, 2013). The exported proteins from PV are found to reside in the Maurer's cleft (Haldar et al., 2002). Knock-down studies of Maurer's cleft residing proteins like Membrane-Associated Histidine-Rich Protein (MAHRP1) and Skeletal binding protein-1 (SBP-1) proved its vitality in protein sorting (Epp and Deitsch, 2006; Maier et al., 2007; Spycher et al., 2008). In addition, most of the known secretory proteins including three antigenic families of parasite proteins (Stevor, Rifin, and Var) are localized in the Maurer's cleft (Cheng et al., 1998) via PTEX export system (de Koning-Ward et al., 2009). PTEX export system, found exclusively in the genus *Plasmodium,* is responsible for the translocation of proteins targeted beyond the vacuolar membrane of the parasite (de Koning-Ward et al., 2009; Desai and Miller, 2014). It is a complex of five proteins including PTEX150, Heat shock protein 101 (HSP101), exported protein 2 (EXP2), PTEX88, and thioredoxin 2 (TRX2). The passage for directing proteins toward the cytosol of host erythrocyte is formed by EXP2 (de Koning-Ward et al., 2009). TRX 2 is found to be involved in unfolding of proteins destined to pass through the PTEX. Inhibition of HSP101 leads to the obstruction in protein export and eventually the accumulation of proteins such as Ring Infected Erythrocyte Surface Antigen (RESA), Ring Exported Protein 3 (REX3), Histidine Rich Protein-1 (HRP1), and KAHRP in parasitic compartment (Beck et al., 2014). It is observed that deletion of PTEX components prevent proteins from crossing PVM, resulting in interference of parasitic growth at the ring and trophozoite stage (Elsworth et al., 2014). However, mode of recognition between proteins to be exported and those to be retained by the PTEX complex still remains unclear. The mechanism of unfolding during protein export is also not defined and therefore it opens a new window of opportunity for scientists to explore and explain the facts related to PTEX system. In addition, the presence of this export system exclusively in *Plasmodium* genus makes it a captivating drug target (de Koning-Ward et al., 2009).

HOST CELL REMODELING

In order to make opportune environment within host, parasite makes substantial modifications in the host erythrocytes (Haldar and Mohandas, 2007). The modifications are predominantly mediated by secretion of parasite proteins across the PVM (Charpian and Przyborski, 2008; Maier et al., 2009; Goldberg and Cowman, 2010; Marti and Spielmann, 2013; Elsworth et al., 2014). The process of erythrocyte remodeling includes.

Cytoadherence

To circumvent immune clearance in spleen, infected erythrocytes get adhered to endothelial wall, which is mediated through various cell adhesion molecules like ICAMs, CD36 on blood vessels (Gardner et al., 1996; Ho and White, 1999; Bhalla et al., 2015). Some events during adhesion process such as rosette formation with fresh erythrocyte (Udomsangpetch et al., 1989), auto-agglutination due to clumping of iRBCs and platelets (Pain et al., 2001) leads to severe disease pathologies (Rowe et al., 1995, 2002; Newbold et al., 1999). *P. falciparum* erythrocytic membrane protein-1 (*Pf*EMP-1) is major virulent factor present on surface of erythrocyte (Magowan et al., 1988; Chen et al., 1998). A study regarding transgenic lines of *P. falciparum* with altered *Pf*EMP-1 expression shows strong immune response targeted against *Pf*EMP-1 (Chan et al., 2012). Alteration in functioning of B-cells during parasite infection comprehends the interaction between cysteine-rich inter-domain region 1α (CIDRα) of *Pf*EMP-1 and B-cells. The complex formed causes the activation of NF-kB pathway resulting in functional impairment of B-cells (Simone et al., 2011). Multiple *Pf*EMP-1 proteins of *P. falciparum* bind to Fc portion of IgM (Jeppesen et al., 2015; Stevenson et al., 2015a) and found to be involved in rosette formation (Stevenson et al., 2015a,b). In addition to *Pf*EMP-1, sub-telomeric variant open reading frame (STEVOR) and RIFIN members also play decisive role in rosette formation (Cheng et al., 1998; Kyes et al., 1999; Niang et al., 2014). The antigenic variation of proteins allows the parasite to escape host immune response (Bull et al., 1998). A protruding structure on the surface of erythrocyte namely 'knob' is found to be essential in adhesion process of iRBCs (Crabb et al., 1997). Some proteins localized to knob interact with surface proteins of erythrocytes. Interactions include binding of KAHRP with ankyrin R and pro-coagulant glucosaminoglycans (Waller et al., 1999; Wickham et al., 2001; Rug et al., 2006; Weng et al., 2014) and binding of *Plasmodium* helical interspersed sub-telomeric domain (PHIST) to *Pf*EMP-1 (Oberli et al., 2014). In case of cerebral malaria, *Pf*14_075, member of PHIST family is found to be highly up-regulated and binds to human brain endothelial cell line (HBEC-5i). The study indicates its mantle in cyto-adherence (Claessens et al., 2012). Proteins such as erythrocyte membrane protein 3 (*Pf*EMP3), Mature parasite-infected Erythrocyte Surface Antigen (MESA; Lustigman et al., 1990), RESA, *Pf*EMP-1 (Sharma, 1997; Horrocks et al., 2005), KAHRP (Rug et al., 2006) and *Pf*EMP3 (Knuepfer et al., 2005) are involved in knob formation. Merozoites Surface Protein-1 (MSP-1), another knob protein, shows interaction with RBC surface proteins like Band 3 and Glycophorin A (GPA). A study of mouse model deficient in GPA-Band3 complex described the role of knob formation in cyto-adherence. (Goel et al., 2003; Baldwin et al., 2015).

Membrane Permeability

Secretory proteins make astonishing alterations in the permeability of iRBCs membrane for ions and nutrient exchange. (Homewood and Neame, 1974; Ginsburg et al., 1983; Kutner et al., 1983). The presence of ion channels such as *Plasmodium* Surface Anion Channel (PSAC) is responsible for induction of drug resistance. The identification of structural composition of this complex would contribute to better understanding of

pathogenic interaction and drug resistance mechanism and therefore suggested for therapeutic intervention (Lisk et al., 2008; Desai, 2012). Cytoadherence-linked antigen3 (Clag3) protein, found on the host membrane is appraised to be associated with PSAC in ion and nutrient transport through channels (Nguitragool et al., 2011; Pillai et al., 2012; Sharma et al., 2015). Secretory proteins involved in regulation of net flux of Na^+, K^+, and other ions are on the focus (Kirk, 2015). For instance, P-type ATP4 (*Pf* ATP4), regulating the transport of Na^+ ions is contemplated as a potential drug target (Spillman et al., 2013). Membrane permeabilization is found to be a necessary event for egress of parasites from iRBCs. Cysteine proteases have been shown to play cardinal role in rupture of erythrocyte membrane for the release of parasite (Hadley et al., 1983; Dluzewski et al., 1986; McKerrow et al., 1993; Raphael et al., 2000; Lee and Fidock, 2008). One of the members of this class, falcipian 2 is responsible for the cleavage of ankyrin and protein 4.1 of erythrocytic cytoskeleton (Dua et al., 2001). It has been evident through the gene disruption studies that expression of *Plasmodium* perforin like protein 2 (PPLP2) is paramount for membrane permeabilization during the gametocyte release from infected erythrocytes. It had been illustrated that gametocytes are unable to release from PPLP2 (−) lines of parasite, thereby reducing the transfer of gametocyte to vector (Wirth et al., 2014). MSP-1 has also been demonstrated to interact with host cytoskeleton spectrin causing the membrane destabilization and thereby enabling the release of merozoites from iRBC (Das et al., 2015).

Membrane Rigidity

Apart from cytoadherence, membrane rigidness or loss of deformability is also responsible for the sequestration of iRBCs (Bull et al., 1998). It has been clarified that knobs are liable for causing stiffness and hardening of the iRBCs (Zhang et al., 2015). Deformability of parasitized RBC is reduced due to association of RESA with spectrin (Mills et al., 2007). *Pf* 332 exported on the membrane is directly involved in membrane rigidity and adhesion (Glenister et al., 2009). The KAHRP along with the membrane skeleton imparts rigidity to infected cell and will eventually obstruct blood flow (Waller et al., 1999; Pei et al., 2005). PHIST protein increases membrane rigidity by binding to membrane skeleton (Parish et al., 2013). Thus, it can be surmised that proteins responsible for rigidity are directly linked to virulence, providing an evidence for secretome in establishment of infection.

SECRETORY PROTEIN EXPORTED BEYOND THE ERYTHROCYTE

Most of the data reported with respect to secretome is related to proteins secreted into the erythrocytes cytosol or membrane. Interestingly, some proteins, which are not restricted to iRBCs membrane rather squeeze out from iRBCs membrane and get secreted out. First experimental evidence (Singh et al., 2009) identified secretion of 27 novel proteins beyond the erythrocyte membrane before it gets ruptured. Immune localization and immune electron microscopic studies confirmed the secretion of proteins beyond iRBC (Singh et al., 2009). Some of them are functionally characterized. The protein containing Sel-1 functional domain is found to be involved in regulating 'Notch signaling pathway' which in turn has been hypothesized to influence the T cell differentiation (Grant and Greenwald, 1996; Singh et al., 2009). In most protozoan parasites, to evade host immune response, common mechanism includes altered T-helper cell differentiation (Zambrano-Villa et al., 2002; Rodrigues et al., 2014). Some proteins, closely associated with highly polymorphic genes, contribute to antigenic determinants of parasite (Singh et al., 2009). Secretory protein with LCCL (Limulus clotting factor C) domain, conserved across apicomplexan parasite, assumed to have role in immune evasion mechanism, (Claudianos et al., 2002; Dessens et al., 2004), defense mechanism and shows binding with lipid A of lipopolysaccharides. CRISPLD2 (Cysteine-Rich Secretory Protein LCCL Domain containing 2), an example of LCCL domain containing protein, has an anti-inflammatory function and is related to disease pathology (Vásárhelyi et al., 2014).

Sequence similarity studies suggested that some proteins viz, virulent immuno-reactive protein (specific to bacteria and virus), PFB0765w (uncharacterized protein), rhoptry neck protein (RON4), moving junction protein and MAL13P1.39 (uncharacterized protein) are involved in modulation of host immune response (Singh et al., 2009). Domain analysis demonstrated the presence of extracellular domain responsible for the interaction with other proteins, speculative of being involved in host–parasite interactions. [**Table 1**: Domains identified by CDD (Conserved Domain Database), NCBI]. It is depicted through flowcytometery and confocal microscopy that translationally controlled tumor protein (TCTP) analog released by *Plasmodium* in host serum is responsible for release of histamine and IL-8 from basophils and eosinophils, respectively, (MacDonald et al., 2001) and reduction in B-cell immune response. In another study, a canonical tyrosyl-tRNA synthetase (*Pf* TyrRS) from *Plasmodium* is evidenced to be secreted out from the iRBCs and involved in non-canonical function of immune cell binding and modulation (Bhatt et al., 2011). Likewise in other intracellular pathogens such as *Mycobacterium tuberculosis* immune modulation ability is found in secretory proteins (Giacomini et al., 2001). During infection, secretome is also charged for causing alteration in functioning of antigen-presenting cells and dendritic cells (Sacks and Sher, 2002; Langhorne et al., 2004; Millington et al., 2006; Sponaas et al., 2006; Teirlinck et al., 2015). The presence of proteins on the surface or in secretion implicate their role in host–parasite interactions and probably in immune modulation for better survival of parasite and it would be fascinating to have information related to the 'Interactome' of the secretory proteins. *In-silico* knock-out studies and graphical analysis of protein–protein interaction network (PPIN) explored newer approach in order to identify the interacting partners vital to parasite during host–parasite interaction (Bhattacharyya and Chakrabarti, 2015). Nevertheless,

TABLE 1 | Some secretory protein exported out from the iRBC.

Sr. No	Gene name	Domain description/protein name	Reference
1	MAL7P1.138	_	Singh et al., 2009
2	MAL8P1.126	Serine protease DegP	Singh et al., 2009
3	MAL13P1.24	–	Singh et al., 2009
4	MAL13P1.39	_	Singh et al., 2009
5	*PF*07_0074	_	Singh et al., 2009
6	*PF*07_0086	Uncharacterized protein with domain 1. TATA element modulatory factor 1 2. DNA repair protein RAD18	Singh et al., 2009
7	*PF*07_0113	_	Singh et al., 2009
8	GBP-*PF*10_0159	1. Glycophorin-binding protein	Singh et al., 2009
9	*PF*10_0318	1. Uncharacterized / ACR, YagE family domain	Singh et al., 2009
10	*PF*10_0380	Trophozoite antigen R45, putative	Singh et al., 2009
11	*PF* EP *PF*11_0139	Protein tyrosine phosphates	Singh et al., 2009
12	RON4 *PF*11_0168	Moving junction protein	Singh et al., 2009
13	TKL-2 *PF*11_0220	Protein Kinase	Singh et al., 2009
14	*PF*11_0324	Uncharacterized protein	Singh et al., 2009
15	*PF*11_0369	Uncharacterized protein	Singh et al., 2009
16	*PF*11_0381	Subtilisin-like protease 2	Singh et al., 2009
18	*PF*13_0198	Reticulocyte-binding protein 2 homolog a	Singh et al., 2009
19	*PF*14_0462	SEL-1 protein, putative	Singh et al., 2009
20	CCP1 *PF*14_0723	LCCL domain containing protein CCP1	Singh et al., 2009
21	*PF*A018w	1. L-seryl-tRNA(Sec) kinase, 2. Predicted nucleotide kinase	Singh et al., 2009
22	*PF*B0190c	Conserved *Plasmodium* protein with domain 1. Sel1-like repeats 2. TPR repeat, SEL1 subfamily	Singh et al., 2009
23	*PF*B0315w	Uncharacterized protein *PF*B0315w	Singh et al., 2009
24	*PF*b0465c	Monocarboxylate transporter, putative with domain 1. The Major Facilitator Super family (MFS) 2. Oxalate/formate antiporter family transporter. 3. Monocarboxylate transporter	Singh et al., 2009
25	*PF*B0655c	Conserved *Plasmodium* protein	Singh et al., 2009
26	*PF*B0750w	Vacuolar protein-sorting protein VPS45, putative	Singh et al., 2009
27	*PF*B0765w	Uncharacterized protein *PF*B0765w with domain 1. Chromosome segregation ATPases 2. Myosin class II heavy chain [Cytoskeleton]	Singh et al., 2009
28	*PF*E0245c	Uncharacterized protein with domain 1. Dos2-interacting transcription regulator of RNA-Pol-II 2. DNA repair/transcription protein Mms19 3. Ultrahigh sulfur keratin-associated protein	Singh et al., 2009
29	*PF*E0440w	Uncharacterized	Singh et al., 2009
30	*PF*L0030c	Erythrocyte membrane protein 1 (*PF*EMP1)	Singh et al., 2009
31	*PF*I1150w	HRP II/I II domain	Singh et al., 2009
32	*PF*L2405c	Chromosome segregation protein SMC (structural maintenance of chromosomes) *PF*G377 protein	Singh et al., 2009
33	*PF*TyrRS	Tyrosyl-tRNA synthetase	Bhatt et al., 2011
34	*PF*TCTP	*Plasmodium falciparum* translationally controlled tumor protein	MacDonald et al., 2001; Calderon-Perez et al., 2014

some more studies are required to understand the role of secretory proteins in regulating host pathways. The role of secretory proteins of the parasite could have larger impact on malaria biology. Besides available knowledge, there is a need to identify signature motif or pattern responsible for secretion of proteins outside iRBCs. The identification of marker responsible for the localization of proteins to infected erythrocyte membrane and their export will be highly beneficial in interaction studies. There is a requirement of classifying secretome in terms of cellular localization and expression during developmental stages of parasite in order to understand its role in better way. It would be interesting to explore trafficking pathway of protein exported beyond the iRBCs membrane.

SECRETOME AS POTENTIAL DRUG/VACCINE TARGET

The intracellular parasite adapts different strategies for protein export in order to survive in host environment. As the secretome is intimately associated with disease pathology and parasite survival is reliant on them, any interference in the secretory pathway or inhibition of secretory proteins itself would jeopardize the parasite. In addition, utilization of information of secretome available shall provide clues to certain strategies involved in host–parasite interaction at molecular level (Ranganathan and Garg, 2009). The function of these proteins can be annotated by comparing with homologous protein of known function in other organisms. Homology modeling of secretory proteins could also provide a starting point for the lead identification in the process of drug development. Vaccine and drug development against the secretory protein is in progress in various other pathogens like *H. pylori* (Lower et al., 2008), Helminths parasite (Hewitson et al., 2009) etc. Till date, various parasite proteins involved in secretory pathway have been characterized and may be critical in anti-malarial drug targeting such as inhibition of PTEX complex. Another important drug target capturing the interest in context of drug development area is PMV (plasmepsin V). Indispensability of PMV in virulence, cytoadherence, and parasitic growth makes PMV an attractive anti-malarial drug target (Sleebs et al., 2014a,b). Structural determination of PMV–WEHI 842 inhibitor complex provides an insight for interaction between active site residue and inhibitor. This study paves the way for developing potent anti-malarial by blocking export machinery of parasite (Hodder et al., 2015).

Taken together, the functional characterization of secretory proteins and proteins involved in their export, implicated in knob formation, involved in trafficking pathway, or those involved in host pathogenic interaction and invasion of host immune system, are all indispensible for parasite survival or pathogenicity. Therefore, complete investigation and characterization of secretome may provide us better understanding to get effective therapies for malaria disease. Allelic replacement and GFP tagging revealed the importance of PMV in protein export and parasite survival thus making it an attractive target for anti malarial drugs.

CONCLUSION

Export of secretory parasite proteins into host cytoplasm will lead to apprehension of host cell functions required for parasite growth and survival by modulating crucial phenomena of malaria biology such as immune evasion and virulence. Deep understanding and investigation of role played by malaria secretome will be not only beneficial in deciphering host–pathogen interactions but it may also lead to better therapeutic intervention for malaria disease.

AUTHOR CONTRIBUTIONS

RS and DS provided data and TB wrote the manuscript.

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REFERENCES

Baldwin, M. R., Li, X., Hanada, T., Liu, S.-C., and Chishti, A. H. (2015). Merozoite surface protein 1 recognition of host glycophorin A mediates malaria parasite invasion of red blood cells. *Blood* 125, 2704–2711. doi: 10.1182/blood-2014-11-611707

Bannister, L. H., Hopkins, J. M., Fowler, R. E., Krishna, S., and Mitchell, G. H. (2000). A Brief illustrated guide to the ultrastructure of *Plasmodium falciparum* asexual blood stages. *Parasitol. Today* 16, 427–433. doi: 10.1016/S0169-4758(00)01755-5

Barnwell, J. (1989). Vesicle-mediated transport of membrane and proteins in malaria-infected erythrocytes. *Blood Cells* 16, 379–395.

Beck, J. R., Muralidharan, V., Oksman, A., and Goldberg, D. E. (2014). PTEX component HSP101 mediates export of diverse malaria effectors into host erythrocytes. *Nature* 511, 592–595. doi: 10.1038/nature13574

Bhalla, K., Chugh, M., Mehrotra, S., Rathore, S., Tousif, S., Prakash Dwivedi, V., et al. (2015). Host ICAMs play a role in cell invasion by *Mycobacterium tuberculosis* and *Plasmodium falciparum*. *Nat. Commun.* 6:6049. doi: 10.1038/ncomms7049

Bhatt, T. K., Khan, S., Dwivedi, V. P., Banday, M. M., Sharma, A., Chandele, A., et al. (2011). Malaria parasite tyrosyl-tRNA synthetase secretion triggers pro-inflammatory responses. *Nat. Commun.* 2:530. doi: 10.1038/ncomms1522

Bhattacharjee, S., Stahelin, R. V., Speicher, K. D., Speicher, D. W., and Haldar, K. (2012). Endoplasmic reticulum PI(3)P lipid binding targets malaria proteins to the host cell. *Cell* 148, 201–212. doi: 10.1016/j.cell.2011.10.051

Bhattacharyya, M., and Chakrabarti, S. (2015). Identification of important interacting proteins (IIPs) in *Plasmodium falciparum* using large-scale interaction network analysis and in-silico knock-out studies. *Malar. J.* 14:70. doi: 10.1186/s12936-015-0562-1

Boddey, J. A., Hodder, A. N., Gunther, S., Gilson, P. R., Patsiouras, H., Kapp, E. A., et al. (2010). An aspartyl protease directs malaria effector proteins to the host cell. *Nature* 463, 627–631. doi: 10.1038/nature08728

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I., and Marsh, K. (1998). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat. Med.* 4, 358–360. doi: 10.1038/nm0398-358

Calderon-Perez, B., Xoconostle-Cazares, B., Lira-Carmona, R., Hernandez-Rivas, R., Ortega-Lopez, J., and Ruiz-Medrano, R. (2014). The *Plasmodium falciparum* translationally controlled tumor protein (TCTP) is incorporated more efficiently into B cells than its human homologue. *PLoS ONE* 9:e85514. doi: 10.1371/journal.pone.0085514

Chan, J.-A., Howell, K. B., Reiling, L., Ataide, R., Mackintosh, C. L., Fowkes, F. J. I., et al. (2012). Targets of antibodies against *Plasmodium falciparum*–infected erythrocytes in malaria immunity. *J. Clin. Invest.* 122, 3227–3238. doi: 10.1172/JCI62182

Chang, H. H., Falick, A. M., Carlton, P. M., Sedat, J. W., DeRisi, J. L., and Marletta, M. A. (2008). N-terminal processing of proteins exported by malaria parasites. *Mol. Biochem. Parasitol.* 160, 107–115. doi: 10.1016/j.molbiopara.2008.04.011

Charpian, S., and Przyborski, J. M. (2008). Protein transport across the parasitophorous vacuole of *Plasmodium falciparum*: into the great wide open. *Traffic* 9, 157–165. doi: 10.1111/j.1600-0854.2007.00648.x

Chen, Q., Barragan, A., Fernandez, V., Sundström, A., Schlichtherle, M., Sahlén, A., et al. (1998). Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. *J. Exp. Med.* 187, 15–23. doi: 10.1084/jem.187.1.15

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., Lanzer, M., et al. (1998). stevor and rif are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. *Mol. Biochem. Parasitol.* 97, 161–176. doi: 10.1016/S0166-6851(98)00144-3

Claessens, A., Adams, Y., Ghumra, A., Lindergard, G., Buchan, C. C., Andisi, C., et al. (2012). A subset of group A-like var genes encodes the malaria parasite ligands for binding to human brain endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1772–E1781. doi: 10.1073/pnas.1120461109

Claudianos, C., Dessens, J. T., Trueman, H. E., Arai, M., Mendoza, J., Butcher, G. A., et al. (2002). A malaria scavenger receptor-like protein essential for parasite development. *Mol. Microbiol.* 45, 1473–1484. doi: 10.1046/j.1365-2958.2002.03118.x

Cooke, B. M., Mohandas, N., and Coppel, R. L. (2001). The malaria-infected red blood cell: structural and functional changes. *Adv. Parasitol.* 50, 1–86. doi: 10.1016/S0065-308X(01)50029-9

Crabb, B. S., Cooke, B. M., Reeder, J. C., Waller, R. F., Caruana, S. R., Davern, K. M., et al. (1997). Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell* 89, 287–296. doi: 10.1016/S0092-8674(00)80207-X

Das, S., Hertrich, N., Perrin A. J., Withers-Martinez, C., Collins, C. R., Jones, M. L., et al. (2015). Processing of *Plasmodium falciparum* merozoite surface protein msp1 activates a spectrin-binding function enabling parasite egress from RBCs. *Cell Host Microbe* 18, 433–444. doi: 10.1016/j.chom.2015.09.007

Deitsch, K. W., and Wellems, T. E. (1996). Membrane modifications in erythrocytes parasitized by *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 76, 1–10. doi: 10.1016/0166-6851(95)02575-8

de Koning-Ward, T. F., Gilson, P. R., Boddey, J. A., Rug, M., Smith, B. J., Papenfuss, A. T., et al. (2009). A novel protein export machine in malaria parasites. *Nature* 459, 945–949. doi: 10.1038/nature08104

Desai, S. A. (2012). Ion and nutrient uptake by malaria parasite-infected erythrocytes. *Cell Microbiol.* 14, 1003–1009. doi: 10.1111/j.1462-5822.2012.01790.x

Desai, S. A., and Miller, L. H. (2014). Malaria: protein-export pathway illuminated. *Nature* 511, 541–542. doi: 10.1038/nature13646

Dessens, J. T., Sinden, R. E., and Claudianos, C. (2004). LCCL proteins of apicomplexan parasites. *Trends Parasitol.* 20, 102–108. doi: 10.1016/j.pt.2004.01.002

Dluzewski, A. R., Rangachari, K., Wilson, R. J. M., and Gratzer, W. B. (1986). *Plasmodium falciparum*: protease inhibitors and inhibition of erythrocyte invasion. *Exp. Parasitol.* 62, 416–422. doi: 10.1016/0014-4894(86)90050-0

Dua, M., Raphael, P., Sijwali, P. S., Rosenthal, P. J., and Hanspal, M. (2001). Recombinant falcipain-2 cleaves erythrocyte membrane ankyrin and protein 4.1. *Mol. Biochem. Parasitol.* 116, 95–99. doi: 10.1016/S0166-6851(01)00306-1

Elsworth, B., Matthews, K., Nie, C. Q., Kalanon, M., Charnaud, S. C., Sanders, P. R., et al. (2014). PTEX is an essential nexus for protein export in malaria parasites. *Nature* 511, 587–591. doi: 10.1038/nature13555

Epp, C., and Deitsch, K. (2006). Deciphering the export pathway of malaria surface proteins. *Trends Parasitol.* 22, 401–404. doi: 10.1016/j.pt.2006.07.002

Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., et al. (2002). A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* 419, 520–526. doi: 10.1038/nature01107

Gardner, J. P., Pinches, R. A., Roberts, D. J., and Newbold, C. I. (1996). Variant antigens and endothelial receptor adhesion in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3503–3508. doi: 10.1073/pnas.93.8.3503

Gehde, N., Hinrichs, C., Montilla, I., Charpian, S., Lingelbach, K., and Przyborski, J. M. (2009). Protein unfolding is an essential requirement for transport across the parasitophorous vacuolar membrane of *Plasmodium falciparum*. *Mol. Microbiol.* 71, 613–628. doi: 10.1111/j.1365-2958.2008.06552.x

Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., et al. (2001). Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J. Immunol.* 166, 7033–7041. doi: 10.4049/jimmunol.166.12.7033

Gilson, P. R., and Crabb, B. S. (2009). Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *Int. J. Parasitol.* 39, 91–96. doi: 10.1016/j.ijpara.2008.09.007

Ginsburg, H., Krugliak, M., Eidelman, O., and Ioav Cabantchik, Z. (1983). New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. *Mol. Biochem. Parasitol.* 8, 177–190. doi: 10.1016/0166-6851(83)90008-7

Glenister, F. K., Fernandez, K. M., Kats, L. M., Hanssen, E., Mohandas, N., Coppel, R. L., et al. (2009). Functional alteration of red blood cells by a megadalton protein of *Plasmodium falciparum*. *Blood* 113, 919–928. doi: 10.1182/blood-2008-05-157735

Goel, V. K., Li, X., Chen, H., Liu, S.-C., Chishti, A. H., and Oh, S. S. (2003). Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5164–5169. doi: 10.1073/pnas.0834959100

Goldberg, D. E., and Cowman, A. F. (2010). Moving in and renovating: exporting proteins from *Plasmodium* into host erythrocytes. *Nat. Rev. Microbiol.* 8, 617–621. doi: 10.1038/nrmicro2420

Grant, B., and Greenwald, I. (1996). The *Caenorhabditis elegans* sel-1 gene, a negative regulator of lin-12 and glp-1, encodes a predicted extracellular protein. *Genetics* 143, 237–247.

Gruring, C., Heiber, A., Kruse, F., Flemming, S., Franci, G., Colombo, S. F., et al. (2012). Uncovering common principles in protein export of malaria parasites. *Cell Host Microbe* 12, 717–729. doi: 10.1016/j.chom.2012.09.010

Gunaratne, R. S., Sajid, M., Ling, I. T., Tripathi, R., Pachebat, J. A., and Holder, A. A. (2000). Characterization of N-myristoyltransferase from *Plasmodium falciparum*. *Biochem. J.* 348(Pt 2), 459–463. doi: 10.1042/bj3480459

Hadley, T., Aikawa, M., and Miller, L. H. (1983). *Plasmodium knowlesi*: studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. *Exp. Parasitol.* 55, 306–311. doi: 10.1016/0014-4894(83)90027-9

Haldar, K., and Mohandas, N. (2007). Erythrocyte remodeling by malaria parasites. *Curr. Opin. Hematol.* 14, 203–209. doi: 10.1097/MOH.0b013e3280f31b2d

Haldar, K., Mohandas, N., Samuel, B. U., Harrison, T., Hiller, N. L., Akompong, T., et al. (2002). Protein and lipid trafficking induced in erythrocytes infected by malaria parasites. *Cell Microbiol.* 4, 383–395. doi: 10.1046/j.1462-5822.2002.00204.x

Heiber, A., Kruse, F., Pick, C., Gruring, C., Flemming, S., Oberli, A., et al. (2013). Identification of new PNEPs indicates a substantial non-PEXEL exportome and underpins common features in *Plasmodium falciparum* protein export. *PLoS Pathog.* 9:e1003546. doi: 10.1371/journal.ppat.1003546

Hewitson, J. P., Grainger, J. R., and Maizels, R. M. (2009). Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. *Mol. Biochem. Parasitol.* 167, 1–11. doi: 10.1016/j.molbiopara.2009.04.008

Hiller, N. L., Bhattacharjee, S., van Ooij, C., Liolios, K., Harrison, T., Lopez-Estrano, C., et al. (2004). A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science* 306, 1934–1937. doi: 10.1126/science.1102737

Hinterberg, K., Scherf, A., Gysin, J., Toyoshima, T., Aikawa, M., Mazie, J. C., et al. (1994). *Plasmodium falciparum*: the Pf332 antigen is secreted from the parasite by a brefeldin A-dependent pathway and is translocated to the erythrocyte membrane via the Maurer's clefts. *Exp. Parasitol.* 79, 279–291. doi: 10.1006/expr.1994.1091

Hisaeda, H., Yasutomo, K., and Himeno, K. (2005). Malaria: immune evasion by parasites. *Int. J. Biochem. Cell Biol.* 37, 700–706. doi: 10.1016/j.biocel.2004.10.009

Ho, M., and White, N. J. (1999). Molecular mechanisms of cytoadherence in malaria. *Am. J. Physiol. Cell Physiol.* 276, C1231–C1242.

Hodder, A. N., Sleebs, B. E., Czabotar, P. E., Gazdik, M., Xu, Y., O'Neill, M. T., et al. (2015). Structural basis for plasmepsin V inhibition that blocks export of

malaria proteins to human erythrocytes. *Nat. Struct. Mol. Biol.* 22, 590–596. doi: 10.1038/nsmb.3061

Homewood, C. A., and Neame, K. D. (1974). Malaria and the permeability of the host erythrocyte. *Nature* 252, 718–719. doi: 10.1038/252718a0

Horrocks, P., Pinches, R. A., Chakravorty, S. J., Papakrivos, J., Christodoulou, Z., Kyes, S. A., et al. (2005). PfEMP1 expression is reduced on the surface of knobless *Plasmodium falciparum* infected erythrocytes. *J. Cell Sci.* 118, 2507–2518. doi: 10.1242/jcs.02381

Jeppesen, A., Ditlev, S. B., Soroka, V., Stevenson, L., Turner, L., Dzikowski, R., et al. (2015). Multiple *Plasmodium falciparum* erythrocyte membrane protein 1 variants per genome can bind IgM via Its Fc fragment fcmu. *Infect. Immun.* 83, 3972–3981.

Kirk, K. (2015). Ion regulation in the malaria parasite. *Annu. Rev. Microbiol.* 69, 341–359. doi: 10.1146/annurev-micro-091014-104506

Knuepfer, E., Rug, M., Klonis, N., Tilley, L., and Cowman, A. F. (2005). Trafficking determinants for PfEMP3 export and assembly under the *Plasmodium falciparum*-infected red blood cell membrane. *Mol. Microbiol.* 58, 1039–1053. doi: 10.1111/j.1365-2958.2005.04895.x

Kutner, S., Ginsburg, H., and Cabantchik, Z. I. (1983). Permselectivity changes in malaria (*Plasmodium falciparum*) infected human red blood cell membranes. *J. Cell. Physiol.* 114, 245–251. doi: 10.1002/jcp.1041140215

Kyes, S. A., Rowe, J. A., Kriek, N., and Newbold, C. I. (1999). Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9333–9338. doi: 10.1073/pnas.96.16.9333

Langhorne, J. R., Albano, F., Hensmann, M., Sanni, L., Cadman, E., Voisine, C., et al. (2004). Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. *Immunol. Rev.* 201, 35–47.

Lanzer, M., Wickert, H., Krohne, G., Vincensini, L., and Breton, C. B. (2006). Maurer's clefts: a novel multi-functional organelle in the cytoplasm of *Plasmodium falciparum*-infected erythrocytes. *Int. J. Parasitol.* 36, 23–36. doi: 10.1016/j.ijpara.2005.10.001

Lee, M. C. S., and Fidock, D. A. (2008). Arresting malaria parasite egress from infected red blood cells. *Nat. Chem. Biol.* 4, 161–162. doi: 10.1038/nchembio0308-161

Lingelbach, K., and Przyborski, J. M. (2006). The long and winding road: protein trafficking mechanisms in the *Plasmodium falciparum* infected erythrocyte. *Mol. Biochem. Parasitol.* 147, 1–8. doi: 10.1016/j.molbiopara.2006.01.014

Lisk, G., Pain, M., Gluzman, I. Y., Kambhampati, S., Furuya, T., Su, X.-Z., et al. (2008). Changes in the plasmodial surface anion channel reduce leupeptin uptake and can confer drug resistance in *Plasmodium falciparum*-infected erythrocytes. *Antimicrob. Agents Chemother.* 52, 2346–2354.

Lower, M., Weydig, C., Metzler, D., Reuter, A., Starzinski-Powitz, A., Wessler, S., et al. (2008). Prediction of extracellular proteases of the human pathogen *Helicobacter pylori* reveals proteolytic activity of the Hp1018/19 protein HtrA. *PLoS ONE* 3:e3510. doi: 10.1371/journal.pone.0003510

Lustigman, S., Anders, R. F., Brown, G. V., and Coppel, R. L. (1990). The mature-parasite-infected erythrocyte surface antigen (MESA) of *Plasmodium falciparum* associates with the erythrocyte membrane skeletal protein, band 4.1. *Mol. Biochem. Parasitol.* 38, 261–270. doi: 10.1016/0166-6851(90)90029-L

Ma, J., Rahlfs, S., Jortzik, E., Schirmer, R. H., Przyborski, J. M., and Becker, K. (2012). Subcellular localization of adenylate kinases in *Plasmodium falciparum*. *FEBS Lett.* 586, 3037–3043. doi: 10.1016/j.febslet.2012.07.013

MacDonald, S. M., Bhisutthibhan, J., Shapiro, T. A., Rogerson, S. J., Taylor, T. E., Tembo, M., et al. (2001). Immune mimicry in malaria: *Plasmodium falciparum* secretes a functional histamine-releasing factor homolog in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10829–10832. doi: 10.1073/pnas.191491498

Magowan, C., Wollish, W., Anderson, L., and Leech, J. (1988). Cytoadherence by *Plasmodium falciparum*-infected erythrocytes is correlated with the expression of a family of variable proteins on infected erythrocytes. *J. Exp. Med.* 168, 1307–1320. doi: 10.1084/jem.168.4.1307

Maier, A. G., Cooke, B. M., Cowman, A. F., and Tilley, L. (2009). Malaria parasite proteins that remodel the host erythrocyte. *Nat. Rev. Microbiol.* 7, 341–354. doi: 10.1038/nrmicro2110

Maier, A. G., Rug, M., O'Neill, M. T., Beeson, J. G., Marti, M., Reeder, J., et al. (2007). Skeleton-binding protein 1 functions at the parasitophorous vacuole membrane to traffic PfEMP1 to the *Plasmodium falciparum*–infected erythrocyte surface. *Blood* 109, 1289–1297. doi: 10.1182/blood-2006-08-043364

Marti, M., Baum, J., Rug, M., Tilley, L., and Cowman, A. F. (2005). Signal-mediated export of proteins from the malaria parasite to the host erythrocyte. *J. Cell Biol.* 171, 587–592. doi: 10.1083/jcb.200508051

Marti, M., Good, R. T., Rug, M., Knuepfer, E., and Cowman, A. F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* 306, 1930–1933. doi: 10.1126/science.1102452

Marti, M., and Spielmann, T. (2013). Protein export in malaria parasites: many membranes to cross. *Curr. Opin. Microbiol.* 16, 445–451. doi: 10.1016/j.mib.2013.04.010

Martin, R. E., Ginsburg, H., and Kirk, K. (2009). Membrane transport proteins of the malaria parasite. *Mol. Microbiol.* 74, 519–528. doi: 10.1111/j.1365-2958.2009.06863.x

McKerrow, J. H., Sun, E., Rosenthal, P. J., and Bouvier, J. (1993). The proteases and pathogenicity of parasitic protozoa. *Annu. Rev. Microbiol.* 47, 821–853. doi: 10.1146/annurev.mi.47.100193.004133

Mendis, K. N., and Carter, R. (1995). Clinical disease and pathogenesis in malaria. *Parasitol. Today* 11, TI1–TI16. doi: 10.1016/0169-4758(95)80143-X

Miller, L. H., Baruch, D. I., Marsh, K., and Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature* 415, 673–679. doi: 10.1038/415673a

Miller, L. H., Good, M. F., and Milon, G. (1994). Malaria pathogenesis. *Science* 264, 1878–1883. doi: 10.1126/science.8009217

Millington, O. R., Di Lorenzo, C., Phillips, R. S., Garside, P., and Brewer, J. M. (2006). Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function. *J. Biol.* 5:5. doi: 10.1186/jbiol34

Mills, J., Diez-Silva, M., Quinn, D., Dao, M., Lang, M., Tan, K., et al. (2007). Effect of plasmodial RESA protein on deformability of human red blood cells harboring *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9213–9217. doi: 10.1073/pnas.0703433104

Mundwiler-Pachlatko, E., and Beck, H.-P. (2013). Maurer's clefts, the enigma of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19987–19994. doi: 10.1073/pnas.1309247110

Newbold, C., Craig, A., Kyes, S., Rowe, A., Fernandez-Reyes, D., and Fagan, T. (1999). Cytoadherence, pathogenesis and the infected red cell surface in *Plasmodium falciparum*. *Int. J. Parasitol.* 29, 927–937. doi: 10.1016/S0020-7519(99)00049-1

Nguitragool, W., Bokhari, A. A. B., Pillai, A. D., Rayavara, K., Sharma, P., Turpin, B., et al. (2011). Malaria parasite clag genes determine nutrient uptake channel activity on infected red blood cells. *Cell* 145, 665–677. doi: 10.1016/j.cell.2011.05.002

Niang, M., Bei A. K., Madnani, K. G., Pelly, S., Dankwa, S., Kanjee, U., et al. (2014). STEVOR is a *Plasmodium falciparum* erythrocyte binding protein that mediates merozoite invasion and rosetting. *Cell Host Microbe* 16, 81–93. doi: 10.1016/j.chom.2014.06.004

Oberli, A., Slater, L. M., Cutts, E., Brand, F., Mundwiler-Pachlatko, E., Rusch, S., et al. (2014). A *Plasmodium falciparum* PHIST protein binds the virulence factor PfEMP1 and comigrates to knobs on the host cell surface. *FASEB J.* 28, 4420–4433. doi: 10.1096/fj.14–256057

Pain, A., Ferguson, D. J., Kai, O., Urban, B. C., Lowe, B., Marsh, K., et al. (2001). Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1805–1810. doi: 10.1073/pnas.98.4.1805

Parish, L. A., Mai, D. W., Jones, M. L., Kitson, E. L., and Rayner, J. C. (2013). A member of the *Plasmodium falciparum* PHIST family binds to the erythrocyte cytoskeleton component band 4.1. *Malar. J.* 12, 160–160. doi: 10.1186/1475-2875-12–160

Parker, P. D., Tilley, L., and Klonis, N. (2004). *Plasmodium falciparum* induces reorganization of host membrane proteins during intraerythrocytic growth. *Blood* 103, 2404–2406. doi: 10.1182/blood-2003-08-2692

Pei, X., An, X., Guo, X., Tarnawski, M., Coppel, R., and Mohandas, N. (2005). Structural and functional studies of interaction between *Plasmodium falciparum* knob-associated histidine-rich protein (KAHRP) and erythrocyte spectrin. *J. Biol. Chem.* 280, 31166–31171. doi: 10.1074/jbc.M505298200

Pillai, A. D., Nguitragool, W., Lyko, B., Dolinta, K., Butler, M. M., Nguyen, S. T., et al. (2012). Solute restriction reveals an essential role for clag3-associated

channels in malaria parasite nutrient acquisition. *Mol. Pharmacol.* 82, 1104–1114. doi: 10.1124/mol.112.081224

Przyborski, J. M., Wickert, H., Krohne, G., and Lanzer, M. (2003). Maurer's clefts—a novel secretory organelle? *Mol. Biochem. Parasitol.* 132, 17–26. doi: 10.1016/S0166-6851(03)00212-3

Ranganathan, S., and Garg, G. (2009). Secretome: clues into pathogen infection and clinical applications. *Genome Med.* 1:113. doi: 10.1186/gm113

Raphael, P., Takakuwa, Y., Manno, S., Liu, S. C., Chishti, A. H., and Hanspal, M. (2000). A cysteine protease activity from *Plasmodium falciparum* cleaves human erythrocyte ankyrin. *Mol. Biochem. Parasitol.* 110, 259–272. doi: 10.1016/S0166-6851(00)00283-8

Rodrigues, V., Cordeiro-da-Silva, A., Laforge, M., Ouaissi, A., Akharid, K., Silvestre, R., et al. (2014). Impairment of T cell function in parasitic infections. *PLoS Negl. Trop. Dis.* 8:e2567. doi: 10.1371/journal.pntd.0002567

Rowe, A., Obeiro, J., Newbold, C. I., and Marsh, K. (1995). *Plasmodium falciparum* rosetting is associated with malaria severity in Kenya. *Infect. Immun.* 63, 2323–2326.

Rowe, J. A., Claessens, A., Corrigan, R. A., and Arman, M. (2009). Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Rev. Mol. Med.* 11:e16. doi: 10.1017/S1462399409001082

Rowe, J. A., Obiero, J., Marsh, K., and Raza, A. (2002). Short report: positive correlation between rosetting and parasitemia in *Plasmodium falciparum* clinical isolates. *Am. J. Trop. Med. Hyg.* 66, 458–460.

Rudzinska, M. A., and Trager, W. (1968). The fine structure of trophozoites and gametocytes in *Plasmodium coatneyi**. *J. Protozool.* 15, 73–88. doi: 10.1111/j.1550-7408.1968.tb02091.x

Rug, M., Prescott, S. W., Fernandez, K. M., Cooke, B. M., and Cowman, A. F. (2006). The role of KAHRP domains in knob formation and cytoadherence of *P. falciparum*-infected human erythrocytes. *Blood* 108, 370–378. doi: 10.1182/blood-2005-11-4624

Russo, I., Babbitt, S., Muralidharan, V., Butler, T., Oksman, A., and Goldberg, D. E. (2010). Plasmepsin V licenses Plasmodium proteins for export into the host erythrocyte. *Nature* 463, 632–636. doi: 10.1038/nature08726

Sacks, D., and Sher, A. (2002). Evasion of innate immunity by parasitic protozoa. *Nat. Immunol.* 3, 1041–1047. doi: 10.1038/ni1102-1041

Sharma, P., Rayavara, K., Ito, D., Basore, K., and Desai, S. A. (2015). A CLAG3 mutation in an amphipathic transmembrane domain alters malaria parasite nutrient channels and confers leupeptin resistance. *Infect. Immun.* 83, 2566–2574. doi: 10.1128/IAI.02966-14

Sharma, Y. D. (1997). Knob proteins in falciparum malaria. *Indian J. Med. Res.* 106, 53–62.

Simone, O., Bejarano, M. T., Pierce, S. K., Antonaci, S., Wahlgren, M., Troye-Blomberg, M., et al. (2011). TLRs innate immunereceptors and *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) CIDR1alpha-driven human polyclonal B-cell activation. *Acta Trop.* 119, 144–150. doi: 10.1016/j.actatropica.2011.05.005

Singh, M., Mukherjee, P., Narayanasamy, K., Arora, R., Sen, S. D., Gupta, S., et al. (2009). Proteome analysis of *Plasmodium falciparum* extracellular secretory antigens at asexual blood stages reveals a cohort of proteins with possible roles in immune modulation and signaling. *Mol. Cell. Proteom.* 8, 2102–2118. doi: 10.1074/mcp.M900029-MCP200

Sleebs, B. E., Gazdik, M., O'Neill, M. T., Rajasekaran, P., Lopaticki, S., Lackovic, K., et al. (2014a). Transition state mimetics of the *Plasmodium* export element are potent inhibitors of plasmepsin V from *P. falciparum* and *P. vivax*. *J. Med. Chem.* 57, 7644–7662. doi: 10.1021/jm500797g

Sleebs, B. E., Lopaticki, S., Marapana, D. S., O'Neill, M. T., Rajasekaran, P., Gazdik, M., et al. (2014b). Inhibition of Plasmepsin V activity demonstrates its essential role in protein export, PfEMP1 display, and survival of malaria parasites. *PLoS Biol* 12:e1001897. doi: 10.1371/journal.pbio.1001897

Spielmann, T., and Gilberger, T.-W. (2010). Protein export in malaria parasites: do multiple export motifs add up to multiple export pathways? *Trends Parasitol.* 26, 6–10. doi: 10.1016/j.pt.2009.10.001

Spielmann, T., Hawthorne, P. L., Dixon, M. W. A., Hannemann, M., Klotz, K., Kemp, D. J., et al. (2006). A cluster of ring stage–specific genes linked to a locus implicated in cytoadherence in *Plasmodium falciparum* codes for PEXEL-negative and PEXEL-positive proteins exported into the host cell. *Mol. Biol. Cell* 17, 3613–3624. doi: 10.1091/mbc.E06-04-0291

Spillman, N. J., Allen, R. J., McNamara, C. W., Yeung, B. K., Winzeler, E. A., Diagana, T. T., et al. (2013). Na^+ regulation in the malaria parasite *Plasmodium falciparum* involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials. *Cell Host Microbe* 13, 227–237. doi: 10.1016/j.chom.2012.12.006

Sponaas, A.-M., Cadman, E. T., Voisine, C., Harrison, V., Boonstra, A., O'Garra, A., et al. (2006). Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells. *J. Exp. Med.* 203, 1427–1433. doi: 10.1084/jem.20052450

Spycher, C., Rug, M., Pachlatko, E., Hanssen, E., Ferguson, D., Cowman, A. F., et al. (2008). The Maurer's cleft protein MAHRP1 is essential for trafficking of PfEMP1 to the surface of *Plasmodium falciparum*-infected erythrocytes. *Mol. Microbiol.* 68, 1300–1314. doi: 10.1111/j.1365-2958.2008.06235.x

Stevenson, L., Huda, P., Jeppesen, A., Laursen, E., Rowe, J. A., Craig, A., et al. (2015a). Investigating the function of Fc-specific binding of IgM to *Plasmodium falciparum* erythrocyte membrane protein 1 mediating erythrocyte rosetting. *Cell Microbiol.* 17, 819–831. doi: 10.1111/cmi.12403

Stevenson, L., Laursen, E., Cowan, G. J., Bandoh, B., Barfod, L., Cavanagh, D. R., et al. (2015b). α 2-Macroglobulin can crosslink multiple *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules and may facilitate adhesion of parasitized erythrocytes. *PLoS Pathog.* 11:e1005022. doi: 10.1371/journal.ppat.1005022

Taraschi, T. F., O'Donnell, M., Martinez, S., Schneider, T., Trelka, D., Fowler, V. M., et al. (2003). Generation of an erythrocyte vesicle transport system by *Plasmodium falciparum* malaria parasites. *Blood* 102, 3420–3426. doi: 10.1182/blood-2003-05–1448

Teirlinck, A. C., Roestenberg, M., Bijker, E. M., Hoffman, S. L., Sauerwein, R. W., and Scholzen, A. (2015). *Plasmodium falciparum* infection of human volunteers activates monocytes and CD16+ dendritic cells and induces upregulation of CD16 and CD1c expression. *Infect. Immun.* 83, 3732–3739. doi: 10.1128/IAI.00473-15

Thavayogarajah, T., Gangopadhyay, P., Rahlfs, S., Becker, K., Lingelbach, K., Przyborski, J. M., et al. (2015). Alternative protein secretion in the malaria parasite *Plasmodium falciparum*. *PLoS ONE* 10:e0125191. doi: 10.1371/journal.pone.0125191

Trager, W., Rudzinska, M. A., and Bradbury, P. C. (1966). The fine structure of *Plasmodium falciparum* and its host erythrocytes in natural malarial infections in man. *Bull. World Health Organ.* 35, 883–885.

Udomsangpetch, R., Wahlin, B., Carlson, J., Berzins, K., Torii, M., Aikawa, M., et al. (1989). *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835–1840. doi: 10.1084/jem.169.5.1835

van Ooij, C., Tamez, P., Bhattacharjee, S., Hiller, N. L., Harrison, T., Liolios, K., et al. (2008). The malaria secretome: from algorithms to essential function in blood stage infection. *PLoS Pathog.* 4:e1000084. doi: 10.1371/journal.ppat.1000084

Vásárhelyi, V., Trexler, M., and Patthy, L. (2014). Both LCCL-domains of human CRISPLD2 have high affinity for lipid A. *Biochimie* 97, 66–71. doi: 10.1016/j.biochi.2013.09.021

Verma, R., Tiwari, A., Kaur, S., Varshney, G. C., and Raghava, G. P. (2008). Identification of proteins secreted by malaria parasite into erythrocyte using SVM and PSSM profiles. *BMC Bioinform.* 9:201. doi: 10.1186/1471-2105-9-201

Waller, K. L., Cooke, B. M., Nunomura, W., Mohandas, N., and Coppel, R. L. (1999). Mapping the binding domains involved in the interaction between the *Plasmodium falciparum* knob-associated histidine-rich protein (kahrp) and the cytoadherence ligand *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). *J. Biol. Chem.* 274, 23808–23813. doi: 10.1074/jbc.274.34.23808

Weng, H., Guo, X., Papoin, J., Wang, J., Coppel, R., Mohandas, N., et al. (2014). Interaction of *Plasmodium falciparum* knob-associated histidine-rich protein (KAHRP) with erythrocyte ankyrin R is required for its attachment to the erythrocyte membrane. *Biochim. Biophys. Acta* 1838, 185–192. doi: 10.1016/j.bbamem.2013.09.014

Wetzel, J., Herrmann, S., Swapna, L. S., Prusty, D., John Peter, A. T., Kono, M., et al. (2015). The role of palmitoylation for protein recruitment to the inner

membrane complex of the malaria parasite. *J. Biol. Chem.* 290, 1712–1728. doi: 10.1074/jbc.M114.598094

World Health Organization [WHO] (2014). *World Malaria Report 2013*. Geneva: World Health Organization.

Wickham, M. E., Rug, M., Ralph, S. A., Klonis, N., McFadden, G. I., Tilley, L., et al. (2001). Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infected human erythrocytes. *EMBO J.* 20, 5636–5649. doi: 10.1093/emboj/20.20.5636

Wirth, C. C., Glushakova, S., Scheuermayer, M., Repnik, U., Garg, S., Schaack, D., et al. (2014). Perforin-like protein PPLP2 permeabilizes the red blood cell membrane during egress of *Plasmodium falciparum* gametocytes. *Cell Microbiol.* 16, 709–733. doi: 10.1111/cmi.12288

Zambrano-Villa, S., Rosales-Borjas, D., Carrero, J. C., and Ortiz-Ortiz, L. (2002). How protozoan parasites evade the immune response. *Trends Parasitol.* 18, 272–278. doi: 10.1016/S1471-4922(02)02289-4

Zhang, Y., Huang, C., Kim, S., Golkaram, M., Dixon, M. W., Tilley, L., et al. (2015). Multiple stiffening effects of nanoscale knobs on human red blood cells infected with *Plasmodium falciparum* malaria parasite. *Proc. Natl. Acad. Sci. U.S.A.* 112, 6068–6073. doi: 10.1073/pnas.1505584112

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REVIEW
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Here, There, and Everywhere: The Ubiquitous Distribution of the Immunosignaling Molecule Lysophosphatidylcholine and Its Role on Chagas Disease

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Chagas disease is a severe illness, which can lead to death if the patients are not promptly treated. The disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is mostly transmitted by a triatomine insect vector. There are 8–10 million people infected with *T. cruzi* in the world, but the transmission of such disease by bugs occurs only in the Americas, especially Latin America. Chronically infected patients will develop cardiac diseases (30%) and up digestive, neurological, or mixed disorders (10%). Lysophosphatidylcholine (LPC) is the major phospholipid component of oxidized low-density lipoproteins associated with atherosclerosis-related tissue damage. Insect-derived LPC powerfully attracts inflammatory cells to the site of the insect bite, enhances parasite invasion, and inhibits the production of nitric oxide by *T. cruzi*-stimulated macrophages. The recognition of the ubiquitous presence of LPC on the vector saliva, its production by the parasite itself and its presence both on patient plasma and its role on diverse host × parasite interaction systems lead us to compare its distribution in nature with the title of the famous Beatles song "Here, There and Everywhere" recorded exactly 50 years ago in 1966. Here, we review the major findings pointing out the role of such molecule as an immunosignaling modulator of Chagas disease transmission. Also, we believe that future investigation of the connection of this ubiquity and the immune role of such molecule may lead in the future to novel methods to control parasite transmission, infection, and pathogenesis.

Keywords: lysophosphatidylcholine, Chagas disease, nitric oxide, MAPK, *Rhodnius prolixus*, macrophage

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INTRODUCTION

Lysophosphatidylcholine (LPC) is a major regulator of several biological processes. It is produced by the hydrolysis of the fatty acid at sn-2 position of phosphatidylcholine (PC) catalyzed by phospholipase A2 (PLA2) or alternatively by its transfer from PC to cholesterol by the action of another enzyme a cholesterol acetyltransferase (1–3). Such molecule was originally believed to play a role exclusively on membrane structure but their involvement on the pathogenesis of several human

diseases is increasingly clear in the last few years. Its recognition as a causative factor has occurred by mainly by two different set of data. The first line of evidence toward a functional and signaling role of LPC is due to the close link between plasma lipid profile and pro-oxidant reactions common in inflammatory diseases, especially in atherosclerosis. The second route of evidences lies on the effects of LPC on cells involved in innate immunity. We will discuss the growing of such concepts and how they overlap and support the role of such lysophospholipid in parasitic diseases as follows.

Lysophosphatidylcholine and the bioactive lipids lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), and sphingosylphosphorylcholine (SPC) modulate a wide number of biological processes on mammalian cells. LPC has been classically involved in various physiological events and is already known as a central molecule in various pathological conditions but is especially present during the deposition and infiltration of inflammatory cells and deposition of atheroma, as discussed above (4–6). Research aimed at LPC increased substantially from the fact that these molecules are involved in atherosclerosis (7). The idea that several phospholipases secreted by circulating leukocytes may participate in this pathology was soon proposed. Thus, the current model suggests that diabetes and hypercholesterolemia contribute to generate a large number of low-density lipoprotein (LDL) particles in plasma, which can undergo oxidation of unsaturated fatty acids, generating an oxidized particle (ox-LDL). Since on average, 50% of LDL fatty acids are arachidonic acid and linoleic acid, the chances of an oxidative event like this are huge. OxLDL is a potential cause of increased expression of inflammatory markers. TNF-α, MCP-1, and MCSF expression are increased by oxLDL and attract and differentiate monocytes to the lesion site. Accordingly, LPC is a powerful chemoattractant molecule to macrophages. It is also generated by cells during apoptosis, as mentioned above. oxLDL particles are recognized by PLA2 secreted by different cells in plasma, including the types IIa, V, and X. In conclusion, plasma LPC is derived from the hydrolysis of PC mainly present in LDL and cell membranes by several different subfamilies of PLA2s usually following and oxidative event on its fatty acids.

Trypanosoma cruzi infects the vertebrate host through skin bites produced by bugs during their feed or by interaction with conjunctival mucosa. This interaction sometimes produces the Romaña signal or chagoma whose histology is defined by a large number of mononuclear cells (8). It is likely that *T. cruzi* stimulates skin cells to produce mediators that trigger a local inflammatory response. Chagas disease displays an acute phase, followed by a chronic phase where the parasites are physically linked to inflammation sites in the heart and esophagus (9–15). The disease is considered today as multifactorial once pathogen and host are continuously interacting throughout the whole patient life (16). Following the first 2–3 weeks of the vector bite, *T. cruzi* infection is manifested by a high load of parasites in the blood and tissues. Acute infection is characterized by a huge activation of the immune system. Such activation includes the production of high levels of cytokines, a large activation of T and B cells, lymphadenopathy, and splenomegaly. Also, it displays a visible inflammation due to the infection in tissue niches. The acute phase leads to the development of acquired immunity. Such mechanism ensures the effective control of parasitemia. The chronic phase takes place throughout the patient's life and is associated with low levels of parasite in the host. The beginning of chronic infection in Chagas disease is asymptomatic in most patients. However, clinical manifestations will range from no symptoms to the involvement of the cardiovascular symptoms and/or gastrointestinal complications as the disease progresses (17, 18). The innate immune system appears to be essential for at least two important aspects of Chagas disease: control of parasite replication in host tissue and the progress of the inflammatory reaction. The inflammatory reaction itself may be a major cause of tissue damage and dysfunction of certain organs in the patient (18). Experiments in *T. cruzi* infection models have shown that a potent Th-1 CD4 and CD8 immune response controls parasitemia during the initial stage of the disease. Such immune response is characterized by the production of IFN-γ, TNF-α, and IL-12, as well as the production of nitric oxide (NO). Additionally, cells of innate immunity, such as "natural killer" (NK) cells, dendritic cells, and macrophages, are also key factors in the initial control parasite replication (11, 17, 19).

In recent years, research on Chagas disease has focused on the investigation of the role of pathogen-associated molecular patterns (PAMPs) protozoa, which are the targets of the innate immune response receptors. Also the problem of identification of relevant receptors for the innate immunity to the parasite during the course of the disease in the host has been addressed by several laboratories. This strategy ultimately aimed at developing therapeutic interventions through the use of derivatives of PAMPs present in the parasites. Glycosyl-phosphatidylinositol (GPI) is the name given to the first glycoconjugate identified in *Trypanosoma brucei*. This molecule was identified with the function of anchoring proteins on the cell surface (20–23). *T. cruzi* express on their surface glycoproteins anchored by GPIs (20–23). GPI anchors from trypomastigotes include mucin-like glycoproteins (mucin-GPI) and are key molecules responsible for stimulating the host's immune system (24–27). These mucin-GPIs activate macrophages and lead to the synthesis of proinflammatory cytokines, chemokines, and NO (28). The innate immune response to *T. cruzi* infection largely results from the activation of signaling pathways triggered by Toll-like receptors (TLRs).

Toll-like receptors recognize conserved structural motifs present on different pathogens. Following their activation, TLRs trigger intracellular signaling cascades that build up the host immune response (18, 29). There are 10 TLRs described in human and 12 in mice (18, 29). GPI-induced stimulus occurs during the initial phase of infection, where macrophages respond to trypomastigotes in a TLR-dependent mechanism. At this point in time the production of IL-12, TNF-α and the activation of CD4 and CD8 responses through IFN-γ will take place (30). TNF-α and IFN-γ-activated macrophages seems to have an important role in the control of parasite growth. Anchors free of proteins or GPI or glicoinositol phospholipids (GIPLs) are also able to stimulate the host immune system. GIPLs are similar to GPIs but contain ceramide in their lipid portion (24, 25). TLRs 2, 4, and 9 are the main TLRs involved in the innate immune response to *T. cruzi* (24, 31–36). TLR2 was found to be activated by mucin-GPI (24, 31–36). The heterodimer

composed of TLR2 and TRL6 receptor is activated by GPI mucin and its co-receptor CD14 (34). *T. cruzi* GIPL activates the inflammatory response *via* TLR4. It induces neutrophil recruitment to the peritoneal cavity of mice and this effect is partly dependent on IL-1β production (32, 36). The *T. cruzi* genomic DNA also plays an important role in the vertebrate host proinflammatory response. Finally, TLR 9 is activated by CpG motifs in non-methylated DNA (34, 35). In the innate immune response mediated by TLRs, *T. cruzi* can also stimulate a TLR-independent response leading to the production of IFN-β and IFN-γ. This is due a rise on intracellular calcium concentration that activates calcineurin and calmodulin-dependent proteins (37–39).

HERE: LPC-MEDIATED HANDLING OF INNATE IMMUNITY AT THE SITE OF PARASITE INFECTION

Our group demonstrated, for the first time, the presence of phospholipids and lysophospholipids in saliva and stool hematophagous organism *Rhodnius prolixus*, a triatomine vector of Chagas disease (40, 41). Such finding engaged salivary LPC into the host plasma environment and uncovered its role as both an anti-hemostatic and immunomodulatory molecule. The main lipids present in the saliva of *R. prolixus* are PC and LPC (40, 41). Salivary LPC is an additional anti-hemostatic molecule that is part of the pharmacological arsenal injected into the bite site to allow the insect feeds. It inhibits platelet aggregation and increases the production of NO in endothelial cells. Thus, the LPC was originally described as a molecule with antiplatelet and vasodilatory activity, and few years later, its effect as an immunomodulator of *T. cruzi* infection has been demonstrated (40, 41) (**Figure 1**).

The role of LPC as a modulator of *T. cruzi* infection is caused by three main mechanisms summarized as follows:

1. LPC is a vector-derived molecule. Following attraction of inflammatory cells to the insect bite site, it enhances the chance of infection by the latter arrival of *T. cruzi* (40, 41).
2. LPC produces a surge on intracellular calcium concentrations in macrophages. This will also enhance parasite invasion. It

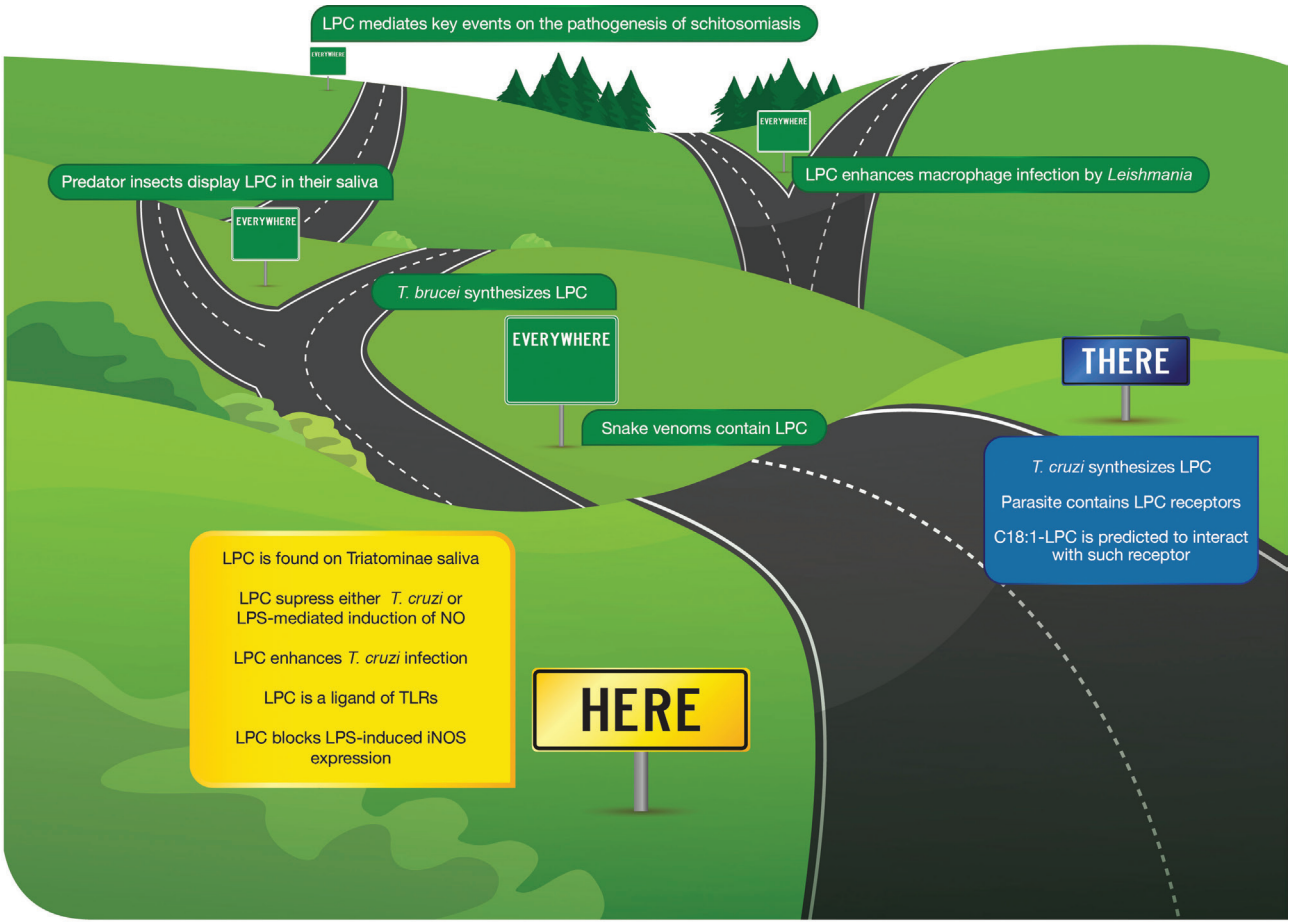

FIGURE 1 | Here, there, and everywhere. This cartoon depicts the major findings in recent years regarding the role of LPC on infectious diseases. Here, originally found as a vector, salivary phospholipid able to modulate Chagas disease through the suppression of NO production. There, LPC was recently described as the product of parasite synthesis. Everywhere, several reports have involved LPC on different aspects of parasitic diseases but also a relevant mediator molecule used to subvert the prey by hunting insects. Finally, its role as a major component of snake venom is also shown.

also modulates the phosphorylation of key signaling molecules such as mitogen-activated kinases (MAPKs) (41, 42).
3. LPC inhibits the production of NO by *T. cruzi*-stimulated macrophages, lipopolysaccharide (LPS), or LPS in the presence of IFN-γ. Thus, it handles the immune system of the vertebrate host (41, 42).

Such findings implied that LPC is a multifactorial signaling molecule, with signaling, anti-hemostatic, and immune effects on the context of Chagas disease transmission (43–46). The establishment of infection by *T. cruzi* depends on the initial invasion of these cells by the parasite (47–49). This leads to the assumption that the salivary LPC can facilitate parasitic infection, favoring not only insect feeding but also the preparation of macrophage susceptibility for the arrival of the parasite, minutes or hours after the initial bite. Salivary LPC injection into the host's skin followed by inoculation of the parasite leaded to an increase of threefold to sixfold on blood parasitemia (41). LPC effect on parasitemia relies on the activation of macrophage chemotaxis and suppression of NO synthesis. We also show an increase in parasite loads within macrophages induced by either 500-fold diluted saliva or LPC. Such results were the first demonstration of a factor able to potentiate the transmission of Chagas disease and the first implication of a lysophospholipid as a modulator of an infectious disease (41, 42).

Activation of TLRs on host cells and the production of TNF-α, IL-12, and NO, required the adapter protein MyD88 (20–24, 30). TLRs 2, 4, and 9 have been implicated in the control of *T. cruzi* infection (20–24, 30). The involvement of TLR2 in the interaction between the parasite and macrophages was also demonstrated (24, 30). TLR2 expression is essential for the induction of IL-12, TNF-α, and NO. This receptor is activated by the parasite molecules, such as GPI anchors, that are isolated from the surface trypomastigotes of *T. cruzi*. Curiously, IL-12 synthesis in macrophages exposed to *T. cruzi* is not affected by saliva (41). Interestingly, in bone marrow derived macrophages derived from TLR2-deficient mice IL-12 production is largely suppressed by LPC (41). These data indicate that in some types of cells this cytokine production can be affected by LPC through a TLR2-independent mechanism.

Furthermore, *T. cruzi* GIPLs are TLR4 agonists with proinflammatory effects (32, 36). We have shown that NO production induced by the parasite or LPS, another ligand of TLR4 or in peritoneal murine macrophages or bone marrow-derived macrophages is blocked in both cases, the LPC even in the presence of IFN-γ vitro (41). The LPC's ability to reverse the induction of NO production in every case, almost regardless of the type of ligand suggests that this bioactive lipid should act on a single pathway. Usually, LPC-sensitive receptors exhibit remarkable overlap in their specificity to the ligands they interact with and vice versa. Different receptors have been proposed for LPC. This includes G2A a G protein-coupled receptor, and GPR4, another important candidate (50). It is noteworthy the low reproducibility of LPC studies where radioactive lipids were used. Together with the capacity of G2A to bind to fatty acids and protons, such findings brought a high complexity to this field (51). Thus, G2A is still recognized as the most known receptor to convey signals mediated by LPC presence in the chemical environment of cells. The redistribution of G2A receptor itself and the exposure of TLR4 on cell surface are highly influenced by LPC metabolism (51, 52). In this latter case, intracellular LPC amount is under control due to the activity of LPC-acyltransferase (LPCAT). This enzyme uses LPC to produce phospholipids. The incubation of monocytes with LPS activates the enzyme and increases the transport of TLR4 to cell-membrane rafts (52). The LPCAT inhibitor, thiophenyl 5-hydroxyethyl 5′3 pyridine (HETP), increases the ratio LPC/PC while it reverses the effect of LPS. Thus, *T. cruzi* may promote a redistribution of G2A and TLR4 on cell surface during infection. This should be investigated in the future by both proteomic and immunological approaches.

In apoptotic cells, LPC is generated by a PLA2- calcium-independent activated caspase-3. LPC then attracts phagocytes to cells and represents a sign of recognition of ongoing apoptosis (50, 53, 54). LPC-induced chemotaxis in Chagas' disease is intriguing once the uptake of apoptotic cells by macrophages infected with *T. cruzi* parasite stimulates their growth (55). Furthermore, the infective stages of *T. cruzi* are capable of generating lipid messengers, including LPC, that modulate the signaling of the host cell (56). LPC activates some protein kinase C isoforms (57). It is probably that different PKC isoforms are activated in different cell types. Also, when combined with different types of TLRs and adapters, the LPC-mediated signaling repertoire may produce a specific and yet to be studied particular mechanism of immunosuppression (**Figure 1**).

In order to evaluate some of these questions, we have addressed the role of LPC on TLR-mediated signaling pathway using HEK 293A cells. Such cells were transfected with TLRs constructs and stimulated with LPCs displaying different fatty acid chain lengths and saturation levels (42). All tested LPCs activated both TLR4 and TLR2-1 signaling pathways. Such results were confirmed through the evaluation of NF-κB activation and IL-8 production. Similar results were obtained when using peritoneal murine macrophages. These cells responded to LPC stimulation by displaying NF-κB translocation. Curiously, when incubated in the presence of LPS, LPC counteracted several features of TLR4 signaling. In this case, NF-κB translocation, NO synthesis, and the expression of inducible nitric oxide synthase (iNOS) were blocked. Such phenotypes occur concomitantly with a hierarchical activation of the MAPKs p38 and JNK, but not ERK, in murine macrophages. Also, LPC blocked LPS-induced ERK activation in peritoneal macrophages but not in TLR-transfected cells. Such results indicated that LPC behaves as a proinflammatory molecule in the absence of traditional TLR ligands. However, when LPS and LPC are present at the same time, a partial silencing of the canonical TLR4 pathway takes place. Such silencing involves the downregulation of ERK pathway and does not affect p32 and JNK. Under these conditions, LPC assumes an anti-inflammatory phenotype through yet unknown ligands on cell surface (42).

THERE: LPC IS SYNTHESIZED BY TRYPANOSOMATID PARASITES

Phosphatidylcholine and LPC are synthesized by *T. cruzi* (58, 59), *T. brucei* (60), *Leishmania* spp. (61, 62), as well as by the malaria parasite, *Plasmodium falciparum* (63). Intriguingly, more than 50% of the total lipids secreted by *T. cruzi* were identified as PC

and LPC (58). Recently, we have purified and structurally characterized a C18:1-LPC in *T. cruzi*, which present a platelet-activating factor (PAF)-like activity, as it aggregates rabbit platelets (59). Comparable to PAF, platelet aggregation was completely blocked by the PAF receptor antagonist, WEB 2086. Considering that increased platelet aggregation related to myocarditis is observed in Chagas disease, it is possible that C18:1-LPC is an important lipid mediator in the progression of this disease. Nevertheless, what functions endogenous *T. cruzi* LPC have in the infection is yet to be unveiled. Such findings enhance our view that during the complete cycle of trypanosomatids transmission LPC will be able to handle surrounding cells, therefore enhancing parasite survival (**Figure 1**).

EVERYWHERE: ROLE OF LPC IN OTHER PARASITIC INFECTIONS

In recent years, our group and others have explored the role of lysophospholipids, and especially LPC on the transmission and pathogenesis of different parasitic diseases. Also, it was demonstrated that LPC plays a role on prey × predator. Such models lead us to the view that LPC is a ubiquitous modulator of host–parasite interaction (**Figure 1**).

Leishmaniasis

A key step on *Leishmania* infection is the invasion of macrophages, which usually occurs through an endocytic process. Such process leads to the formation of the parasitophorous vacuole (PV) that will harbor the parasite. PV was isolated from *Leishmania*-infected macrophages, and the analysis of its phospholipid composition was conducted (64). The levels of LPC increased as compared with non-infected cells. Such results were obtained by allowing either the promastigote or amastigote forms of the parasite to interact with macrophages previously labeled with radioactive phosphate. Thus, these findings indicate that following invasion there is a remodeling of host cell phospholipids, which enhances the production of LPC. The results obtained in the *T. cruzi* model (41, 42), where LPC was pointed out as a negative modulator of NO production suggest that *Leishmania*-induced remodeling of PV may handle the microbicide production and protects the parasite. If this mechanism is essential to allow parasite escape from PV should be investigated in the future. It is important to consider that the use of miltefosine, the first orally active anti-leishmanial drug when directly used to treat *Leishmania donovani* promastigotes, induced at higher doses the increase on LPC production on total parasite membrane extract, probably due to the activation of PLA2s (65). Curiously, such effect is confined to some specific membranar structure of the parasite since it is lost when the plasma membrane is evaluated separately. The involvement on LPC on *Leishmania*-infected was recently busted by the finding that such molecule in BALB/c mouse-derived DC infection (66). LPC enhanced by 92% the infection index was correlated to a delay on cell maturation as evaluated by the expression of $CD86^{+}/CD11c^{+}$. Treatment of such cells with LPS displayed the opposite effects. Also, the authors found that LPC reversed the immune balance between IL-10, TNF-α, and IL-6 once it induced a huge increase on the first cytokine while not affecting of slightly reducing the proinflammatory ones (66). Finally, they observed that LPC is also able to selectively increase arginase1 expression by 395%. This enzyme is commonly induced by Th2 cytokine response set and enhances parasite proliferation. Such effect is paralleled by a suppression of iNOS activation, which keeps the levels of the NO quite low in *Leishmania*-infected cells. Indoleamine 2,3-dioxygenase (IDO) catalyzes tryptophan catabolism through kynurenine pathway. The depletion of tryptophan blocks parasite proliferation. *Leishmania* and LPC induced IDO activation, which usually leads to the accumulation of kynurenine metabolites blocking Th1 response. Also, the increase on arginase expression and downregulation of iNOS production depletes invaded cells of GSH, which induced acute oxidative stress. LPC has recently been described as a major inducer of macrophage polarization toward the M2 phenotype (67–69). In monocytes obtained from patients with Post Kala-Azar Dermal Leishmanisis (68), it was noticed a strong downregulation of TLR2/4 receptors and an increased expression of PPAR-γ and arginase-1. Also, IL-4, IL-10, and IL-13 were significantly raised as compared to healthy individuals. Finally, it increased classical M2 markers CD206, arginase-1, and PPARG in monocytes. Such findings strongly demonstrate that M2 polarization of macrophages is a hallmark of *Leishmania* infection *in vivo* and suggest that a bridge to the LPC-induced effects is the next step toward the development of drugs that modulate disease chronicity. These drugs should be anti-leishmanial molecules that ensure the repolarization of M2 macrophages toward the M1 proinflammatory and protective phenotype (68).

Human African Trypanosomiasis

A global metabolic profiling of plasma of patients carrying *T. brucei rhodesiense* [human African trypanosomiasis (HAT)] was performed and differences in the lipid, amino acid and metabolite profiles were identified. The most striking difference between the HAT patients and the control group was that six significantly altered amounts of LPC species were lower in patients than in controls (69). The interpretation of these data suggests that even though *T. brucei rhodesiense* is known to synthesize LPC *de novo*, the parasite probably scavenges several lipids from the host, as needed. Bearing in mind the results from our group on the key roles LPC play in *T. cruzi* infection, it is not surprising that LPC most likely engage crucial functions in other diseases caused by trypanosomatids.

Schistosomiasis

The first connection between LPC and the biology of schistosomiasis was noticed by the modifications on lipid plasma composition in infected patients (70). Curiously, in the plasma of infected hamsters, the levels of LPC are similar in both control and infected patients but major differences were found in the composition of the fatty acid acyl chains (71). LPC fatty acid composition showed a decrease on 18:2 and 20:5, but less marked changes in 20:4 (71). Usually, human red blood cells (RBCs) are lysed by schistosomula of *Schistosoma mansoni*. LPC-derived from the parasite is used to lyse RBCs (72). The source of this LPC is probably the parasite itself once schistosomula synthesize LCP and

release it in the culture medium as evaluated with experiments using [^{3}H]-phospholipids (72). The dynamics of such process was further through the use of precursors to label phospholipid and neutral lipid with the use of acyl chains ([^{3}H]palmitic acid and [^{3}H]oleic acid) or phospholipid polar head groups ([^{3}H]choline and [^{14}C]ethanolamine) (73). Schistosomal-derived lipids and LPC were shown to stimulate macrophages *in vitro* and induced TLR2-dependent NF-κB activation and cytokine production (74). Administration of LPC induced eosinophil recruitment and cytokine production, in a mechanism largely dependent on TLR2. Thus, parasite-derived LPC mediated key events on the pathogenesis and lethality of schistosomiasis.

Everywhere

The use of LPC on host × parasite interaction seems to be a ubiquous strategy to allow the handling of intracellular signaling pathways. Indeed, LPC may be a handful molecule also used by predators while hunting their daily meal. Snakes and bugs contain lysophospholipids in their saliva (75), *Belostoma anurum* saliva. This predator insect injects its saliva into the prey, which becomes immobilized (76). LPC isolated from saliva is neuroparalytic *in vitro* and *in vivo* (76), and it is highly likely that induce membrane depolarization and calcium entry into sensory nerves (76, 77). Indeed, LPC represents 25% of the total phospholipids present in the saliva of such insect. Since *Belostoma* employs extra-oral digestion LPC also helps in the digestion of preys larger than this insect such as amphibians and small fishes. Thus, it was shown that LPC have paralytic activity in zebrafish, which was the first evidence that lysophospholipids might play an important role in prey immobilization (76). Lysophospholipids and specially LPC paralyze the neuromuscular junction and are also present on snake venoms (78, 79). LPC can also modulate the activity of NK cells (79). Indeed, snake presynaptic phospholipase A2 neurotoxins are commonly present on snake venoms. Mass spectromic analysis of the lipids resulting from SPAN action demonstrated several forms of LPC including myristoyl-LPC and fatty acids (77). Altogether the above finding suggest that LPC use both as a immune and neuromodulator is an ancient biochemical and pharmacological use for such molecule, which probably arose from the eventual leak of modified phospholipids from membranes of interacting organisms whether in the mammalian × protozoan model or in the insect × protozoan model (80, 81).

CONCLUSION

In the future, drugs that target LPC of the signaling systems conveying LPC-mediated signals may largely affect the transmission and pathogenesis of several of the above mentioned diseases. The development of such drugs has been hampered by the yet unknown true LPC receptor. A functional analysis of novel LPC receptor candidates and the test of hypothesis regarding the parasite receptor recently described will provide a wider view of the potential of such molecule to trigger investigations in this area. It is quite interesting that the potential possibility of LPC modulates the several steps of TLR-mediated signaling or the interaction of this cascade with downstream effectors. Due to its major role as a suppressor of NO production in the presence of LPS, it is likely that LPC mechanism of action provides a novel system to understand the functionality of still unknown transcriptional factors as well as the hierarchical mechanism of MAPK regulation. It is quite challenging to imagine that the previously known structural phospholipids are yet a potential source of yet not completed understood signaling modules. The seminal discover of lysophospholipid role in human atherosclerosis combined with its potential selection as an immunosupressor, pharmacological, and neurological modulator by bugs, parasites, worms, and snakes leads to an increase interest on how far the ubiquity of such molecules has been shaped during evolution. The detailed investigation of such models at the molecular level in the future will certainly provide in the near future several novel targets for blocking the transmission and pathogenesis of such diseases.

AUTHOR CONTRIBUTIONS

MS-N, AL, and GA discussed the data and wrote the paper. MS-N and GA are both the corresponding authors.

REFERENCES

1. Kabarowski JH, Xu Y, Witte ON. Lysophosphatidylcholine as a ligand for immunoregulation. *Biochem Pharmacol* (2002) **64**:161–7. doi:10.1016/S0006-2952(02)01179-6
2. Xu H, Valenzuela N, Fai S, Figeys D, Bennett SA. Targeted lipidomics – advances in profiling lysophosphocholine and platelet-activating factor second messengers. *FEBS J* (2013) **280**:5652–67. doi:10.1111/febs.12423
3. Drzazga A, Sowińska A, Koziołkiewicz M. Lysophosphatidylcholine and lysophosphatidylinosiol – novel promissing signaling molecules and their possible therapeutic activity. *Acta Pol Pharm* (2014) **71**:887–99.
4. Wang J, Zhang Y, Wang H, Han H, Nattel S, Yang B, et al. Potential mechanisms for the enhancement of HERG K^+ channel function by phospholipid metabolites. *Br J Pharmacol* (2004) **141**:586–99. doi:10.1038/sj.bjp.0705646
5. Wang L, Radu CG, Yang LV, Bentolila LA, Riedinger M, Witte ON. Lysophosphatidylcholine-induced surface redistribution regulates signaling of the murine G protein-coupled receptor G2A. *Mol Biol Cell* (2005) **16**:2234–47. doi:10.1091/mbc.E04-12-1044
6. Drobnik W, Liebisch G, Audebert FX, Frohlich D, Gluck T, Vogel P, et al. Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *J Lipid Res* (2003) **44**:754–61. doi:10.1194/jlr.M200401-JLR200
7. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* (1990) **344**:160–2. doi:10.1038/344160a0
8. Dias JC. Cecilio Romana, Romana's sign and Chagas' disease. *Rev Soc Bras Med Trop* (1997) **30**:407–13.
9. Andrade ZA. Pathogenesis of Chagas' disease. *Res Immunol* (1991) **142**:126–9. doi:10.1016/0923-2494(91)90021-A
10. Tarleton RL. Chagas disease: a role for autoimmunity ? *Trends Parasitol* (2003) **19**:447–51. doi:10.1016/j.pt.2003.08.008
11. Golgher D, Gazzinelli RT. Innate and acquired immunity in the pathogenesis of Chagas disease. *Autoimmunity* (2004) **37**:399–409. doi:10.1080/08916930410001713115
12. Andrade LO, Andrews NW. The *Trypanosoma cruzi*-host-cell interplay: location, invasion, retention. *Nat Rev Microbiol* (2005) **3**:819–23. doi:10.1038/nrmicro1249

13. Jones EM, Colley DG, Tostes S, Lopes ER, Vnencak-Jones CL, McCurley TL. Amplification of a *Trypanosoma cruzi* DNA sequence from inflammatory lesions in human chagasic cardiomyopathy. *Am J Trop Med Hyg* (1993) **48**:348–57.
14. Vago AR, Macedo AM, Oliveira RP, Andrade LO, Chiari E, Galvão LM, et al. Kinetoplast DNA signatures of *Trypanosoma cruzi* strains obtained directly from infected tissues. *Am J Pathol* (1996) **149**:2153–9.
15. Benvenuti LA, Roggério A, Freitas HF, Mansur AJ, Fiorelli A, Higuchi ML. Chronic American trypanosomiasis: parasite persistence in endomyocardial biopsies is associated with high-grade myocarditis. *Ann Trop Med Parasitol* (2008) **102**:481–7. doi:10.1179/136485908X311740
16. Dutra WO, Gollob KJ. Current concepts in immunoregulation and pathology of human Chagas disease. *Curr Opin Infect Dis* (2008) **21**:287–92. doi:10.1097/QCO.0b013e3282f88b80
17. Rassi A Jr, Rassi A, Marin-Neto JA. Chagas disease. *Lancet* (2010) **375**:1388–402. doi:10.1016/S0140-6736(10)60061-X
18. Junqueira C, Caetano B, Bartholomeu DC, Melo MB, Ropert C, Rodrigues MM, et al. The endless race between *Trypanosoma cruzi* and host immunity: lessons for and beyond Chagas disease. *Expert Rev Mol Med* (2010) **12**:e29. doi:10.1017/S1462399410001560
19. Brener Z, Gazzinelli RT. Immunological control of *Trypanosoma cruzi* infection and pathogenesis of Chagas' disease. *Int Arch Allergy Immunol* (1997) **114**:103–10. doi:10.1159/000237653
20. Ferguson MA, Low MG, Cross GA. Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. *J Biol Chem* (1985) **260**:14547–55.
21. Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J Cell Sci* (1999) **112**:2799–809.
22. Almeida IC, Camargo MM, Procópio DO, Silva LS, Mehlert A, Travassos LR, et al. Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. *EMBO J* (2000) **19**:1476–85. doi:10.1093/emboj/19.7.1476
23. Almeida IC, Gazzinelli RT. Proinflammatory activity of glycosylphosphatidylinositol anchors derived from *Trypanosoma cruzi*: structural and functional analyses. *J Leukoc Biol* (2001) **70**:467–77.
24. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procópio DO, et al. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J Immunol* (2001) **167**:416–23. doi:10.4049/jimmunol.167.1.416
25. Coelho PS, Klein A, Talvani A, Coutinho SF, Takeuchi O, Akira S, et al. Glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from *Trypanosoma cruzi* trypomastigotes induce *in vivo* leukocyte recruitment dependent on MCP-1 production by IFN-gamma-primed-macrophages. *J Leukoc Biol* (2002) **71**:837–44.
26. Aliberti JC, Machado FS, Souto JT, Campanelli AP, Teixeira MM, Gazzinelli RT, et al. beta-Chemokines enhance parasite uptake and promote nitric oxide-dependent microbiostatic activity in murine inflammatory macrophages infected with *Trypanosoma cruzi*. *Infect Immun* (1999) **67**:4819–26.
27. Shoda LK, Kegerreis KA, Suarez CE, Roditi I, Corral RS, Bertot GM, et al. DNA from protozoan parasites *Babesia bovis*, *Trypanosoma cruzi*, and *T. brucei* is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. *Infect Immun* (2001) **69**:2162–71. doi:10.1128/IAI.69.4.2162-2171.2001
28. Gutierrez FR, Mineo TW, Pavanelli WR, Guedes PM, Silva JS. The effects of nitric oxide on the immune system during *Trypanosoma cruzi* infection. *Mem Inst Oswaldo Cruz* (2009) **1**:236–45. doi:10.1590/S0074-02762009000900030
29. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* (2010) **140**:805–20. doi:10.1016/j.cell.2010.01.022
30. Campos MA, Closel M, Valente EP, Cardoso JE, Akira S, Alvarez-Leite JI, et al. Impaired production of proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeloid differentiation factor 88. *J Immunol* (2004) **172**:1711–8. doi:10.4049/jimmunol.172.3.1711
31. Martin D, Tarleton R. Generation, specificity, and function of CD8+ T cells in *Trypanosoma cruzi* infection. *Immunol Rev* (2004) **201**:304–17. doi:10.1111/j.0105-2896.2004.00183.x
32. Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, Golenbock DT, et al. Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with *T. cruzi*. *J Immunol* (2004) **173**:5688–96. doi:10.4049/jimmunol.173.9.5688
33. Ropert C, Gazzinelli RT. Regulatory role of Toll-like receptor 2 during infection with *Trypanosoma cruzi*. *J Endotoxin Res* (2004) **10**:425–30. doi:10.1177/09680519040100060801
34. Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol* (2006) **6**:895–906. doi:10.1038/nri1978
35. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. *J Immunol* (2006) **177**:3515–9. doi:10.4049/jimmunol.177.6.3515
36. Medeiros MM, Peixoto JR, Oliveira AC, Cardilo-Reis L, Koatz VL, Van Kaer L, et al. Toll-like receptor 4 (TLR4)-dependent proinflammatory and immunomodulatory properties of the glycoinositolphospholipid (GIPL) from *Trypanosoma cruzi*. *J Leukoc Biol* (2007) **82**:488–96. doi:10.1189/jlb.0706478
37. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. Toll-like receptor recognizes bacterial DNA. *Nature* (2000) **408**:740–5. doi:10.1038/35047123
38. Chessler AD, Ferreira LR, Chang TH, Fitzgerald KA, Burleigh BA. A novel IFN regulatory factor 3-dependent pathway activated by trypanosomes triggers IFN-beta in macrophages and fibroblasts. *J Immunol* (2008) **181**:7917–24. doi:10.4049/jimmunol.181.11.7917
39. Chessler AD, Unnikrishnan M, Bei AK, Daily JP, Burleigh BA. *Trypanosoma cruzi* triggers an early type I IFN response in vivo at the site of intradermal infection. *J Immunol* (2009) **182**:2288–96. doi:10.4049/jimmunol.0800621
40. Golodne DM, Monteiro RQ, Graca-Souza AV, Silva-Neto MA, Atella GC. Lysophosphatidylcholine acts as an anti-hemostatic molecule in the saliva of the blood-sucking bug *Rhodnius prolixus*. *J Biol Chem* (2003) **278**:27766–71. doi:10.1074/jbc.M212421200
41. Mesquita RD, Carneiro AB, Bafica A, Gazos-Lopes F, Takiya CM, Souto-Padron T, et al. *Trypanosoma cruzi* infection is enhanced by vector saliva through immunosuppressant mechanisms mediated by lysophosphatidylcholine. *Infect Immun* (2008) **76**:5543–52. doi:10.1128/IAI.00683-08
42. Carneiro AB, Iaciura BM, Nohara LL, Lopes CD, Veas EM, Mariano VS, et al. Lysophosphatidylcholine triggers TLR2- and TLR4-mediated signaling pathways but counteracts LPS-induced NO synthesis in peritoneal macrophages by inhibiting NF-κB translocation and MAPK/ERK phosphorylation. *PLoS One* (2013) **8**:e76233. doi:10.1371/journal.pone.0076233
43. Murugesan G, Sandhya Rani MR, Gerber CE, Mukhopadhyay C, Ransohoff RM, Chisolm GM, et al. Lysophosphatidylcholine regulates human microvascular endothelial cell expression of chemokines. *J Mol Cell Cardiol* (2003) **35**:1375–84. doi:10.1016/j.yjmcc.2003.08.004
44. Radu CG, Yang LV, Riedinger M, Au M, Witte ON. T cell chemotaxis to lysophosphatidylcholine through the G2A receptor. *Proc Natl Acad Sci U S A* (2004) **101**:245–50. doi:10.1073/pnas.2536801100
45. Yang LV, Radu CG, Wang L, Riedinger M, Witte ON. Gi-independent macrophage chemotaxis to lysophosphatidylcholine via the immunoregulatory GPCR G2A. *Blood* (2005) **105**:1127–34. doi:10.1182/blood-2004-05-1916
46. Silva-Neto MA, Carneiro AB, Silva-Cardoso L, Atella GC. Lysophosphatidylcholine: a novel modulator of *Trypanosoma cruzi* transmission. *J Parasitol Res* (2012) **2012**:625838. doi:10.1155/2012/625838
47. Connelly MC, Kierszenbaum F. Modulation of macrophage interaction with *Trypanosoma cruzi* by phospholipase A2-sensitive components of the parasite membrane. *Biochem Biophys Res Commun* (1984) **121**:931–9. doi:10.1016/0006-291X(84)90766-6
48. Kollien AH, Schaub GA. The development of *Trypanosoma cruzi* in triatominae. *Parasitol Today* (2000) **16**:381–7. doi:10.1016/S0169-4758(00)01724-5
49. Denkers EY, Butcher BA. Sabotage and exploitation in macrophages parasitized by intracellular protozoans. *Trends Parasitol* (2005) **21**:35–41. doi:10.1016/j.pt.2004.10.004
50. Peter C, Waibel M, Radu CG, Yang LV, Witte ON, Schulze-Osthoff K, et al. Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *J Biol Chem* (2008) **283**:5296–305. doi:10.1074/jbc.M706586200

51. Kabarowski JH. G2A and LPC: regulatory functions in immunity. *Prostaglandins Other Lipid Mediat* (2009) **89**:73–81. doi:10.1016/j.prostaglandins.2009.04.007
52. Jackson SK, Abate W, Parton J, Jones S, Harwood JL. Lysophospholipid metabolism facilitates Toll-like receptor 4 membrane translocation to regulate the inflammatory response. *J Leukoc Biol* (2008) **84**:86–92. doi:10.1189/jlb.0907601
53. Kim SJ, Gershov D, Ma X, Brot N, Elkon KB. I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. *J Exp Med* (2002) **196**:655–65. doi:10.1084/jem.20020542
54. Lauber K, Bohn E, Kröber SM, Xiao YJ, Blumenthal SG, Lindemann RK, et al. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* (2003) **113**:717–30. doi:10.1016/S0092-8674(03)00422-7
55. Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, et al. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* (2000) **403**:199–203. doi:10.1038/35003208
56. Belaunzarán ML, Wainszelbaum MJ, Lammel EM, Gimenez G, Aloise MM, Florin-Christensen J, et al. Phospholipase A1 from *Trypanosoma cruzi* infective stages generates lipid messengers that activate host cell protein kinase c. *Parasitology* (2007) **134**:491–502. doi:10.1017/S0031182006001740
57. Oishi K, Raynor RL, Charp PA, Kuo JF. Regulation of protein kinase C by lysophospholipids. Potential role in signal transduction. *J Biol Chem* (1988) **263**:6865–71.
58. Agusti R, Couto AS, Alves MJ, Colli W, Lederkremer RM. Lipids shed into the culture medium by trypomastigotes of *Trypanosoma cruzi*. *Mem Inst Oswaldo Cruz* (2000) **95**:97–102. doi:10.1590/S0074-02762000000100016
59. Gazos-Lopes F, Oliveira MM, Hoelz LV, Vieira DP, Marques AF, Nakayasu ES, et al. Structural and functional analysis of a platelet-activating lysophosphatidylcholine of *Trypanosoma cruzi*. *PLoS Negl Trop Dis* (2014) **8**:e3077. doi:10.1371/journal.pntd.0003077
60. Werbovetz KA, Englund PT. Lipid metabolism in *Trypanosoma brucei*: utilization of myristate and myristoyllysophosphatidylcholine for myristoylation of glycosyl phosphatidylinositols. *Biochem J* (1996) **318**:575–81. doi:10.1042/bj3180575
61. Ramos RG, Libong D, Rakotomanga M, Gaudin K, Loiseau PM, Chaminade P. Comparison between charged aerosol detection and light scattering detection for the analysis of *Leishmania* membrane phospholipids. *J Chromatogr A* (2008) **1209**:88–94. doi:10.1016/j.chroma.2008.07.080
62. Zheng L, T'Kind R, Decuypere S, von Freyend SJ, Coombs GH, Watson DG. Profiling of lipids in *Leishmania donovani* using hydrophilic interaction chromatography in combination with Fourier transform mass spectrometry. *Rapid Commun Mass Spectrom* (2010) **24**:2074–82. doi:10.1002/rcm.4618
63. Asahi H. *Plasmodium falciparum*: chemically defined medium for continuous intraerythrocytic growth using lipids and recombinant albumin. *Exp Parasitol* (2009) **121**:22–8. doi:10.1016/j.exppara.2008.09.009
64. Henriques C, Atella GC, Bonilha VL, de Souza W. Biochemical analysis of proteins and lipids found in parasitophorous vacuoles containing *Leishmania amazonensis*. *Parasitol Res* (2003) **89**:123–33. doi:10.1007/s00436-002-0728-y
65. Rakotomanga M, Blanc S, Gaudin K, Chaminade P, Loiseau PM. Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* (2007) **51**:1425–30. doi:10.1128/AAC.01123-06
66. Tounsi N, Meghari S, Moser M, Djerdjouri B. Lysophosphatidylcholine exacerbates *Leishmania major*-dendritic cell infection through interleukin-10 and a burst in arginase1 and indoleamine 2,3-dioxygenase activities. *Int Immunopharmacol* (2015) **25**:1–9. doi:10.1016/j.intimp.2015.01.006
67. Ishihara K, Kuroda A, Sugihara K, Kanai S, Nabe T, Akiba S. Regulation of macrophage differentiation and polarization by group IVC phospholipase A_2. *Biochem Biophys Res Commun* (2011) **416**:325–30. doi:10.1016/j.bbrc.2011.11.034
68. Mukhopadhyay D, Mukherjee S, Roy S, Dalton JE, Kundu S, Sarkar A, et al. M2 polarization of monocytes-macrophages is a hallmark of Indian post kala-azar dermal leishmaniasis. *PLoS Negl Trop Dis* (2015) **9**:e0004145. doi:10.1371/journal.pntd.0004145
69. Lamour SD, Gomez-Romero M, Vorkas PA, Alibu VP, Saric J, Holmes E, et al. Discovery of infection associated metabolic markers in human African trypanosomiasis. *PLoS Negl Trop Dis* (2015) **9**:e0004200. doi:10.1371/journal.pntd.0004200
70. Gillett MP, Besterman EM. Plasma and erythrocyte lysolecithin *Schistosomiasis mansioni*. *Atherosclerosis* (1977) **26**:389–90. doi:10.1016/0021-9150(77)90094-6
71. Allan D, Evans WH. Changes in unsaturated fatty acids in serum lipids of hamsters infected with schistosomes (*S. mansoni*). *Biochim Biophys Acta* (1987) **919**:199–202. doi:10.1016/0005-2760(87)90208-6
72. Golan DE, Brown CS, Cianci CM, Furlong ST, Caulfield JP. Schistosomula of *Schistosoma mansoni* use lysophosphatidylcholine to lyse adherent human red blood cells and immobilize red cell membrane components. *J Cell Biol* (1986) **103**:819–28. doi:10.1083/jcb.103.3.819
73. Furlong ST, Caulfield JP. *Schistosoma mansoni*: synthesis and release of phospholipids, lysophospholipids, and neutral lipids by schistosomula. *Exp Parasitol* (1989) **69**:65–77. doi:10.1016/0014-4894(89)90172-0
74. Magalhães K, Almeida PE, Atella G, Maya-Monteiro CM, Castro-Faria-Neto H, Pelajo-Machado M, et al. Schistosomal-derived lysophosphatidylcholine are involved in eosinophil activation and recruitment through toll-like receptor-2-dependent mechanisms. *J Infect Dis* (2010) **202**:1369–79. doi:10.1086/656477
75. Caccin P, Magnabosco A, Tedesco E, Silva-Cardoso L, Atella GC, Montecucco C. Lysophospholipids are evolutionary ancient venom components. *Toxicon* (2010) **56**:1525–7. doi:10.1016/j.toxicon.2010.06.025
76. Silva-Cardoso L, Caccin P, Magnabosco A, Patrón M, Targino M, Fuly A, et al. Paralytic activity of lysophosphatidylcholine from saliva of the waterbug *Belostoma anurum*. *J Exp Biol* (2010) **213**:3305–10. doi:10.1242/jeb.041954
77. Rigoni M, Caccin P, Gschmeissner S, Koster G, Postle AD, Rossetto O, et al. Equivalent effects of snake PLA2 neurotoxins and lysophospholipid-fatty acid mixtures. *Science* (2005) **310**:1678–80. doi:10.1126/science.1120640
78. Caccin P, Rigoni M, Bisceglie A, Rossetto O, Montecucco C. Reversible skeletal neuromuscular paralysis induced by different lysophospholipids. *FEBS Lett* (2006) **580**:6317–21. doi:10.1016/j.febslet.2006.10.039
79. Fuly AL, Machado AL, Castro P, Abrahão A, Redner P, Lopes UG, et al. Lysophosphatidylcholine produced by the phospholipase A2 isolated from *Lachesis muta* snake venom modulates natural killer activity as a protein kinase C effector. *Toxicon* (2007) **50**:400–10. doi:10.1016/j.toxicon.2007.04.008
80. Qin X, Qiu C, Zhao L. Lysophosphatidylcholine perpetuates macrophage polarization toward classically activated phenotype in inflammation. *Cell Immunol* (2014) **289**:185–90. doi:10.1016/j.cellimm.2014.04.010
81. Taniguchi K, Hikiji H, Okinaga T, Hashidate-Yoshida T, Shindou H, Ariyoshi W, et al. Essential role of lysophosphatidylcholine acyltransferase 3 in the induction of macrophage polarization in PMA-treated U937 cells. *J Cell Biochem* (2015) **116**:2840–8. doi:10.1002/jcb.25230

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Differential Expression of miRNA Regulates T Cell Differentiation and Plasticity During Visceral Leishmaniasis Infection

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Visceral leishmaniasis (VL) is a tropical neglected disease caused by *Leishmania donovani*, results in significant mortality in the Indian subcontinent. The plasticity of T cell proliferation and differentiation depends on microRNA mediated gene regulation which leads Th1/Th2 or Th17/Treg type of immune response during human VL. This study depicts the identification of target immune signaling molecule and transcription factors, which play a role in T-cell proliferation and differentiation followed by the identification of miRNA controlling their gene expression using three web servers' viz., TargetScan, miRPath and miRDB. This study provides the bioinformatics evidences that seed region present in the miRNAs miR-29-b, miR-29a, have the putative binding site in the 3′-untranslated region (UTR) of TBX21 transcription factor of $CD4^+$ T helper (Th1), which may suppress the Th1 specific protective immune response. Development of Th2 type specific immune response can be suppressed by binding of miR-135 and miR-126 miRNAs over the 3′-UTR region of GATA-3 transcription factor of Th2 specific $CD4^+$ T helper cells. MiRNA identified against Th2/Treg immune cells are important and their over expression or administration can be used for developing the Th1/Th17 type of protective immune response during VL infection. This study indicates that miRNAs have the capacity to regulate immune signaling, cytokine production and immune cell migration to control the VL infection in human. This observation warrants further investigation for the development of miRNA based therapy controlling T cell differentiation in human VL.

Keywords: MicroRNAs, *Leishmania donovani*, visceral leishmaniasis, Th1/Th2, $CD4^+$ T cell, Th17/Treg

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INTRODUCTION

Leishmaniasis is a tropical neglected diseases which affect 350 million people worldwide living in 98 countries and three territories of five continents (Alvar et al., 2012). The increasing incidence of leishmaniasis show different faces such as visceral, cutaneous and muco-cutaneous and post kala-azar dermal leishmaniasis. Among all these form of leishmaniasis, visceral leishmaniasis (VL) is most severe which affects 58,200 individuals yearly worldwide. It causes absolute death if untreated and is clearly a poverty-related disease which accounts over 30,000 deaths annually (Prajapati et al., 2012). During VL infection, serum plays an important role in binding of promastigotes to macrophage through adsorption of opsonins (antibody/complement protein) to the parasite and

subsequent binding to Fc receptor of the macrophage followed by endocytosis (Sharma and Singh, 2009). Therefore, parasites escape the humoral immune response of host by residing in the phagolysosome of macrophage and subsequently antibodies have no effect on the infection which may be harmful to the host (Unanue and Allen, 1987).

Leishmania donovani infection and its presentation by conventional antigen presenting cells induce different pro-inflammatory cytokine response. Naive $CD4^+$ T cell can initiates differentiation into specific lineages such as Th1, Th2, Th17 and regulatory T (Treg) cells depending upon pro-inflammatory response and expression of specific transcription factors. Each T cell lineage has distinct effectors mechanism along with specific cytokine profiles. In response of IFN-γ and IL-12 pro-inflammatory cytokines, naïve $CD4^+$ T cells differentiate into Th1 cell through controlled process and activity of STAT1, STAT4 and T-bet transcription factors. The STAT4 transcription factor is required to initiate IL-12 signaling along with IFN-γ and inducible nitric oxide synthase expression. Th1 immune cells are the main player of parasite clearance during VL by producing a large amount of IFN-γ and play a critical role to protect human against intracellular parasite by the activation of macrophages (Romagnani, 1999). On the other way, IL-4 promotes the differentiation of naïve $CD4^+$ T cell into Th2 type immune cell through the activation of STAT6 and transcription factor GATA3. In case of VL, Th2 cells help in parasite survival by down regulation of IL-12 via IL-4, to maintain the homeostasis between Th1 and Th2 (Sundar et al., 1997). The differentiation of $CD4^+$ T cells to Th17 cells is carried out in response to IL6 and TGF-β. Initially, IL-6 activates STAT3, then the combined effect of IL-6 and TGF-β signaling leads to the expression of retinoid related orphan receptor (ROR-γt and ROR-α) transcription factor resulting in the initiation of Th17 differentiation. During VL, *L. donovani* induces differentiation of Th17 cells and the resulting IL-17 and IL-22 cytokines are associated with resistance to VL (Pitta et al., 2009). Treg cells differentiation requires same TGF-β to promote the expression of transcription factor Foxp3 but in the absence of IL-6, blocks the expression of ROR-γt and ROR-α transcription factor which results in differentiation of naïve T cell into Treg cells (Zhou et al., 2008).

MiRNAs are evolutionary conserved, non-protein coding, small silencing RNA with size ranges from 20 to 24 nucleotides. The seed sequence of miRNA (7–8 nucleotide) makes base pair with 3′-untranslated region (UTR) of target mRNA results in either translation inhibition or/and mRNA degradation (Bartel, 2009). Till date, several miRNAs have shown the capacity to regulate biological processes like cell differentiation, proliferation, and apoptosis (Miska, 2005; Ruan et al., 2009). It also contributes to the process of thymic T cell maturation and differentiation. The controlled expression of miR-181a contributes to clonal deletion of auto-reactive T cells by the modulation of TCR signaling threshold and survival of low affinity peptide-specific T cell (Li et al., 2007). MiR-155, miR-181c, miR-9, and miR-31 also play an important role in T cell activation by regulating IL-2 signaling pathway. MiR132/212 cluster induces Th17 cells differentiation and its deficiency lowers the frequencies of Th1 and Th17cells due to inhibition of experimental autoimmune encephalitis development (Nakahama et al., 2013). While the over-expression of miR-26a leads to increased Treg expression which play an important role in Th1 and Th17 differentiation (Zhang et al., 2015). In this article, we have presented whether miRNA expression can regulate the $CD4^+$ T cell differentiation during VL infection. Here, we have identified transcription factors and important cytokines playing a role in T cell differentiation and favoring the condition for the development of VL disease in human. Using computational study, we predicted up-regulation of putative miRNAs regulating key transcription factor and guiding conversion of Th_0 cell into Th2 type of immune cells which results in VL infection.

MATERIALS AND METHODS

Identification of Target Immune Signaling Molecules

Visceral leishmaniasis infection in human is associated with activation of Th2 and suppression of Th1 immune response. Suppression of Th1 immune response is associated with decreased level of IFN-γ, IL-12 and higher production of IL-4 and IL-13 cytokines to maintain the Th1/Th2 plasticity. Involvement of Th17 immune cells for the production of IL-17, IL-22 cytokines and to recruit neutrophil is also important factor to provide the protection from VL infection. Development of protective immune response against *L. donovani* parasites depends upon the pro inflammatory immune response which is mediated by antigen presenting cells. In response to pro inflammatory cytokines $CD4^+$ T cells converts into either Th1 or Th2 type of the immune cell. Conversion of immune cells in Th1/Th2 is dependent upon the formation of key transcription factors and important cytokines. We have identified key transcription factors and cytokines playing an important role in T cell differentiation and plasticity during the development of protection from VL disease (**Table 1**).

Resources Used for the miRNA Prediction

MiRNAs are small RNA molecule which down regulate the target gene expression at post transcription level. There are many resources available which can be used to predict the most potential miRNAs against target genes. Here, we have tabulated the name of web servers which is freely available for public use and can be used to predict miRNAs (**Table 2**). For this study, we used three web servers viz., TargetScan, miRPath and miRDB to predict miRNAs against key transcription factors and important cytokines regulating $CD4^+$ T cell differentiation during VL infection.

MiRNAs Prediction Against Transcription Factors Controlling T Cell Differentiation During Visceral Leishmaniasis Infection

$CD4^+$ T cell differentiation follows different pathways in human VL. When *L. donovani* parasites enters in human peripheral

TABLE 1 | Key transcription factors regulating CD4+ T cell differentiation and maturation.

S. No.	Name	Category	Function	Reference
1	TBX21	Transcription factor	Induce IFN-γ production	Szabo et al., 2000
	GATA-3	Transcription factor	Master regulator of Th2 cell differentiation	Zheng and Flavell, 1997
2	STAT-1	STAT protein	Induces TBX21 expression	Afkarian et al., 2002
3	STAT-4	STAT protein	Induces IFN-γ production and expression of TBX21	Usui et al., 2003
4	STAT-5	STAT protein	Th2 differentiation	Zhu et al., 2003
5.	STAT-6	STAT protein	Induce GATA-3	Takeda et al., 1996
6	Runx-3/Eomes	Transcription repressor	Induces IFN-γ expression	Naoe et al., 2007
7	IRF-1	IFN regulated factor	Induces IFN-γ expression	Kano et al., 2008
8	IRF-4	IFN regulated factor	Up regulate GATA-3 expression	Lohoff et al., 2002
9	HLX	Other factor	Enhances TBX21 mediated IFN-γ production	Mullen et al., 2002
10	Ets-1	Other factor	Cofactor for TBX21	Grenningloh et al., 2005
11	GFi-1	Transcription repressor	Promote Th2 cell differentiation	Li et al., 1999
12	IKaros	Other transcription factor	Suppress Th1 cell differentiation	Quirion et al., 2009
13	cMaf	Other transcription factor	Enhances IL-4 production	Kim et al., 1999
14	JunB	Other transcription factor	Enhances IL-4 production	Li et al., 1999
15	Dec2	Other transcription factor	Induce GATA-3 expression	Yang et al., 2009
16	Blimp-1	Other transcription factor	Suppresses expression of IL-2 and IFN-γ	Martins and Calame, 2008
17	IL-12	Cytokines	Induction of Th1 cell differentiation	Hsieh et al., 1993
18	IFN-γ	Cytokines	Induction of Th1 cell differentiation	Lighvani et al., 2001
19	IL-4	Cytokines	Induction of Th2 cell differentiation	Swain et al., 1990; Seder et al., 1992
20	IL-2	Cytokines	Induction of Th1 cell differentiation	Le Gros et al., 1990
21	ROR-γt	Transcription factor	Induction of Th17 cell differentiation	Park et al., 2005
22	ROR-α	Transcription factor	Promote Th17 differentiation	Park et al., 2005
23	STAT3	STAT protein	Induction of Th17 cell differentiation	Park et al., 2005
24	IL-6	Cytokine	Induction of Th17 cell differentiation	Harrington et al., 2005
25	TGF-β	Transcription factor	Induction of both Th17and Treg cell differentiation	Bettelli et al., 2006
26	Foxp3	Transcription factor	Induction of Treg cell differentiation	Zhou et al., 2008

TABLE 2 | List of resources used for microRNA prediction.

S. No.	Availability	Name of tool	Resource (URL)	Reference
1	Online search	TargetScan	http://www.targetscan.org/	Thomson et al., 2011
2		miRPath	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r = mirpath/index	Huang et al., 2014
3		miRDB	http://mirdb.org/miRDB/	Wang and El Naqa, 2008
4		miRTrail	http://mirtrail.bioinf.uni-sb.de/mirtrail.php	Yang et al., 2011
5		miRanda	http://www.microrna.org/microrna/home.do	Thomson et al., 2011
6		miRwalk	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/	Dweep et al., 2013
7		miRGen	http://carolina.imis.athena-innovation.gr/index.php?r=mirgenv3	Al-Nakhle et al., 2010
8		Pictar	http://pictar.mdc-berlin.de	Thomson et al., 2011

blood, $CD4^+$ T cells (Th_0) transformation started in response to pro inflammatory cytokines. Pro inflammatory cytokines released from antigen presenting cells play an important role in the conversion of Th_0 cell into different lineages such as Th1, Th2, Th17 and Treg etc. Induction of IL-4/IL-13 cytokine signaling from antigen presenting cells induces the expression of GATA3 transcription factor which leads to conversion of Th_0 cells into Th2 cells. Using potentially used webservers TargetScan, miRPath and miRDB web servers, we have identified putative miRNAs which subsequently down regulate the expression of transcription factor to control Th_0 cell differentiation. In these web servers, we submitted the NCBI gene ID of key transcription factors and important genes to get the potential miRNAs.

RESULTS

MiRNAs Regulating Th1/Th2 Differentiation and Plasticity

MiRNAs regulating Th1/Th2 signaling were predicted using three foremost web servers viz., TargetScan, (Agarwal et al., 2015) miRPath (Paraskevopoulou et al., 2013) and miRDB (Wong and Wang, 2015). TBX21 protein is a Th1 immune

cell-specific transcription factor regulates Th1 immune signaling specific cytokines. By using TargetScan, we speculated that miR-29a, miR-29b, and miR-29c were found to be potential miRNA with the high probability of conserved targeting (P_{CT}) value which can bind at position 245–251 of TBX21 3′-UTR. While, the miRNA obtained from miRPath and miRDB have shown that miR-548ah-5p, miR-526b-5p, and miR-4726-5p have the same target binding site at 3′-UTR of TBX21 gene. These miRNAs have the potential to down regulate the expression of TBX21 gene and subsequently inhibit the conversion of Th_0 cells into Th1 cells. STAT1 and STAT4 transcription factors were also submitted for miRNA prediction since they play an important role in the development of Th1 cell and IFN-γ production. By using all three web servers, miR-1252, miR-4697, miR-4724, and miR-495 were found to be potential miRNA against STAT1 gene. Similarly, TargetScan and miRDB prediction have shown that miR-200a have the putative binding sites at position 237–244 of STAT4 gene with high P_{CT} values (**Table 3**). Entry of *L. donovani* parasites inside human peripheral blood induces a number of signaling in the $CD4^+$ T cells which develops an environment to induce above miRNAs to negatively regulate Th1 immune cell conversion.

Simultaneously, we have identified miRNAs against Th2 immune signaling molecules, which is responsible for *L. donovani* replication and VL disease development. GATA3 protein is T cell specific transcription factor which induces the conversion of Th_0 into Th2 immune cells and IL-4/IL-13 signaling for developing a Th2 immune response during VL disease. GATA3 protein has multiple transcripts but for NM_001002295 transcript, by using all three web servers we found that miR-135 has the binding site at the 3′-UTR of GATA3 gene (position 207–213). MiR-135 has shown high P_{CT} value against GATA3 gene and has the potential to inhibit the expression of this gene and Th2 immune cell conversion. While miR-126 was predicted by miRPath and miRDB as a negative regulator of GATA3. STAT6 transcription factor induced by IL-4 has shown multiple transcripts with different 3′-UTR length. STAT6 gene with 3′-UTR length of 1168 nucleotides were found to show miR-135 binding at position 1100–1107. To control the VL infection, IL-4 and IL-13 pro-inflammatory cytokines released from antigen presenting cells can be inhibited by miR-1272 and miR-155 (**Figure 1**). Signature cytokines secreted by Th1/Th2 cell were also submitted for putative miRNA prediction, since they have important role in the maintenance of Th1/Th2 plasticity. MiRNAs against key transcription factor and important cytokines controlling Th1 and Th2 specific immune signaling events are listed in the **Tables 3** and **4** respectively.

TABLE 3 | MiRNAs regulating Th1 cell differentiation and plasticity.

Serial No.	Gene	miRDB	miRPath	TargetScan
1	TBX21	miR-548ah-5p	miR-548ah-5p	miR-29a
		miR-526b-5p	miR-526b-5p	miR-29b
		miR-4726-5p	miR-4726-5p	miR-29c
2	STAT1	miR-1252	miR-1252	miR-1252
		miR-4697	miR-4697	miR-4697
		miR-4724	miR-4724	miR-4724
		miR-495	miR-495	miR-495
3	STAT4	miR-200a	–	miR-200a
4	IFNG	miR-24	miR-24	miR-24
		miR-29	–	–
5	IL12	miR-21	miR-21	miR-21
		miR-590-5p	–	miR-590-5p
6	RunX3	miR-130b	miR-130b	–
		miR-106b	miR-106b	–
		miR-130a	miR-130a	–
7	EOMES	miR-182	miR-182	miR-182
		miR-29a	miR-29a	–
		miR-29b	miR-29b	–
8	IRF1	–	miR-635	miR-635
		–	miR-4747-5p	miR-4747-5p
		miR-4708-3p	–	miR-4708-3p
		–	miR-4667	miR-4667
		miR-4483	–	miR-4483
		–	miR-23	–

MiRNAs Regulating Th17/Treg Differentiation and Plasticity

Subpopulations of $CD4^+$ T cells producing IL-17 cytokines were capable to inducing IFN-γ in STAT4 dependent manner. During VL infection, it has been shown that IL-17 and IL-22 cytokine level increases in PBMC culture. Using TargetScan, miRPath and miRDB web servers, we have collected the series of miRNAs activated in response to *L. donovani* infection and controlling Th17/Treg differentiation. TargetScan and miRPath analysis have shown that miR-4500, let-7a, let-7b, and let-7c have the putative binding site at position 407-413 of 3′-UTR for RORC gene which can subsequently negatively regulate the expression of this gene for Th17 differentiation. RORC gene encodes for RORγt transcription factors. MiR-106a and miR-106b have shown putative binding site at the position 252–258 of 3′-UTR for STAT3 gene, when analyzed by all three web servers, which have the potential to down regulate the expression of this gene. MiR-124 was also found to be a negative regulator of STAT3 gene, by miRDB and miRPath and literature (Lu et al., 2013).

A small population of $CD4^+$ T cells showing $CD4^+$ $CD25^+$ $Foxp3^+$ characteristic termed as Treg cell and this subpopulation help in the *Leishmania* parasitic growth and development. TargetScan and miRDB prediction gave that miR-3622b-5p has shown putative binding site at position 796–802 of 3′-UTR for Foxp3 gene. MiR-940 and miR-1827 were predicted by TargetScan and miRPath, have shown binding site at position 413–419 and 305–315 respectively of 3′-UTR for the same gene, to down regulate the Treg-specific differentiation (**Figure 2**). Interestingly, all the predicted miRNAs for Foxp3 gene have shown different binding at 3′-UTR of Foxp3 gene. Th17/Treg plasticity is equally important for the *L. donovani* parasite to raise VL infection. In presence of Treg-specific miRNA

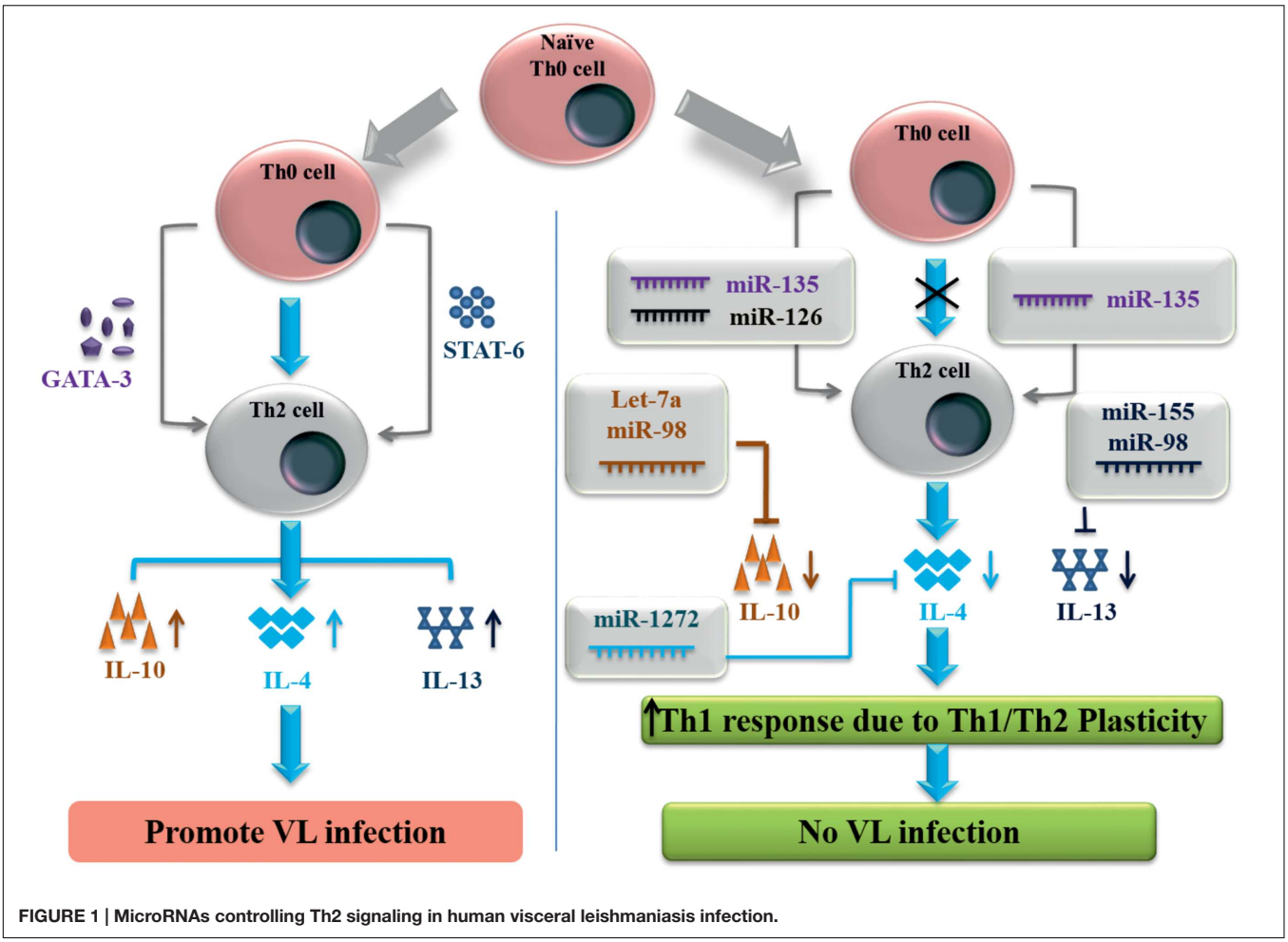

FIGURE 1 | MicroRNAs controlling Th2 signaling in human visceral leishmaniasis infection.

environment, Th17 cell differentiation followed by IL-17 and IL-22 cytokine production, may further inhibit the growth of *L. donovani* parasites. Predicted miRNAs for the key transcription factors and important cytokines related to Th17 and Treg cells have been explained in the **Table 5**.

DISCUSSION

Human VL is associated with increased Th2 and Treg immune responses which induce the elevation of disease signature cytokines such as IL-6, IL-8, IL-10 and TGF-β. While a protective immune response against *L. donovani* is associated with elevated IFN-γ and IL-12 level, which leads to Th1 immune response. T cells developed from lymphoid progenitor stem cell in bone marrow and their maturation takes place in the thymus. MiRNAs have shown significant role in $CD4^+$ T cell differentiation, maturation and to develop plasticity during cancer and infectious diseases (Liu et al., 2013). An immunological study indicates that Th1/Th2 paradigm is not solely responsible to controlling or favoring VL disease in the human model. Suppression of signature cytokines associated with Th1 immune response and elevation of Th2 type immune response cytokines have opened the window for development of immunotherapy against VL disease. Here, in this study we have predicted miRNAs as the regulatory molecule to control the $CD4^+$ T cell differentiation to protect or favor VL disease. MiRNAs are an important player at the molecular level in cells which controls immune response in the human by controlling immune signaling at the post-transcriptional level. MiRNAs are emerging as a key controller for $CD4^+$ T cell differentiation and for maintaining plasticity during VL infection. We targeted $CD4^+$ T cell differentiation controlling transcription factor molecule for miRNA prediction which can regulate T cell differentiation phenomenon.

In our computational study, we observed that miR-548ah, miR-526b, miR-4726-5p, miR-29C, miR-29b, and miR-29a putatively control the expression of TBX21 protein expression in $CD4^+$ T cell. During VL infection in Th2 type immune environment, negative regulation of TBX21 transcription factor by aforementioned miRNAs leads to raise the VL infection. Our computational study states that the elevated level of miR-1252, miR-4697, miR-4724, and miR-495, negatively regulates

TABLE 4 | MiRNAs regulating Th2 cell differentiation and plasticity.

S. No.	Gene	miRDB	miRPath	TargetScan
1	GATA3	miR-135	miR-135	miR-135
		miR-126	miR-126	–
2	IL4	miR-1272	miR-1272	miR-1272
3	STAT6	miR-135	–	miR-135
4	IL13	–	let-7d	miR-155
		–	let-7e	–
		–	let-7f	–
		–	let-7g	–
		–	let-7i	–
		–	miR-98	–
5	IL10	–	miR-98	miR-98
		–	miR 4500	miR 4500
		–	miR-4458	miR-4458
		–	let-7a	let-7a
		–	let-7b	let-7b
		–	let-7c	let-7c
		–	let-7d	let-7d
6	IL2	miR-181a	–	miR-181a-5p
		miR-181b	–	miR-181b-5p
		miR-181c	–	miR-181c-5p
		miR-181d	–	miR-181d-5p
7	STAT5	mir-4663	–	mir-4663
		–	miR-548k	miR-548k
		–	miR-3941	miR-3941
		–	miR-4672	miR-4672
		–	miR-4719	miR-4719
		–	miR-524-5p	miR-524-5p
		–	miR-520d-5p	miR-520d-5p
8	IRF4	miR-128	miR-128	miR-128
		miR-125b	miR-125b	–
9	GFI1	miR-142-3p	–	miR-142-3p
10	CMAF	–	miR-182	miR-182
		–	miR-143	–
		–	miR-301a	–
11	JunB	miR-199a-5p	–	miR-199a-5p
		miR-199b-5p	–	miR-199b-5p

the STAT1 signaling. It was also noted that miR-200a expression in $CD4^+$ T cells has the binding sites at the position 237-244 of 3′-UTR for STAT4 gene which can subsequently negatively regulate the Th1 type of immune response during VL infection (Huang et al., 2011).

In human VL infection, Th2 type of immune response activates to favor the replication of *L. donovani* in macrophages cells. Using TargetScan, miRPath and miRDB web servers, we identified miRNAs against the key transcription factor which has shown the potential to down regulate Th2 immune response. MiR-135 has a putative binding site to regulate GATA3 transcription factor which control the Th2 immune cell differentiation from $CD4^+$ T cells. We also identified that miR-135 have the great potential to down regulate the expression of the STAT6 transcription factor at the post-transcriptional level in $CD4^+$ T cells. Our finding indicates that let-7e, let-7c and miR-98 have the putative binding site at the 3′-UTR of IL-10 gene and negatively regulates the functional expression of IL-10. Our study states that miR-1272 and miR-155 have the potential to down regulate the IL-4/IL-13 signaling to check the Th2 response during VL infection. In the Th1 type of immune response micro-environment miR-1272 and miR-155 activated which subsequently inhibit the IL-4/IL-13 signaling pathway to suppress the Th2 type of immune response in human. Immune signaling polarization toward Th2 immune response is one of the main streams for the development of VL disease. In the presence of miRNAs investigated against GATA3, STAT6 transcription factors and IL-4, IL-13, IL-10 cytokines have the great potential to down regulate the Th2 immune response which subsequently induces the production of IFN-γ by Th1/Th2 plasticity phenomenon in human during VL infection. Furthermore, in the presence of elevated IFN-γ response, reactive oxygen species and nitric oxide signaling induces the parasitic clearance and protection from *L. donovani* parasites.

Mature T cells in peripheral blood of human consist of Th17 immune cells characterized by the production of IL-17, IL-21 and IL-22 cytokines. Protective role of IL-17 and IL-22 cytokines have been associated with the protection of human in response to *L. donovani* infection to suppress VL infection. Th17 immune cell differentiation from $CD4^+$ T cell takes place by the expression of ROR-γt transcription factor. MiR-4500 and let-7c were found to have a putative binding site at the 3′-UTR of RORC gene, which negatively regulates the maturation and differentiation of Th17 cells during VL infection. In the condition of elevated expression of miR-93 and miR-124, miR-106a, and miR-106b, Th17 immune diminished and *L. donovani* parasite finds the favorable condition to replicate in the macrophages of human. Regulatory T cells are the subpopulation of $CD4^+$ T cells and characterized by the expression of $CD4^+$ $CD25^+$ $Foxp3^+$ in human VL and associated with the production of TGF-β cytokines to favor the parasite replication in VL disease. Our study indicates that miR-744 suppresses the expression of TGF-β cytokine which subsequently have the potential to inhibit the Treg cell differentiation and maturation in VL disease (Butz et al., 2012).

Our study highlights the importance of miRNAs in the $CD4^+$ T cell differentiation, maturation and their functional aspects toward the development of protection from *L. donovani* parasites. This computational study generates the signaling mechanism controlled and regulated by miRNAs in human VL infection. MiRNA developed against Th2 and Treg immune cells are important and their over expression can be used for developing Th1 and Th17 type of protection specific immune response during VL infection. In cancer disease, many miRNA based therapy is under clinical trial and have shown excited efficacious response by developing a protective immune response. By this study, we have postulated that miRNAs against key transcription factors and important cytokines can be used to design miRNA based therapy to develop Th1 and Th17 type of protective immune response to generate IFN-γ and nitric oxide signaling to kill *L. donovani* parasites. By understanding miRNA regulatory network to control $CD4^+$ T cell differentiation, we can develop less toxic accurate

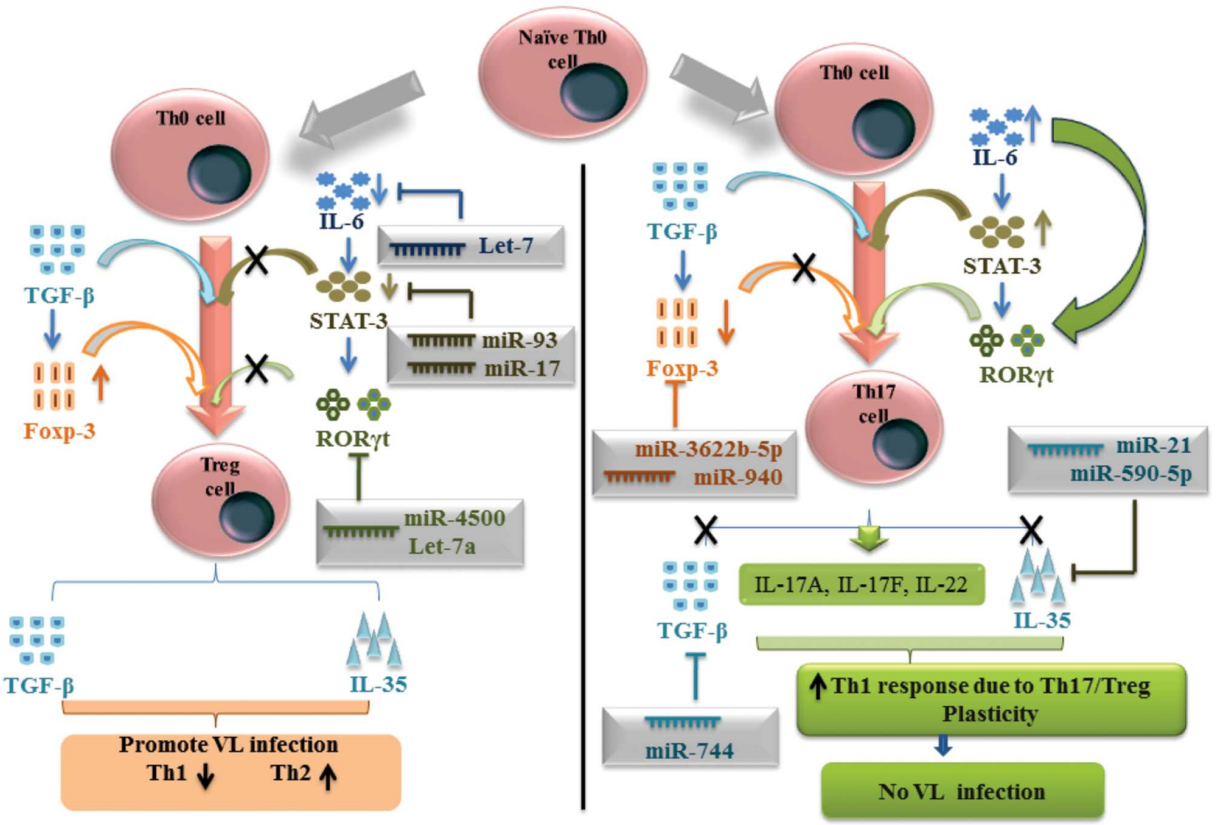

FIGURE 2 | MicroRNAs mediated plasticity between Thl7 and Treg cells in human visceral leishmaniasis.

TABLE 5 | MiRNAs regulating Th17 and Treg cell differentiation and plasticity.

Serial No.	Gene	miRDB	miRPath	TargetScan
1	RORC	–	let-7a-5p	let-7a-5p
		–	let-7b-5p	let-7b-5p
		–	let-7c	let-7c
		–	miR-4500	miR-4500
2	STAT3	miR-93	miR-93	miR-93
		miR-519d	miR-519d	miR-519d
		miR-17	miR-17	miR-17
		miR-106b	miR-106b	miR-106b
		miR-106a	miR-106a	miR-106a
		miR-124	miR-124	–
3	TGFβ	–	–	miR-744
4	IL6	–	–	let-7
5	Foxp3	miR-3622b-5p	–	miR-3622b-5p
		–	miR-940	miR-940
		–	miR-1827	miR-1827
6	IL35	–	–	miR-21
		–	–	miR-590-5p

and targeted therapy against VL disease. By this fascinating bioinformatics based study one can provide the intricate mechanism to control $CD4^+$ T cell differentiation and to develop gene regulation mechanism to inhibit *L. donovani* growth and replication. This study further develops the deep concern for the development of miRNA based therapy in VL disease.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RKP, SS, and VKP. Performed the experiments: RKP and VKP. Analyzed the data: RKP and VKP. Contributed reagents/materials/analysis tools: RKP and VKP. Wrote the paper: RKP, SS, and VKP.

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REFERENCES

Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., et al. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD^{4+} T cells. *Nat. Immunol.* 3, 549–557. doi: 10.1038/ni794

Agarwal, V., Bell, G. W., Nam, J. W., and Bartel, D. P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 4:e05005. doi: 10.7554/eLife.05005

Al-Nakhle, H., Burns, P. A., Cummings, M., Hanby, A. M., Hughes, T. A., Satheesha, S., et al. (2010). Estrogen receptor β1 expression is regulated by miR-92 in breast cancer. *Cancer Res.* 70, 4778–4784. doi: 10.1158/0008-5472.CAN-09-4104

Alvar, J., Velez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE* 7:e35671. doi: 10.1371/journal.pone.0035671

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., et al. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238. doi: 10.1038/nature04753

Butz, H., Rácz, K., Hunyady, L., and Patócs, A. (2012). Crosstalk between TGF-β signaling and the microRNA machinery. *Trends Pharmacol. Sci.* 33, 382–393. doi: 10.1016/j.tips.2012.04.003

Dweep, H., Sticht, C., and Gretz, N. (2013). In-silico algorithms for the screening of possible microrna binding sites and their interactions. *Curr. Genomics.* 14, 127–136. doi: 10.2174/1389202911314020005

Grenningloh, R., Kang, B. Y., and Ho, I. C. (2005). Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. *J. Exp. Med.* 201, 615–626. doi: 10.1084/jem.20041330

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., et al. (2005). Interleukin 17-producing CD^{4+} effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6, 1123–1132. doi: 10.1038/ni1254

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'garra, A., and Murphy, K. M. (1993). Development of TH1 CD^{4+} T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260, 547–549. doi: 10.1126/science.8097338

Huang, C., Xiao, X., Chintagari, N. R., Breshears, M., Wang, Y., and Liu, L. (2014). MicroRNA and mRNA expression profiling in rat acute respiratory distress syndrome. *BMC Med. Genomics* 7:46. doi: 10.1186/1755-8794-7-46

Huang, Y., Lei, Y., Zhang, H., Hou, L., Zhang, M., and Dayton, A. I. (2011). MicroRNA regulation of STAT4 protein expression: rapid and sensitive modulation of IL-12 signaling in human natural killer cells. *Blood* 118, 6793–6802. doi: 10.1182/blood-2011-05-356162

Kano, S., Sato, K., Morishita, Y., Vollstedt, S., Kim, S., Bishop, K., et al. (2008). The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD^{4+} T cells. *Nat. Immunol.* 9, 34–41. doi: 10.1038/ni1538

Kim, J. I., Ho, I. C., Grusby, M. J., and Glimcher, L. H. (1999). The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10, 745–751. doi: 10.1016/S1074-7613(00)80073-4

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D., and Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J. Exp. Med.* 172, 921–929. doi: 10.1084/jem.172.3.921

Li, B., Tournier, C., Davis, R. J., and Flavell, R. A. (1999). Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. *EMBO J.* 18, 420–432. doi: 10.1093/emboj/18.2.420

Li, Q. J., Chau, J., Ebert, P. J., Sylvester, G., Min, H., Liu, G., et al. (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129, 147–161. doi: 10.1016/j.cell.2007.03.008

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., et al. (2001). T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15137–15142. doi: 10.1073/pnas.261570598

Liu, J., Wu, C. P., Lu, B. F., and Jiang, J. T. (2013). Mechanism of T cell regulation by microRNAs. *Cancer Biol. Med.* 10, 131–137. doi: 10.7497/j.issn.2095-3941.2013.03.002

Lohoff, M., Mittrucker, H. W., Prechtl, S., Bischof, S., Sommer, F., Kock, S., et al. (2002). Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11808–11812. doi: 10.1073/pnas.182425099

Lu, Y., Yue, X., Cui, Y., Zhang, J., and Wang, K. (2013). MicroRNA-124 suppresses growth of human hepatocellular carcinoma by targeting STAT3. *Biochem. Biophys. Res. Commun.* 441, 873–879. doi: 10.1016/j.bbrc.2013.10.157

Martins, G., and Calame, K. (2008). Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu. Rev. Immunol.* 26, 133–169. doi: 10.1146/annurev.immunol.26.021607.090241

Miska, E. A. (2005). How microRNAs control cell division, differentiation and death. *Curr. Opin. Genet. Dev.* 15, 563–568. doi: 10.1016/j.gde.2005.08.005

Mullen, A. C., Hutchins, A. S., High, F. A., Lee, H. W., Sykes, K. J., Chodosh, L. A., et al. (2002). Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nat. Immunol.* 3, 652–658.

Nakahama, T., Hanieh, H., Nguyen, N. T., Chinen, I., Ripley, B., Millrine, D., et al. (2013). Aryl hydrocarbon receptor-mediated induction of the microRNA-132/212 cluster promotes interleukin-17-producing T-helper cell differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11964–11969. doi: 10.1073/pnas.1311087110

Naoe, Y., Setoguchi, R., Akiyama, K., Muroi, S., Kuroda, M., Hatam, F., et al. (2007). Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. *J. Exp. Med.* 204, 1749–1755. doi: 10.1084/jem.20062456

Paraskevopoulou, M. D., Georgakilas, G., Kostoulas, N., Reczko, M., Maragkakis, M., Dalamagas, T. M., et al. (2013). DIANA-LncBase: experimentally verified and computationally predicted microRNA targets on long non-coding RNAs. *Nucleic Acids Res.* 41, D239–D245. doi: 10.1093/nar/gks1246

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., et al. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6, 1133–1141. doi: 10.1038/ni1261

Pitta, M. G., Romano, A., Cabantous, S., Henri, S., Hammad, A., Kouriba, B., et al. (2009). IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*. *J. Clin. Invest.* 119, 2379–2387. doi: 10.1172/JCI38813

Prajapati, V. K., Mehrotra, S., Gautam, S., Rai, M., and Sundar, S. (2012). In vitro antileishmanial drug susceptibility of clinical isolates from patients with Indian visceral leishmaniasis–status of newly introduced drugs. *Am. J. Trop. Med. Hyg.* 87, 655–657. doi: 10.4269/ajtmh.2012.12-0022

Quirion, M. R., Gregory, G. D., Umetsu, S. E., Winandy, S., and Brown, M. A. (2009). Cutting edge: Ikaros is a regulator of Th2 cell differentiation. *J. Immunol.* 182, 741–745. doi: 10.4049/jimmunol.182.2.741

Romagnani, S. (1999). Th1/Th2 cells. *Inflamm. Bowel Dis.* 5, 285–294. doi: 10.1002/ibd.3780050410

Ruan, K., Fang, X., and Ouyang, G. (2009). MicroRNAs: novel regulators in the hallmarks of human cancer. *Cancer Lett.* 285, 116–126. doi: 10.1016/j.canlet.2009.04.031

Seder, R. A., Paul, W. E., Davis, M. M., and De St Groth, B. (1992). The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD^{4+} T cells from T cell receptor transgenic mice. *J. Exp. Med.* 176, 1091–1098. doi: 10.1084/jem.176.4.1091

Sharma, U., and Singh, S. (2009). Immunobiology of leishmaniasis. *Indian J. Exp. Biol.* 47, 412–423.

Sundar, S., Reed, S. G., Sharma, S., Mehrotra, A., and Murray, H. W. (1997). Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis. *Am. J. Trop. Med. Hyg.* 56, 522–525.

Swain, S. L., Weinberg, A. D., English, M., and Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145, 3796–3806.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655–669.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., et al. (1996). Essential role of Stat6 in IL-4 signalling. *Nature* 380, 627–630. doi: 10.1038/380627a0

Thomson, D. W., Bracken, C. P., and Goodall, G. J. (2011). Experimental strategies for microRNA target identification. *Nucleic Acids Res.* 39, 6845–6853. doi: 10.1093/nar/gkr330

Unanue, E. R., and Allen, P. M. (1987). The basis for the immunoregulatory role of macrophages and other accessory cells. *Science* 236, 551–557. doi: 10.1126/science.2437650

Usui, T., Nishikomori, R., Kitani, A., and Strober, W. (2003). GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. *Immunity* 18, 415–428. doi: 10.1016/S1074-7613(03)00057-8

Wang, X., and El Naqa, I. M. (2008). Prediction of both conserved and nonconserved microRNA targets in animals. *Bioinformatics* 24, 325–332. doi: 10.1093/bioinformatics/btm595

Wong, N., and Wang, X. (2015). miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res.* 43, D146–D152. doi: 10.1093/nar/gku1104

Yang, R., Dai, Z., Chen, S., and Chen, L. (2011). MicroRNA-mediated gene regulation plays a minor role in the transcriptomic plasticity of cold-acclimated zebrafish brain tissue. *BMC Genomics* 12:605. doi: 10.1186/1471-2164-12-605

Yang, X. O., Angkasekwinai, P., Zhu, J., Peng, J., Liu, Z., Nurieva, R., et al. (2009). Requirement for the basic helix-loop-helix transcription factor Dec2 in initial TH2 lineage commitment. *Nat. Immunol.* 10, 1260–1266. doi: 10.1038/ni.1821

Zhang, R., Tian, A., Wang, J., Shen, X., Qi, G., and Tang, Y. (2015). miR26a modulates Th17/T reg balance in the EAE model of multiple sclerosis by targeting IL6. *Neuromol. Med.* 17, 24–34. doi: 10.1007/s12017-014-8335-5

Zheng, W., and Flavell, R. A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89, 587–596. doi: 10.1016/S0092-8674(00)80240-8

Zhou, L., Lopes, J. E., Chong, M. M., Ivanov, I. I., Min, R., Victora, G. D., et al. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453, 236–240. doi: 10.1038/nature06878

Zhu, J., Cote-Sierra, J., Guo, L., and Paul, W. E. (2003). Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19, 739–748. doi: 10.1016/S1074-7613(03)00292-9

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A Model to Study the Impact of Polymorphism Driven Liver-Stage Immune Evasion by Malaria Parasites, to Help Design Effective Cross-Reactive Vaccines

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Malaria parasites engage a multitude of strategies to evade the immune system of the host, including the generation of polymorphic T cell epitope sequences, termed altered peptide ligands (APLs). Herein we use an animal model to study how single amino acid changes in the sequence of the circumsporozoite protein (CSP), a major target antigen of pre-erythrocytic malaria vaccines, can lead to a reduction of cross reactivity by T cells. For the first time in any APL model, we further compare different inflammatory adjuvants (Montanide, Poly I:C), non-inflammatory adjuvants (nanoparticles), and peptide pulsed dendritic cells (DCs) for their potential capacity to induce broadly cross reactive immune responses. Results show that the capacity to induce a cross reactive response is primarily controlled by the T cell epitope sequence and cannot be modified by the use of different adjuvants. Moreover, we identify how specific amino acid changes lead to a one-way cross reactivity: where variant-x induced responses are re-elicited by variant-x and not variant-y, but variant-y induced responses can be re-elicited by variant-y and variant-x. We discuss the consequences of the existence of this one-way cross reactivity phenomenon for parasite immune evasion in the field, as well as the use of variant epitopes as a potential tool for optimized vaccine design.

Keywords: circumsporozoite protein, CD8 epitope, altered peptide ligands, polymorphism, cross reactivity, vaccines

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INTRODUCTION

Immune evasion by parasites is a major obstacle to overcome for vaccine development. One of the mechanisms employed by malaria parasites is the development of variant epitope sequences, termed altered peptide ligands (APLs). APLs contain multiple or single amino acid changes that can affect their immunogenicity or ability to be recognized by pre-existing immune responses (Plebanski et al., 1997). There are many different APLs in regional populations of parasites, and one possible way to increase cross reactive immunity to APL epitopes is through vaccine design against multiple variant antigens.

Vaccines to malaria, including the recently approved RTS,S (Mosquirix) vaccine, tend to focus on the pre-erythrocytic stage of malaria to prevent infection progressing to the clinically symptomatic erythrocytic stage (Duffy et al., 2012). Liver stage malaria infection by sporozoites would benefit from a CD8 T cell response to eliminate parasitized

hepatocytes (Doolan and Martinez-Alier, 2006). Eliciting such a CD8 T cell response is a challenge for vaccine design in particular due to the inability of most adjuvants to induce high levels of CD8 T cells. Furthermore, in addition to high levels of CD8 T cell responses, prospective malaria vaccines would need to induce broad cross reactivity to multiple variant epitopes to ensure protective immunity.

Vaccine adjuvants have the capacity to enhance recognition, and potentially cross reactivity, to vaccine antigens. Nonetheless, this question has not been widely addressed experimentally as yet in any vaccine model. Traditionally adjuvants enhance immune responses by eliciting inflammatory cytokine production. Whilst the most common adjuvant, Alum, induces Th2 biased responses (McKee et al., 2009), certain inflammatory type experimental adjuvants, such as the water-in-oil emulsion, Montanide, and the toll like receptor 3 (TLR3) agonist, Poly I:C, are well regarded for their ability to elicit CD8 T cells to short peptide epitopes (Aucouturier et al., 2002; Elliott et al., 2008; Herrera et al., 2011; Cho et al., 2013). However, not all adjuvant systems need to induce inflammatory cytokines to elicit potent responses. *Ex vivo* peptide pulsed DCs are naturally immunogenic, and do not require the addition of an adjuvant, as they can be directly loaded *ex vivo* with peptides and transferred back to a host, to induce substantial immune responses. Furthermore, novel polystyrene nanoparticles (PSNPs), in the viral size range of 40–50 nm, with covalently bound peptide are capable of inducing robust CD8 T cell responses, in the absence of conventional inflammatory signals (Xiang et al., 2013). In our previous studies the non-inflammatory PSNP vaccines induced comparable levels of CD8 T cell immune responses to peptide delivered with the inflammatory adjuvant Montanide (Wilson et al., 2015). Alongside adjuvant selection, target antigen choice for vaccines is an important consideration for the induction of optimal antigen-specific immunity.

One such target antigen for *Plasmodium falciparum* liver stage malaria vaccine development, the circumsporozoite protein (CSP), displays a wide range of APL variant epitopes. CSP from *P. falciparum* contains two main variable T cell regions, T helper region 2 and 3 (Th2R and Th3R), both of which contain highly polymorphic nested CD4 and CD8 T cell epitopes (Plebanski et al., 1997). In the case of the human leukocyte antigen B35 (HLA-B35) binding region within Th3R, only two of the multiple polymorphic variants bind major histocompatibility complex (MHC) molecules, and naturally induced CD8 T cell reactivity to these two variants is not cross reactive (Gilbert et al., 1998; Plebanski et al., 1999). The current leading pre-erythrocytic vaccine, RTS,S induces CD4 but not CD8 T cells in humans (Lalvani et al., 1999; Reece et al., 2004). It is relevant to note that this vaccine showed better efficacy against the parasites in the population bearing the allelic variant of CSP also present in the vaccine (Neafsey et al., 2015), highlighting the importance of considering antigen polymorphism during vaccine design. Polymorphic antigens pose numerous problems, however, for complex pathogens, such as malaria, polymorphic antigen candidates may not be easily avoided. Therefore, devising strategies to overcome immune evasion and complications with polymorphic antigens for vaccine design is an important question to address in this field.

Mouse models of variant epitopes are a useful tool to understand and demonstrate the impact of amino acid changes to T cell epitopes, even though these epitopes cannot be directly used in human vaccines. The known protective immunodominant CD8 T cell epitope of the murine strain *P. berghei* CSP is SYIPSAEKI (also named pb9, or KI). Notably, position 8 along this peptide has been shown to be an important position for T cell recognition and T cell activation (Maryanski et al., 1993; Kessler et al., 1998), and thus an important residue to study the impact of potential variant amino acid changes. Herein, we show that single amino acid changes to the T cell receptor (TCR) contact residue, position 8 (lysine), of the SYIPSAEKI epitope can lead to a loss of recognition by T cells. We further assess the impact of utilizing diverse adjuvants, representing both inflammatory and non-inflammatory adjuvant systems, to affect the magnitude and breadth of cross-reactivity of the immune responses.

MATERIALS AND METHODS

Animals

BALB/c mice (6–8 weeks old) were purchased from Monash Animal Services (MAS, Melbourne, VIC, Australia). All animals were used under ethics approval by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee.

Ex Vivo DC Pulsing with Peptide

Hematopoietic stem cells were harvested from the femur and tibia of BALB/c mice, and seeded at 0.5×10^6 cells/ml in complete media (CM) containing RPMI 1640 supplemented with 10% fetal calf serum (FCS, Gibco, Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), 2 mM L-Glutamine (Gibco), 1 M Hepes, and 0.1 mM 2-mercaptoethanol (Sigma–Aldrich, St Louis, MO, USA), and additionally a final of 10 ng/ml Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA) and 5 ng/ml Interleukin 4 (IL-4) for 6 days (note that half of the media was replaced with replenished cytokines on day 3) to induce the differentiation of DCs. On day 6, DCs were pulsed with 5 μg/ml SYIPSAEKI peptide for 1–2 h in the same culture media, and then washed with phosphate buffered saline (PBS) and re-suspended at 1×10^7 cells/ml in PBS for immunizations.

Peptide Conjugation to Nanoparticles

Nanovaccine formulations were prepared by conjugation of peptides SYIPSAEKI, SYIPSAERI, SYIPSAEVI, SYIPSAEAI, SYIPSAEEI, or SYIPSAEDI to carboxylated PSNPs (Polysciences Inc., Warrington, PA, USA) based on the previously outlined method (Xiang et al., 2013; Wilson et al., 2015). Briefly, PSNPs (∼40–50 nm) at a final of 1% solids were activated using a 2-*N*-morpholino-ethanesulfonic acid (MES; 50 mM final, pH = 7) buffered solution of 1-ethyl-3-(3-dimethylaminopropryl) carbodiimide hydrochloride (EDC; 4 mg/ml final, Sigma–Aldrich), together with the malaria peptides (SYIPSAEKI or the

variants SYIPSAERI, SYIPSAEVI, SYIPSAEAI, SYIPSAEEI, and SYIPSAEDI, Mimotopes, Melbourne, VIC, Australia; 1 mg/ml final) on a rotary wheel, for 4 h at room temperature (RT). Following incubation, the conjugation reaction was stopped and unbound reactive sites coated by the addition of glycine (7 mg/ml final, Sigma–Aldrich) and further incubated as above for 30 min. Unconjugated peptide and excess glycine and other reagents were removed by dialysing in dialysis membrane (10–14 kDa molecular weight cutoff; Viskase, Darien, USA), against PBS (pH = 7.2–7.4) overnight at 4°C. Conjugation efficiency was determined by Bicinchoninic Assay (BCA^{TM}; Thermo Fisher Scientific, Rockford, IL, USA) following manufacturer's instruction. Final size of the formulation was determined by dynamic light scattering instruments (Zetasizer; Malvern Instruments, Worcestershire, UK).

Vaccine Adjuvants and Immunizations

Vaccines consisted of the following formulations in PBS; SYIPSAEKI (final 5 μg/ml) pulsed DCs (1×10^6 cells/mouse in 100 μl PBS) with or without the addition of PSNPs (50 μl at 2% solids mixed in); SYIPSAEKI (final 25 μg/mouse) mixed with the adjuvants Montanide ISA 720 (70% v/v with PBS; Tall Bennet Group, USA), or Polyinosinic-polycytidylic acid sodium salt (Poly I:C; 25 μg/mouse final, Sigma–Aldrich). Nanovaccines contained either SYIPSAEKI (∼25 μg/mouse) simply mixed with PSNPs (∼1% final solids), or peptides covalently conjugated to the PSNPs, including SYIPSAEKI or the variant peptides SYIPSAERI, SYIPSAEVI, SYIPSAEAI, SYIPSAEEI, and SYIPSAEDI (target 25 μg/mouse, ∼0.65–1.47% final solids to achieve target peptide loading per mouse). Mice were immunized with respective formulations, either once, or twice, 2 weeks apart, intradermally at the base of tail. Approximately 13–14 days after the last immunization (unless otherwise stated) mice were humanely euthanized and splenocytes harvested and assessed for IFN-γ production by ELISpot assay.

ELISpot

Antigen specific and cross reactive CD8 T cell responses were assessed by IFN-γ ELISpot assay. Ninty-six well multiscreen plates (MSIP; Millipore, Billerica, USA) were coated with 5 μg/ml anti-mouse IFN-γ (AN18; MABTech, Stockholm, Sweden), 100 μl/well in PBS, and incubated at 4°C overnight. Following incubation, all wells were washed five times with PBS and blocked with CM for a minimum of 1 h at 37°C. Splenocytes harvested from immunized mice were added to wells in triplicate (1×10^7 cells/ml, 50 μl/well), and co-incubated with the different recall antigens (SYIPSAEKI, SYIPSAERI, SYIPSAEVI, SYIPSAEAI, SYIPSAEEI, SYIPSAEDI; Mimetopes, Melbourne, Australia; final 2.5 μg/ml), as well as a media alone control, and concanavalin A (ConA; Amersham Biosciences, Uppsala, Sweden; final 1 μg/ml) as positive control. Splenocytes and antigens were co-incubated in CM at 37°C, with 6% $C0_2$ for 12–16 h. Following incubation, plates were washed with PBS and the anti-IFN-γ biotinylated detection antibody was added (R4-6A2-biotin, MABTech; 1 μg/ml final in PBS/0.5% FCS), 100 μl/well, and incubated at RT for 2 h. Plates were washed as above before addition of streptavidin-alkaline phosphate enzyme conjugate (ALP; final 1 μg/ml in PBS/0.5% FCS), 100 μl/well, for 1.5 h at RT. Plates were washed with PBS as above and given a final wash with reverse osmosis (RO) water. Spots were developed using an AP colorimetric kit (Bio-Rad, Philadelphia, PA, USA), following manufacturer's instructions. Plates were left to dry overnight at RT before spots were counted using an AID ELISpot reader system (AutoImmune Diagnostika GmbH, Germany).

Binding Assay

For determination of peptide binding ability to MHC class I (MHCI) (H2-Kd), a binding assay using a MHCI (H2-Kd) transfected RMA-S cell line was performed. Briefly, RMA-S-Kd cells were cultured in CM further supplemented with 0.8 mg/ml G418 disulphide salt (Sigma–Aldrich), until confluent. Cells were seeded into 48 well plates (0.5×10^6 cells/ml) and incubated at 28–29°C overnight with 6% $C0_2$, allowing cells to express H2-Kd. Following incubation peptides (SYIPSAEKI, SYIPSAERI, SYIPSAEVI, SYIPSAEAI, SYIPSAEEI, and SYIPSAEDI) were added in triplicate wells (at final concentrations of 0, 0.025, 0.25, and 2.5 μg/ml), and further incubated at 28–29°C with 6% $C0_2$ for 1 h. Plates were then transferred to 37°C, 6% $C0_2$, for an additional 3 h so that empty H2-Kd molecules could be recycled.

Cells were transferred to 96 well 'V' bottom plates for flow cytometry staining and stained with biotinylated anti-H2-Kd mAb (clone SF1-1.1; Biolegend, San Diego, CA, USA), or isotype control biotinylated anti-mouse IgG2aκ (Biolegend), at pre-determined dilutions, in PBS/2% FCS (30 μl/well) for 15 min at RT. Following incubation cells were washed with PBS/2% FCS (100 μl/well), and centrifuged before addition of streptavidin-AF700 (Thermo-Fisher) in PBS/2% FCS (30 μl/well) for 15 min at RT. Stained cells were given a final wash with PBS/2% FCS and fixed with 1% (v/v) paraformaldehyde (PFA; Sigma–Aldrich). Cells were acquired using an LSRII flow cytometer (BD Biosciences, USA), located at the AMREP Flow Cytometry Core Facility (Melbourne, Australia). Data was analyzed using FlowJo software (version 10; Treestar, USA).

Statistics

Statistical analysis was conducted using two way analysis of variance (ANOVA), with *post hoc* Dunnetts (or Tukey where applicable), multiple comparison tests, using Graphpad Prism software (v.6. San Diego, CA, USA). Statistical significance was set as $p < 0.05$. Values are expressed as mean ± standard deviation (SD), and group sizes are indicated in the figure legends.

RESULTS

Ex Vivo Peptide Pulsed DCs as Natural Adjuvants Induce Low Levels of Cross Reactivity to APLs of the CD8 Epitope SYIPSAEKI

APLs of the *P. berghei* immunodominant CD8 T cell epitope SYIPSAEKI (herein termed KI) were created by substituting

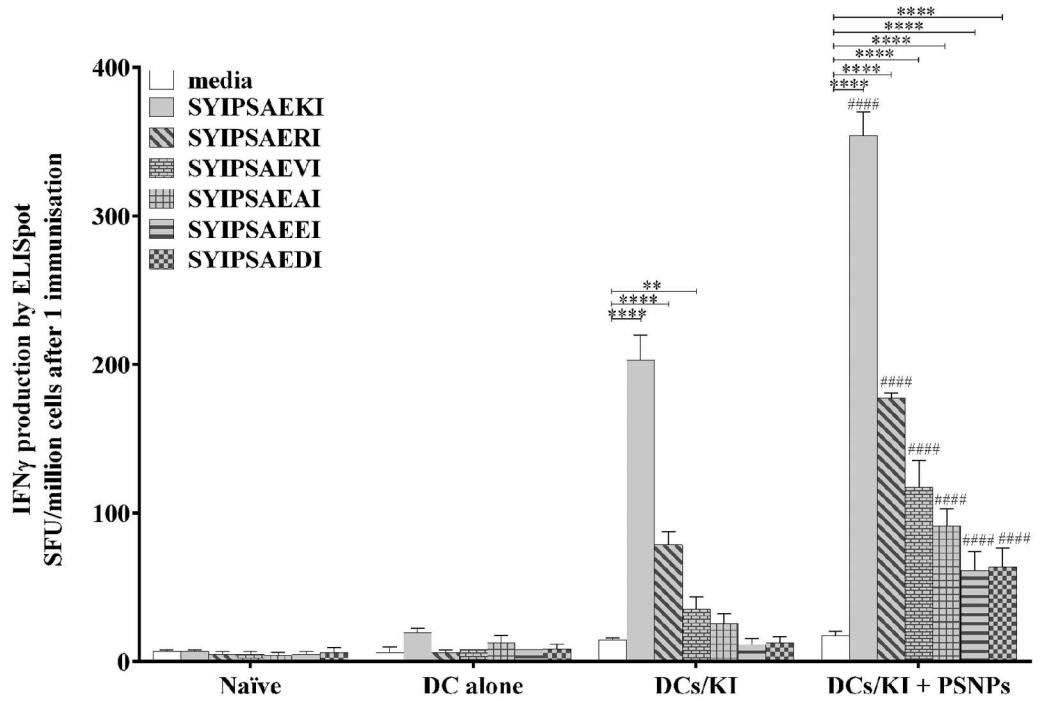

FIGURE 1 | Cross reactivity to APLs when adjuvanted by KI pulsed DCs, with or without nanoparticles (PSNPs) co-injection. BALB/c mice were immunized once with naïve control (PBS alone), DCs alone (1×10^6 cells in PBS), or peptide pulsed DCs (1×10^6 cells pulsed with 5 μg/ml KI, DCs/KI), with or without co-injected PSNPs (1×10^6 cells pulsed with 5 μg/ml KI co-injected with a final 0.67% PSNPs, DCs/KI + PSNPs). Thirty five days after the immunization splenocytes were harvested and antigen specific or cross reactive IFN-γ production was measured via ELISpot assay. Data shown as mean ± SD of SFU/million cells (pooled cells from four mice per group) from triplicate wells. $^{**}p < 0.01$, $^{****}p < 0.0001$, ####$p < 0.0001$ between DCs/KI and DCs/KI + PSNPs groups.

the lysine at position 8 with amino acids carrying different charges. Five altered peptides were created; SYIPSAERI (RI), arginine substitution at position 8; SYIPSAEVI (VI), valine substitution; SYIPSAEAI (AI), alanine substitution; SYIPSAEEI (EI), glutamic acid substitution; and SYIPSAEDI (DI), aspartic acid substitution. Additionally, the substituted amino acids alter the charge of the position 8 amino acid, KI and RI carry a positive side chain charge, VI and AI carry a neutral side chain charge, and EI and DI carry a negative side chain charge. Notably, side chain charge may not alter substantially the overriding charge of the peptide. Cross reactivity to these APLs was initially examined using various adjuvant systems immunized with the index peptide KI.

Ex vivo peptide pulsed DCs are naturally immunogenic and are advantageous due to their ability to effectively prime antigen specific T cells, without requiring an additional adjuvant. Bone marrow was extracted from BALB/c mice and cultured for 6 days, with the cytokines GM-CSF and IL-4, and cultured DCs were pulsed with KI for 1–2 h prior to immunization (DCs/KI). Previous studies have shown that non-inflammatory nanoparticles (PSNPs) further enhance immune responses when mixed with micro-particulate vaccines (Fifis et al., 2004; Xiang et al., 2015). Therefore, KI pulsed DCs were additionally co-injected with PSNPs alone (DCs/KI + PSNPs), to examine if a potential similar enhancement would also have an effect on the pattern of cross reactivity to APL variants. BALB/c mice were immunized, once with the above formulations, and 35 days after the immunization splenocytes were harvested and analyzed for IFN-γ production via ELISpot assay.

Figure 1 shows that KI pulsed DCs induced significantly higher levels of KI specific IFN-γ to the peptide itself, compared to the media alone control ($p < 0.0001$, **Figure 1**), and a moderate level of cross reactivity to the other APL variants, in particular significant responses above media for RI ($p < 0.0001$), and VI ($p < 0.01$). The ranking of APL responses showed that RI (positive charge at position 8, the same charge as the lysine in KI) was the most cross reactive, followed by VI and AI (neutral charge at position 8), with EI and DI (negative charge at position 8) the least cross reactive to the index peptide KI (**Figure 1**). Interestingly, KI pulsed DCs co-injected with PSNPs showed significantly higher KI specific IFN-γ responses for all peptides, apart from media alone, compared to the DCs/KI group ($p < 0.0001$ for each peptide), as well as significantly increased magnitude of responses, compared to media, to all five cross reactive APLs tested ($p < 0.0001$ for all APLs). Notably, the pattern of IFN-γ response to the APLs remained the same, suggesting that PSNPs may not be directly affecting the breadth of the T cell response, but may enhance the number of reactive T cells, which could proportionally enhance the magnitude of responses.

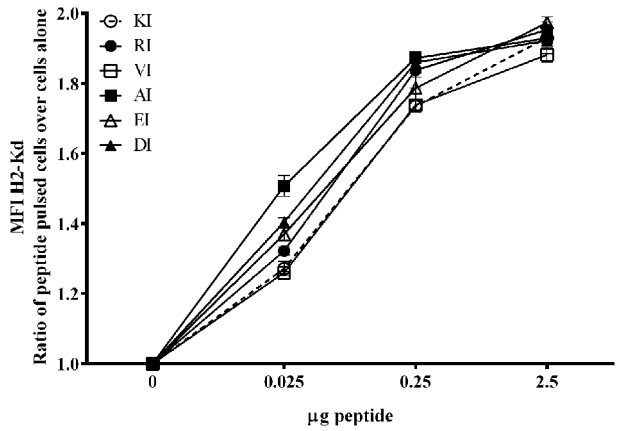

FIGURE 2 | Binding ability of the index peptide, KI, or APLs to the MHC molecule H2-Kd. H2-Kd transfected RMA-S cells were cultured *in vitro* overnight to allow expression of the MHC, and subsequently co-cultured KI, or with each of the altered peptides, in triplicate at three different concentrations, for peptide-MHC complex stabilization at the cell surface. Cells were stained with anti-mouse H2-Kd and the mean fluorescent intensity (MFI) of H2-Kd for each condition was determined (0 is equivalent to the MFI for RMA-S-H2-Kd cells alone). Data shown as MFI of H2-Kd expression (Ratio of peptide pulsed cells over cells alone) ±SD from triplicate wells. Dotted line represents the index peptide KI.

Limited APL Cross Reactivity is not a Consequence of Lack of Peptide Binding to MHCI

The low level of cross reactive responses to certain epitopes, particularly DI and EI, could be explained if these APL variants had a lower binding capacity to MHCI than KI. To assess this formally, we performed a binding assay which utilizes the cell line, RMA-S, deficient in MHCI loading molecules, which has been transfected with H2-Kd, creating a cell line reported to have a heat sensitive expression of MHCI. In temperature ranges of 26–30°C H2-Kd is expressed on the cell surface, allowing peptide binding and stabilization of the peptide-MHC complex (Ljunggren et al., 1990; Rock et al., 1992). At higher temperatures (37°C) empty MHC molecules start to be recycled back into the cell, unless they are stabilized on the surface by exogenously binding peptide. Detection of surface peptide-MHCI complexes is achieved through flow cytometry staining for H2-Kd. Of interest to this study was the relative comparison of peptide binding ability of the APLs to the index peptide, KI. As can be seen in **Figure 2**, all variant peptides achieved an equal, or greater, level of binding compared to KI at all three concentrations of peptide loading tested (0.025, 0.25, and 2.5 μg/ml). This suggests that the limited cross reactive responses observed were not due to a lack of binding ability to MHCI for these variant peptides.

The Adjuvant Montanide Elicits a Similar Pattern of Cross Reactivity Compared to Peptide Pulsed DCs

A high magnitude of antigen specific IFN-γ responses have previously been shown to the KI peptide *in vivo* by using the conventional inflammatory adjuvant Montanide, with lower responses inducible with Poly I:C (Wilson et al., 2015). To examine the cross reactivity of the five APLs mentioned above we examined the responses induced using Montanide or Poly I:C as the vaccine adjuvant. BALB/c mice were immunized either once, or twice, with the peptide alone (KI alone), or mixed with Montanide or Poly I:C (Montanide + KI and Poly I:C + KI, respectively), and 14 days after the last immunization IFN-γ production was assessed via ELISpot assay.

After one immunization (**Figure 3A**), Montanide + KI induced the highest IFN-γ production to the index epitope, KI, significantly higher compared to the media alone background ($p < 0.0001$). Furthermore, moderate compared to KI, but

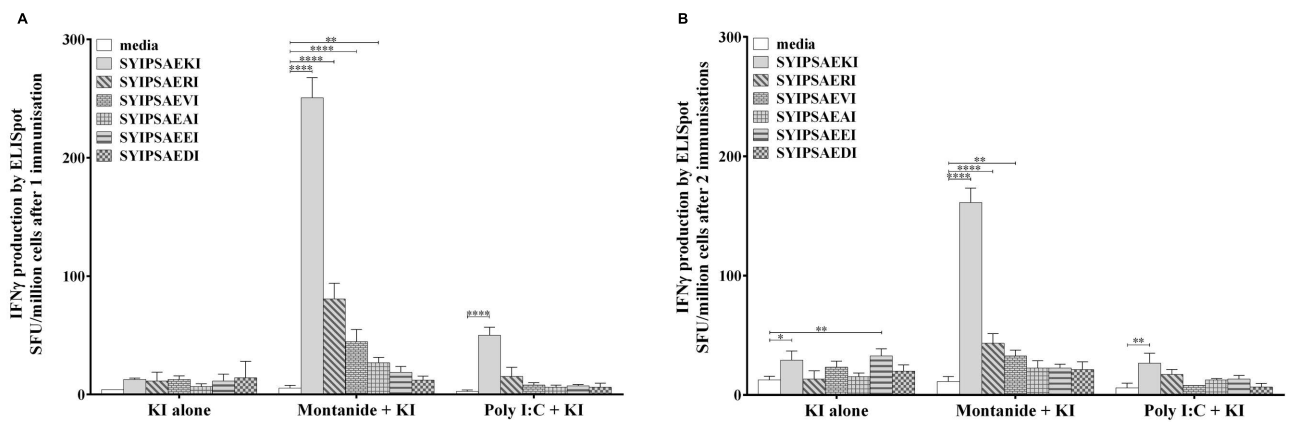

FIGURE 3 | Cross reactivity to APLs of the peptide SYIPSAEKI induced by the conventional adjuvants Montanide or Poly I:C. BALB/c mice were immunized once **(A)**, or twice **(B)** with KI peptide alone (25 μg /mouse), or mixed with Montanide (25 μg peptide mixed with 70% v/v Montanide in PBS /mouse, Montanide + KI) or Poly I:C (25 μg peptide and 25 μg Poly I:C /mouse, Poly I:C + KI). Fourteen days after the last immunization splenocytes were harvested and antigen specific or cross reactive IFN-γ production was measured via ELISpot assay. Data shown as mean ± SD of SFU/million cells (pooled cells from 3 to 4 mice per group) from triplicate wells. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

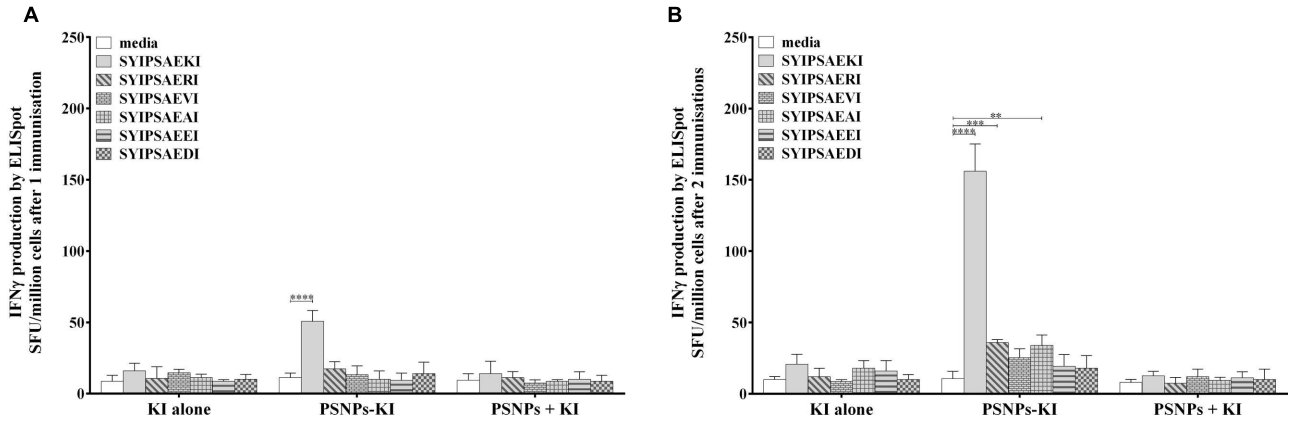

FIGURE 4 | Cross reactivity to APLs of the peptide SYIPSAEKI induced by nanovaccine systems. BALB/c mice were immunized once **(A)**, or twice **(B)** with KI peptide alone, or either mixed with, or conjugated to, PSNPs (25 μg peptide, 1% PSNPs /mouse for both mixed in and conjugated groups). Fourteen days after the last immunization splenocytes were harvested and antigen specific or cross reactive IFN-γ production was measured via ELISpot assay. Data shown as mean ± SD of SFU/million cells (pooled cells from three mice per group) from triplicate wells. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

significant, cross reactivity was seen with this group to the APLs RI and VI ($p < 0.0001$), as well as to AI ($p < 0.01$), similar to the pattern observed with *ex vivo* pulsed DCs. Poly I:C + KI only induced significant levels of IFN-γ response to KI ($p < 0.0001$), showing a corresponding decreased ability to induce detectable cross reactive T cells. A further boost immunization displayed a similar pattern of results for the Montanide + KI group, with a significantly high amount of KI specific CD8 T cell responses ($p < 0.0001$), and low to moderate cross reactivity to the APLs, in particular significant cross reactivity to RI ($p < 0.0001$), and VI ($p < 0.01$), with overall magnitude of responses lower, but ranked similar, compared to one immunization (**Figure 3B**). The boost immunization did not enhance responses induced by the Poly I:C + KI group.

Conjugated PSNPs Induce Little Cross Reactivity to APLs of the CD8 Epitope SYIPSAEKI

Previously it has also been observed that peptide conjugated to, but not mixed with, PSNPs induces high levels of KI specific CD8 T cell responses (Wilson et al., 2015). Following this, cross reactivity to APLs of KI were investigated after one or two immunizations with KI mixed with, or conjugated to, PSNPs. After one immunization, there were low numbers of KI specific CD8 T cell responses induced by the PSNPs-KI conjugated group, albeit significant compared to media alone ($p < 0.0001$), and cross reactive CD8 T cell activity was not detectable (**Figure 4A**).

A boost immunization enhanced the KI specific IFN-γ response to the PSNPs-KI group approximately threefold larger compared to one immunization, with a slight boost to the small cross reactive responses, resulting in the detection of significant responses also to RI ($p < 0.001$), and AI ($p < 0.01$, **Figure 4B**), compared to media alone. Overall little cross reactivity was seen to the APL variants (**Figure 4B**), albeit the ranking of cross reactivity to the different variants remained consistent, regarding side chain charge, to that seen with Montanide and *ex vivo* pulsed DCs. Also consistent with previous findings, the peptide needed to be conjugated to the PSNPs to elicit a response, as the peptide alone or peptide mixed with PSNPs did not induce detectable KI specific or cross reactive responses after a single or boost immunization (**Figures 4A,B**).

The APL DI Conjugated to PSNPs Induces a Broader Pattern of Cross Reactive Responses Compared to that Induced by the Other Variants

Across the previous adjuvants examined the most cross reactivity was observed to APLs carrying a positive or neutral side chain charge. The lowest levels of cross reactive responses were consistently seen to the APLs carrying a negative charged residue at position 8, namely EI and DI. To test whether one of the APL variants could possibly enhance the peptide specific or cross reactive responses, we further conjugated each APL to PSNPs using the method described previously, and examined the pattern of response by IFN-γ ELISpot 13 days after one immunization. Nanovaccine formulations were chosen as the preferred carrier system, to investigate whether an improvement in cross reactivity could be seen when immunizing with a variant compared to the index KI. Furthermore, there are advantages to using non-inflammatory adjuvants/carriers in vaccine formulations.

Consistent with previous data, after one immunization the PSNPs-KI immunized group showed significant IFN-γ production to the KI epitope ($p < 0.0001$, **Figure 5**), however, no significant cross reactive responses were observed to other variants. Immunization with PSNPs-RI induced significant self-reactive responses to RI, as well as significant cross reactivity to KI ($p < 0.0001$). PSNPs-VI induced reactivity to VI, and cross reactivity to AI ($p < 0.0001$), but no cross reactivity to KI. Likewise immunizing with PSNPs-AI showed no reactivity to the index KI, or any other epitope, besides significant reactivity to itself ($p < 0.0001$). Interestingly, the PSNPs-EI group induced significant CD8 T cell responses to EI itself ($p < 0.0001$), as

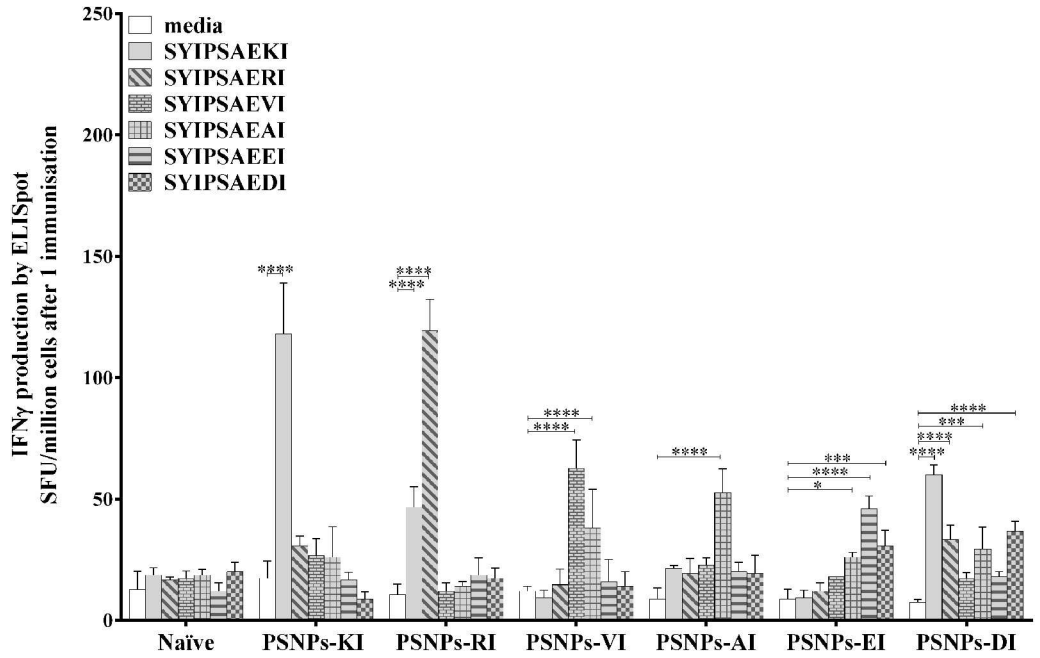

FIGURE 5 | Antigen specific and cross reactive CD8 T cell responses induced by APL conjugated PSNPs. BALB/c mice were immunized once with the conjugated APLs SYIPSAEKI (25 μg peptide, 0.76% PSNPs/mouse), SYIPSAERI (25 μg peptide, 0.65% PSNPs/mouse), SYIPSAEVI (25 μg peptide, 0.86% PSNPs/mouse), SYIPSAEAI (25 μg peptide, 1.09% PSNPs/mouse), SYIPSAEEI (25 μg peptide, 1.47% PSNPs/mouse), and SYIPSAEDI (25 μg peptide, 1.02% PSNPs/mouse) conjugated to PSNPs. Thirteen days after the immunization splenocytes were harvested and antigen specific or cross reactive IFN-γ production was measured via ELISpot assay. Data shown as mean ± SD of SFU/million cells (pooled cells from 3 mice per group) from triplicate wells. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

well as DI ($p < 0.001$), and AI ($p < 0.05$), but no significant responses were detected to VI, RI, or KI. Surprisingly, PSNPs-DI immunization induced significant reactivity to DI ($p < 0.001$), and cross -reactivity to AI ($p < 0.001$), RI ($p < 0.0001$), and KI ($p < 0.0001$), with the response to KI even greater than that to DI itself. Of the variants tested, only two, RI and DI, were identified as being cross-reactive to the index peptide epitope KI in addition to the response to the priming epitope itself. This implies a 'one-way' cross reactivity, where PSNPs-KI cannot induce a significant cross reactive response to DI or RI after one immunization, though conversely, PSNPs-DI and PSNPs-RI can induce a cross reactive response to KI. Overall, PSNPs-DI induced a substantially different pattern of cross-reactivity, to RI, AI, and KI, after one immunization compared to the responses induced by the other conjugated APLs. It would be of interest to investigate in future the breadth of response to DI incorporated with other powerful adjuvant systems, considering that in this study the adjuvant system employed did not substantially alter reactivity, though impacted the overall magnitude of response.

DISCUSSION

The current study presents an *in vivo* model to investigate how polymorphism and variability in protective CD8 T cell epitopes from infectious pathogens, such as malaria, can possibly limit the ability of the vaccines to eradicate them. We have focused on modifications of amino acid position 8 of the protective CD8 T cell epitope of *P. berghei*, KI, since this position has been previously demonstrated to physically interact with affinity modulating regions of a cognate T cell receptor (TCR; Maryanski et al., 1993; Kessler et al., 1998; Guillaume et al., 2003). Furthermore, modifications at this amino-acid position did not alter binding affinity to the MHCI molecule (**Figure 2**). This model was therefore used to investigate for the first time whether the use of different adjuvants, including conventional inflammation promoting adjuvants and novel non-inflammatory nanoparticle based carrier systems, could broaden the repertoire of T cells induced by vaccines. Additionally, we explored whether these adjuvants could potentially be used to generate broad patterns of cross-reactivity capable of recognizing populations of highly variable pathogens, simulating those naturally present in malaria endemic regions.

Vaccination with the immunodominant CD8 T cell epitope KI indicated that reactivity to APLs was unable to be enhanced by the various adjuvant systems. The results showed that the induced responses were largely specific to the immunizing KI epitope itself, with only limited cross-reactivity observed to other variants. The low to moderate level of reactivity that was observed to variant epitopes of KI was not due to a simple lack of binding ability, as all tested variants bound as strongly, or even stronger, to MHCI than the index peptide KI. The cross reactivity of KI was focused primarily on the variant RI, which was the most highly related variant to KI (both positively charged at position 8).

Furthermore, the KI induced responses failed to elicit substantial cross-reactivity to other variants with a neutral (AI and VI) or negative (EI and DI) charge at position 8, regardless of the adjuvant systems used. Cross-reactive responses, when found, were directly related to the magnitude of the immune response induced to the index KI T cell epitope. Importantly, there was no direct relationship between the intrinsic nature of the adjuvant system being utilized, whether this was based on vaccines with water-in-oil emulsions (Montanide), 'danger signals' (Poly I:C), non-inflammatory nano-carriers (PSNPs) or *ex vivo* peptide pulsed DCs. These data sets indicate that for vaccines to be truly broadly cross reactive, it is necessary to look beyond enhancing the magnitude of the index response. Simply modifying the type of adjuvant is unlikely to modify the process of priming or boosting immunity sufficiently to provide broad cross reactive immune responses.

The fact that DI and EI were unable to elicit strong effector T cell reactivity *in vitro*, from cells primed with KI, could indicate that the naïve T cell repertoire of the animals was inherently limited in recognizing such variants, for example, due to holes in the TCR repertoire produced from cross reactivity to self-antigens. Alternatively, it could have meant that these variants may be immunogenic themselves, but did not cross react with KI because their recognition requires a different set of TCRs from that induced by these variants. To distinguish between these possibilities, we immunized with each APL conjugated to the nanoparticle vaccine carrier PSNPs. What we found was that EI and DI were indeed immunogenic, eliciting antigen specific T cells, and hence the inability of KI to induce cross reactive responses was not due to holes in the TCR repertoire. Moreover, DI was capable of inducing a broader pattern of T cell reactivity, inducing KI specific CD8 T cells to comparable, or even higher levels as to its own DI specific T cells. This "one-way" cross reactivity observation was specific to the DI and RI variants, whilst the other immunized APLs predominantly induced homologous responses to the immunization peptide itself. Though RI immunized mice elicited CD8 T cell reactivity to RI and KI, DI immunized mice were the only group capable of inducing cross-reactive responses that were broader than the antigen specific and index peptide reactive responses induced to the immunized epitope. There are intriguing consequences to the identification of the existence of one-directional cross reactivity. For polymorphic pathogens, it suggests that some natural variants may have the advantage of not being recognized by immune responses induced by other variants, whilst being able themselves to elicit immunity which eliminates other strains in the same populations.

For vaccine development, these findings show it may be possible in future to rationally re-engineer polymorphic antigens to enable them to recognize multiple relevant variants in a given population of pathogens. Thus acknowledging the fact that there is extensive research required before the use of re-engineered polymorphic antigens can commonly be used in vaccine design, especially for malaria vaccine development, which already carries a host of inherent challenges. Moreover, although murine models cannot directly be applied to human studies, the basic knowledge and understanding of interactions between APLs and reactivity induced by modifying the peptide, or by using various adjuvant systems, could be tested against known human variants. Importantly, this study has shown that for vaccine design it is relevant to consider immune responses elicited to APLs, and that it is possible to alter the response by modifying polymorphic epitopes, instead of simply varying the adjuvant system used. Given that broader responses were only seen with certain variants, it would be crucial to screen potential targets for their reactivity to evaluate their value as a vaccine candidate. We envisage such approaches would be able to be taken to tackle the many polymorphic pathogens for which there are still no effective broadly reactive vaccines, and particularly help the development of potent liver-stage malaria vaccines.

AUTHOR CONTRIBUTIONS

KW designed and performed experiments, analyzed data, wrote the manuscript. SX helped design experiments and reviewed the manuscript. MP conceived the project, planned experiments, analyzed data, funded the project and wrote the manuscript.

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REFERENCES

Aucouturier, J., Dupuis, L., Deville, S., Ascarateil, S., and Ganne, V. (2002). Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev. Vaccines* 1, 111–118. doi: 10.1586/14760584.1.1.111

Cho, H. I., Barrios, K., Lee, Y. R., Linowski, A. K., and Celis, E. (2013). BiVax: a peptide/poly-IC subunit vaccine that mimics an acute infection elicits vast and effective anti-tumor CD8 T-cell responses. *Cancer Immunol. Immunother.* 62, 787–799. doi: 10.1007/s00262-012-1382-6

Doolan, D. L., and Martinez-Alier, N. (2006). Immune response to pre-erythrocytic stages of malaria parasites. *Curr. Mol. Med.* 6, 169–185. doi: 10.2174/156652406776055249

Duffy, P. E., Sahu, T., Akue, A., Milman, N., and Anderson, C. (2012). Pre-erythrocytic malaria vaccines: identifying the targets. *Expert Rev. Vaccines* 11, 1261–1280. doi: 10.1586/erv.12.92

Elliott, S. L., Suhrbier, A., Miles, J. J., Lawrence, G., Pye, S. J., Le, T. T., et al. (2008). Phase I trial of a CD8+ T-cell peptide epitope-based vaccine for infectious mononucleosis. *J. Virol.* 82, 1448–1457. doi: 10.1128/JVI.01409-07

Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G. A., Li, J., Mottram, P. L., et al. (2004). Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J. Immunol.* 173, 3148–3154. doi: 10.4049/jimmunol.173.5.3148

Gilbert, S. C., Plebanski, M., Gupta, S., Morris, J., Cox, M., Aidoo, M., et al. (1998). Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* 279, 1173–1177. doi: 10.1126/science.279.5354.1173

Guillaume, P., Legler, D. F., Boucheron, N., Doucey, M. A., Cerottini, J. C., and Luescher, I. F. (2003). Soluble major histocompatibility complex-peptide octamers with impaired CD8 binding selectively induce Fas-dependent apoptosis. *J. Biol. Chem.* 278, 4500–4509. doi: 10.1074/jbc.M208863200

Herrera, S., Fernandez, O. L., Vera, O., Cardenas, W., Ramirez, O., Palacios, R., et al. (2011). Phase I safety and immunogenicity trial of *Plasmodium vivax* CS derived long synthetic peptides adjuvanted with montanide ISA 720 or montanide ISA 51. *Am. J. Trop. Med. Hyg.* 84, 12–20. doi: 10.4269/ajtmh.2011.09-0516

Kessler, B., Hudrisier, D., Schroeter, M., Tschopp, J., Cerottini, J. C., and Luescher, I. F. (1998). Peptide modification or blocking of CD8, resulting in weak TCR signaling, can activate CTL for Fas- but not perforin-dependent cytotoxicity or cytokine production. *J. Immunol.* 161, 6939–6946.

Lalvani, A., Moris, P., Voss, G., Pathan, A. A., Kester, K. E., Brookes, R., et al. (1999). Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant *Plasmodium falciparum* malaria vaccine. *J. Infect. Dis.* 180, 1656–1664. doi: 10.1086/315074

Ljunggren, H. G., Stam, N. J., Ohlen, C., Neefjes, J. J., Hoglund, P., Heemels, M. T., et al. (1990). Empty MHC class I molecules come out in the cold. *Nature* 346, 476–480. doi: 10.1038/346476a0

Maryanski, J. L., Luthy, R., Romero, P., Healy, F., Drouet, C., Casanova, J. L., et al. (1993). The interaction of antigenic peptides with the H-2Kd MHC class I molecule. *Semin. Immunol.* 5, 95–104. doi: 10.1006/smim.1993.1013

McKee, A. S., Munks, M. W., MacLeod, M. K., Fleenor, C. J., Van Rooijen, Kappler, N., et al. (2009). Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. *J. Immunol.* 183, 4403–4414. doi: 10.4049/jimmunol.0900164

Neafsey, D. E., Juraska, M., Bedford, T., Benkeser, D., Valim, C., Griggs, A., et al. (2015). Genetic diversity and protective efficacy of the RTS,S/AS01 Malaria Vaccine. *N. Engl. J. Med.* 373, 2025–2037. doi: 10.1056/NEJMoa1505819

Plebanski, M., Lee, E. A. M., Hannan, C. M., Flanagan, K. L., Gilbert, S. C., Gravenor, M. B., et al. (1999). Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. *Nat. Med.* 5, 565–571. doi: 10.1038/8444

Plebanski, M., Lee, E. A., and Hill, A. V. (1997). Immune evasion in malaria: altered peptide ligands of the circumsporozoite protein. *Parasitology* 115(Suppl.), S55–S66. doi: 10.1017/S0031182097002035

Reece, W. H., Pinder, M., Gothard, P. K., Milligan, P., Bojang, K., Doherty, T., et al. (2004). A CD4 (+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. *Nat. Med.* 10, 406–410. doi: 10.1038/nm1009

Rock, K. L., Rothstein, L., and Benacerraf, B. (1992). Analysis of the association of peptides of optimal length to class I molecules on the surface of cells. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8918–8922. doi: 10.1073/pnas.89.19.8918

Wilson, K. L., Xiang, S. D., and Plebanski, M. (2015). Montanide, Poly I:C and nanoparticle based vaccines promote differential suppressor and effector cell expansion: a study of induction of CD8 T cells to a minimal *Plasmodium berghei* epitope. *Front. Microbiol.* 6:29. doi: 10.3389/fmicb.2015.00029

Xiang, S. D., Kong, Y. Y., Hanley, J., Fuchsberger, M., Crimeen-Irwin, B., and Plebanski, M. (2015). Nanoparticles modify dendritic cell homeostasis and induce non-specific effects on immunity to malaria. *Trans. R. Soc. Trop. Med. Hyg.* 109, 70–76. doi: 10.1093/trstmh/tru182

Xiang, S. D., Wilson, K., Day, S., Fuchsberger, M., and Plebanski, M. (2013). Methods of effective conjugation of antigens to nanoparticles as non-inflammatory vaccine carriers. *Methods* 60, 232–241. doi: 10.1016/j.ymeth.2013.03.036

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REVIEW
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Ambivalent Outcomes of Cell Apoptosis: A Barrier or Blessing in Malaria Progression

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The life cycle of *Plasmodium* in two evolutionary distant hosts, mosquito, and human, is a complex process. It is regulated at various stages of developments by a number of diverged mechanisms that ultimately determine the outcome of the disease. During the development processes, *Plasmodium* invades a variety of cells in two hosts. The invaded cells tend to undergo apoptosis and are subsequently removed from the system. This process also eliminates numerous parasites along with these apoptotic cells as a part of innate defense against the invaders. *Plasmodium* should escape the invaded cell before it undergoes apoptosis or it should manipulate host cell apoptosis for its survival. Interestingly, both these phenomena are evident in *Plasmodium* at different stages of development. In addition, the parasite also exhibits altruistic behavior and triggers its own killing for the selection of the best 'fit' progeny, removal of the 'unfit' parasites to conserve the nutrients and to support the host survival. Thus, the outcomes of cell apoptosis are ambivalent, favorable as well as unfavorable during malaria progression. Here we discuss that the manipulation of host cell apoptosis might be helpful in the regulation of *Plasmodium* development and will open new frontiers in the field of malaria research.

Keywords: cell apoptosis, *Plasmodium*, mosquito, midgut epithelium, hepatocytes, erythrocytes

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INTRODUCTION

Arthropods are infamous vectors for numerous human diseases that are major public health hazards throughout the world. The causative agents of these diseases include helminths, protozoa, bacteria, and viruses. Among the large number of vector-borne infections, malaria is included in the list of top five infectious diseases. It is caused by an apicomplexan parasite *Plasmodium* and transmitted by the *Anopheles* mosquito among humans. This disease is reported in over 90 countries primarily in tropical and subtropical regions in sub-Saharan Africa, Central and South America, the Caribbean island of Hispaniola, the Middle East, the Indian subcontinent, South-East Asia, and Oceania. On an average more than 198 million people are infected and 584,000 people die due to malaria every year worldwide (World Health Organization [WHO], 2014).

The malaria parasite *Plasmodium* completes its life cycle in two evolutionary distant hosts; mosquito and a vertebrate such as human. The sexual life cycle is completed in female *Anopheles* mosquito (a definitive primary host) and asexual life cycle predominates in human (a secondary host). The mosquito ingests *Plasmodium* gametocytes stages that undergo fertilization and form zygote inside the midgut lumen. Further, in the same compartment, the formation of ookinetes

from zygote takes place near about 15h after ingestion. Ookinete traverses the midgut epithelium around 24h of ingestion and develops into an oocyst in the space between midgut epithelium and basal lamina (**Figure 1**). Oocyst matures approximately after 10 days and then releases 1000s of sporozoites into the mosquito haemocoel. Sporozoites circulate throughout the hemolymph and some of them end up in salivary glands. Sporozoites entered the salivary gland further undergo maturation and are ready to be

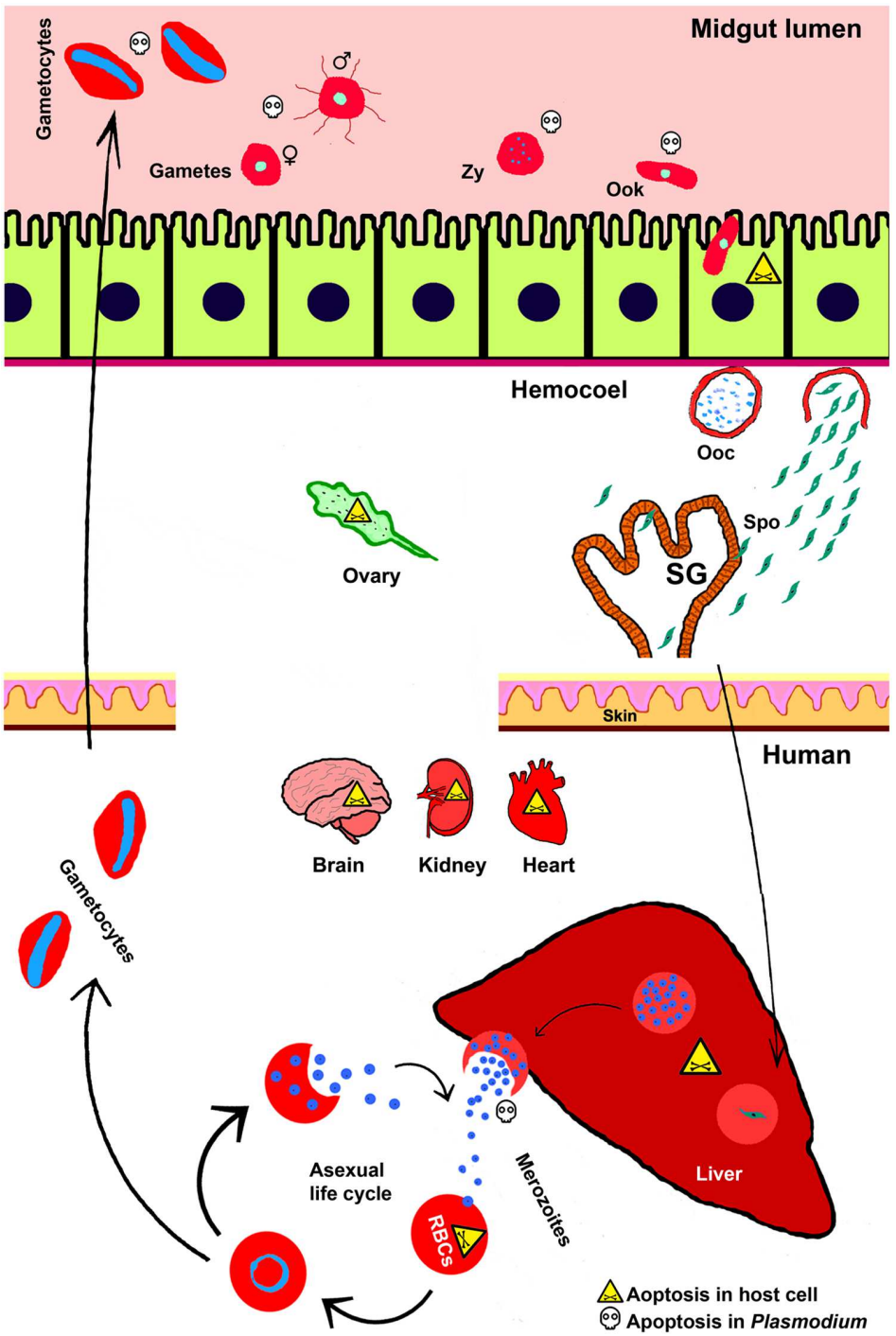

FIGURE 1 | Apoptosis during *Plasmodium* life cycle in invertebrate and vertebrate hosts. Life cycle of *Plasmodium* is completed in two hosts. The sexual life cycle occurs in mosquito and asexual life cycle takes place in the vertebrate host such as, human. Various stages of *Plasmodium* development and the phenomena of cell death in both the hosts and parasites are depicted here. Zy, Zygote; Ook, ookinetes; Ooc, oocysts; Spo, sporozoites; SG, salivary gland.

injected into the new human host during the subsequent feeding (Zheng, 1997; Beier, 1998; Sinden, 2002).

Plasmodium cycle in human host begins with the entry of mosquito-injected sporozoites into the circulating blood. These sporozoites migrate toward the liver and initiate pre-erythrocytic cycle in hepatocytes that continue approximately for 6–15 days. During this phase, *Plasmodium* undergoes extensive growth and division and at the end, 1000s of merozoites are released into the blood. These merozoites further invade red blood cells (RBCs) and initiate erythrocytic cycle (Frevert, 2004). This asexual erythrocytic cycle further produces more merozoites and at 48 or 72 h, depending on the *Plasmodium* species, they are released from the RBC and immediately invade new erythrocytes. In continuation of the erythrocytic cycle some merozoites differentiate into gametocytes and after ingestion by another female mosquito they continue the sexual cycle as discussed above (Cowman et al., 2012).

It is noteworthy to mention that in both the hosts, *Plasmodium* development takes place in compartments-specific manner and during this process parasite interacts with diverse cell types. During these interactions, apoptosis takes place in the parasites as well as the host cells. Apoptosis is a genetically orchestrated type of cell death that involves numerous distinguishable morphological and cellular alterations (Kerr et al., 1972; Cowman et al., 2012). Generally, the advanced stages of apoptosis are associated with the removal of dying cell from the body system and its replacement by a new cell. Studies revealed that pathogens are eliminated along with the dying cell in case of several diseases, including malaria (Williams, 1994; Sinai et al., 2004).

Cell apoptosis plays an important role in establishment of host–pathogen relationships. The present review identifies the sites of apoptosis in malaria parasite and host cells during *Plasmodium* life cycle. It also highlights the effect of apoptosis on the parasite or host as a strategy by which the parasites proliferate in a healthy environment or a self-defense/repair mechanism by the host. Here, apoptosis of host cells, *Plasmodium* and the outcome of this phenomenon in disease progression has been discussed. Apoptosis of immune cells in both the hosts is not considered as part of this review.

APOPTOSIS IN *Plasmodium* DURING SEXUAL STAGES OF DEVELOPMENT

Plasmodium encounters a harsh environment inside the mosquito gut that creates somewhat a bottleneck situation to the parasite numbers at different stages of development such as, gamete, zygote, and ookinete stages (Chose et al., 2003). Majority of gametocytes that arrive in the midgut lumen fail to develop further and on an average approximately 80% of them undergo apoptosis (Sinden, 1999; Sinden and Billingsley, 2001). Studies analyzed the mode of cell death in *Plasmodium berghei* (mouse malaria) sexual stages found that zygotes and ookinetes exhibit the characteristic apoptotic cell morphology revealed by DNA fragmentation, chromation condensation and phosphatidylserine (PS) translocation (Al-Olayan et al., 2002).

The apoptosis in *Plasmodium* may be a natural way to select the most potent or best 'fit' parasite that can carry forward the malaria cycle in mosquito where it may also reduce the parasite burden for better survival of the insect host. This indicates that an increased load of *Plasmodium* infection might have deleterious effects in the mosquito host. Similar parasitic behavior is also reported in *Leishmania* where promostigote stage reveals apoptotic features during their development inside the sand fly gut. Apoptotic parasites die and show philanthropic behavior toward the viable parasites. This phenomenon maintains a vital association for the survival of both parasites and sand fly (Shaha, 2006; Van Zandbergen et al., 2006).

It is also possible that mosquito internal environment factors such as, immune components of the ingested blood, the natural gut symbionts or mosquito innate immunity might be regulating the process of apoptosis in *Plasmodium*. The immune components in mosquito ingested blood include white blood cells (WBCs), complement system proteins, cytokines and reactive oxygen or nitrogen species (Lensen et al., 1998; Ramiro et al., 2011; Simon et al., 2013). Previous reports found that WBCs have phagocytic activity against *P. falciparum* and *P. berghei* gametocytes/gametes in *Anopheles gambiae* midgut. The removal of WBCs form the ingested blood significantly reduced the number of apoptotic ookinetes. In addition, the nitric oxides (NO) generated by activated WBCs also induce parasite death (Lensen et al., 1997; Muniz-Junqueira et al., 2001). Cytokines such as, TNF-α and TGF-β1 present in the ingested blood meal also have anti-plasmodial effects. TNF-α inhibits the male gamete formation (called exflagellation) in *P. berghei* that is mediated through leucocytes generated reactive nitrogen species (RNS; Naotunne et al., 1993; Ali et al., 2010; Ramiro et al., 2011). TGF-β1 in fact induces *A. stephensi* nitric oxide synthase (NOS) at low concentrations and suppresses the parasite numbers (Luckhart et al., 2003). The human complement system remains active up to 6 h in the mosquito midgut. A direct effect of the complement system in preventing gametogenesis has been observed in case of *P. falciparum* development. However, oocysts and zygotes have learned to nullify the complement-mediated lysis through surface binding of factor H that inactivates complement effector protein C3b. This seems to be a co-evolved mechanism of protection in these complement-sensitive stages (Simon et al., 2013). The role of mosquito gut flora in the regulation of *Plasmodium* development is reported by several laboratories. In a simple way, the reduction of microbial communities in antibiotics fed *Anopheles* mosquitoes increases their susceptibility to malaria parasite infection (Boissière et al., 2012; Minard et al., 2013; Kajla et al., 2015a). These observations are important in terms of manipulating the endogenous microbial flora of the mosquito to control their vectorial capacity and this area is under investigation by many researchers.

Gene silencing studies revealed that a number of mosquito immune genes regulate parasite load. For example, clip-domain serine protease (CLIPC2) plays an anti-plasmodial role (Blumberg et al., 2013). Similarly, thioester containing protein 1 (TEP1), an opsonin, induces parasite melanization and inhibits *Plasmodium* development (Blandin et al., 2004; Blumberg et al., 2013). In addition, mosquito generated NO

and reactive oxygen species (ROS) have been reported to trigger apoptosis in *Plasmodium* ookinetes. Studies demonstrated that *in vitro* exposure of *Plasmodium* ookinetes with sodium nitroprusside, the NO producer, induced caspase-like activity (Ali et al., 2010). On the other hand, feeding L-NAME (*N*-nitro-L-arginine methyl ester), the inhibitor of NO producing key enzyme NOS, along with *P. falciparum* infected blood reduced the levels of apoptotic ookinetes and ultimately increased the number of developing oocysts (Luckhart et al., 1998). In addition, the reduction of NOS gene expression after silencing the mosquito STAT, produced similar effect on *Plasmodium* as observed above in case of L-NAME treatment (Gupta et al., 2009). Thus, we can conclude that the regulation of *Plasmodium* development inside the mosquito midgut is a multifaceted phenomenon and has been exploited by a number of transmission blocking strategies. For example, the mosquito midgut specific molecules such as alanyl amino peptidase 1 (APN1), carboxypeptidase B (CPB) and a heme peroxidase HPX15 are proposed to design transmission blocking vaccines because inhibition of these molecules suppressed *Plasmodium* development (Lavazec et al., 2007; Atkinson et al., 2015; Kajla et al., 2015b).

Apoptosis in Ookinete-Invaded Midgut Epithelial Cells is Beneficial for the Mosquito Host

Plasmodium ookinetes are motile and cross the midgut epithelium around 20 h after ingestion and targeted by the mosquito immune system. Numerous mosquito immune pathways such as, peptidoglycan recognition proteins (PGRP), scavenger receptors (SCRs), C-type lectins (CTLs), and the genes regulating melanization cascade are reported to induce apoptosis in ookinetes during their traversal to the mosquito midgut epithelium (Michel and Kafatos, 2005). Ookinete invasion-induced expression of NOS and peroxidases mediate the nitration of invaded midgut cells. This nitration process activates mosquito complement TEP1 that mediate ookinete death (Kumar et al., 2004; De Almeida Oliveira et al., 2012; Garver et al., 2013). Ookinete invasion also induces apoptosis in midgut epithelial cells that is revealed by characteristic apoptotic features and commonly observed in *A. gambiae- P. berghei*, *A. gambiae-P. falciparum*, and *Aedes aegypti- P. gallinaceum* combinations. At the last stage of apoptosis, the invaded cells extrude from the epithelial layer into the gut lumen and are replaced by the new cells (Han et al., 2000; Zieler and Dvorak, 2000; Kumar et al., 2004; Gupta et al., 2005). It is important that for a successful invasion the traversing ookinetes must escape unharmed before the invaded cells extrude into the lumen.

The apoptosis of midgut cells is beneficial for the mosquito host because it tends to remove the 'slow moving ookinetes' that are entrapped inside the damaged cells. Studies found that in case of *A. stephensi*, the bulged out epithelial cells are found in midgut lumen and *P. falciparum* ookinete are entrapped inside them (Baton and Ranford-Cartwright, 2004, 2005). Our observations also revealed that in the same mosquito midgut cells undergo apoptosis soon after they are invaded by the *P. falciparum* ookinetes (Kumar and Barillas-Mury, 2005). Although the *P. berghei* ookinete invasion induces cell death in mosquito midgut epithelium, however, in this case the ookinetes entrapped inside the dead cells are rarely observed. It may be simply due to the reason that *P. falciparum* infected mosquitoes are maintained at 28°C and *P. berghei* infected mosquitoes at 20°C. This lower temperature maintenance might slow down the midgut cell death processes as reported in case of some mammalian cells (Sakurai et al., 2005).

The invasion of *Anopheles* mosquito midgut by *P. falciparum* and *P. berghei* ookinetes is also different in many ways. In the former case, generally less number of ookinetes successfully completes the invasion and develops into the oocysts. However, in the case of *P. berghei* the rate of ookinete invasion is mostly higher in comparison to the *P. falciparum* infection (Smith et al., 2014). The rapid turnover of mosquito midgut epithelial cells in case of *P. falciparum*, due to their maintenance at 28°C, may be associated with less successful invasion events. In this case, the damaged cells undergo a rapid removal from the epithelial surface and are replaced by the new cells. These dead cells also take away the pathogens that invade them as the part of an intrinsic defense mechanism. The phenomenon of rapid epithelial turnover in reducing the infection by microbial intruders has been observed in other enteric infections. For example, in case of gastrointestinal habitants *Helicobacter pylori* and *Trichuris muris* nematode infections, the gastric epithelial cells undergo apoptosis and expulsion. The increased rate of epithelial turnover makes the mouse resistant against the worm infection (Moss et al., 1996; Kim et al., 1998; Cliffe et al., 2005).

It is of note that the mosquito midgut has few hundred cells; thus, the death in large extend of these cells during ookinete invasion may also be deleterious to the insect host. In other words, the higher density of *Plasmodium* infectious stages in the ingested blood might have deleterious effects on mosquito host. Thus, the balanced regulation of midgut epithelium apoptosis in these mosquitoes is important for their survival after *Plasmodium* infection.

Ookinete Tends to Suppress Midgut Cell Apoptosis for Its Own Survival

It is clear from the above discussion that to establish infection in the mosquito midgut, ookinetes should escape before they are destroyed or extruded along with the damaged epithelial cell. We believe that in this situation *Plasmodium* must be manipulating either the midgut epithelial immunity or apoptosis in the target cell. Studies carried in *A. gambiae* mosquito revealed that the ookinete protein Pfs47 prevents the activation of caspases and inhibits Jun-N-terminal kinase-mediated activation of apoptosis in invaded mosquito midgut cells (Ramphul et al., 2015). In this study, the wild type parasites regulated broad changes in gene expression profile of the mosquito midgut, however, the Pfs47 mutants failed to do so. Evidences obtained from the above study revealed that the *Plasmodium* death reduced the parasite load in mosquito midgut to support the survival of the vector, completion of sexual life cycle and disease transmission to the vertebrate host. On the other hand, *Plasmodium* mediated

interference to the apoptosis of mosquito midgut epithelium also supports its own survival. It manipulates the expression of peroxidase/oxidase system responsible for the nitration of ookinetes, which causes parasite lysis by TEP1 (Ramphul et al., 2015).

The Death of Oocysts is Mediated by the Mosquito Late Phase Immunity

The ookinete that successfully traverses the midgut are further transformed into the oocyst. The phenomenon of cell apoptosis is not observed in oocysts. However, recent studies reveal that in *A. gambiae* the oocysts encounter a late phase immunity that is mediated through STAT pathway. Silencing of STAT gene in these mosquitoes increased the number of oocysts survival against controls, which was mediated through the reduced expression of NOS, an effector gene of STAT pathway (Gupta et al., 2009). In the same mosquito, an LPS-induced TNFα transcription factor (LITAF)-like 3 (LL3) is also reported to mediate the late phase immunity. LL3 binds to the promoter region of an anti-plasmodial gene termed as serine protease inhibitor 6 (SRPN6) and modulates its expression. Silencing of LL3 gene restricts the differentiation of hemocytes and their responses to parasite infection that results in the increased number of oocysts in the silenced mosquitoes. Thus, LL3 implicates late-phase immunity against *Plasmodium* oocysts through hemocytes (Smith et al., 2012, 2015). These observations might call upon to understand the correlation between the STAT and SRPN 6 pathways in terms of regulating the late phase immunity against *Plasmodium* oocysts.

Death Process in the Sporozoites

Sporozoites released from the oocyst after several rounds of mitotic divisions although undergo a death process; however, this is a type of non-apoptotic death. They are released in 1000s and 10–20% of them finally invade the salivary glands. Rests of the sporozoites are cleared from the mosquito circulation by hemocytes-mediated phagocytosis (Foley, 1978; Hernández-Martínez et al., 2002; Hillyer et al., 2003, 2007). In salivary glands, *Plasmodium* uptake occurs via a receptor-mediated endocytosis or through a specific ligand interaction. No cytoskeleton rearrangement or apoptosis has been observed in the gland epithelium (Ghosh and Jacobs-Lorena, 2009; Mueller et al., 2010).

Plasmodium entry in salivary gland epithelial layer is dissimilar to the midgut epithelium invasion. Salivary glands mount an acute immune response against invading sporozoites. Studies found that some common genes exhibit similar regulation in both epithelia. For example, *Plasmodium* invasion downregulates a fatty acid synthase (AGAP009176) however, GTP-binding nuclear protein, lysosomal thioreductase precursor, and SRPN6 were up-regulated in both the tissues (Rosinski-Chupin et al., 2007). Interestingly, the silencing of *SRPN6* gene in mosquito midgut and salivary gland increased the number of parasites in the respective organ (Abraham et al., 2005; Pinto et al., 2008). This indicates that mosquito innate immunity regulates *Plasmodium* number at different stages of development. This provides an opportunity to manipulate mosquito immunity to control the vectorial competence.

Plasmodium Infection Mediated Apoptosis in Non-target Mosquito Cells

Plasmodium does not directly interact with some mosquito cells; however, the infection exhibits an indirect effect on the apoptosis of some other host cells. For example, *P. yoelii nigeriensis* infection in *A. stephensi* resulted in characteristic apoptosis of cells in ovaries or the follicular epithelium and reduced egg production by the gravid females (Hopwood et al., 2001; Ahmed and Hurd, 2006). In addition, the follicular epithelial cell apoptosis is also the major trigger of follicular reabsorption. The follicles showing reabsorption are detected at 12 h that gradually increases to maximum at 24 h post infected blood feeding. Interestingly, at 12 h post feeding the *Plasmodium* development taking place in midgut bolus and at 24 h the ookinete invades the midgut (Han et al., 2000; Kumar et al., 2010). These findings might indicate the advanced detection of *Plasmodium*-induced factors by the mosquito system. In parallel, the anti-plasmodial immunity of mosquito may be responsible for follicular apoptosis through the generation of ROS or RNS. These assumptions are supported by experimental findings where high levels of NO, nitrites/nitrates or ROS are found in *Plasmodium* infected mosquito midgut as well as hemolymph (Luckhart et al., 1998; Crampton and Luckhart, 2001; Kumar et al., 2003). This may be also possible that during the ookinete invasion some bolus bacteria are exposed to the mosquito immune system and the reactive products of innate immunity (ROS and RNS) mediate the degenerative effects in ovaries. The induction of mosquito immunity after lipopolysaccharides (LPS) inoculation into the hemocoel also caused follicular resorption and reduction of fecundity (Ahmed et al., 2002; Hurd, 2003). On the other hand, *Plasmodium* might have developed some mechanisms to induce follicular resorption and channelizing the eggs stored energy for its own development. The understanding of these mechanisms requires further investigations and it can provide a ground to control mosquito fecundity and their population.

Plasmodium ASEXUAL LIFE CYCLE AND CELL APOPTOSIS IN VERTEBRATE HOST

Plasmodium sporozoites initiate a silent infectious phase called pre-erythrocytic stage inside the liver cells (hepatocytes) and ultimately develop into exo-erythrocytic merozoites (Prudêncio et al., 2011). The sporozoites delivered after mosquito bite in the vertebrate host travel through blood stream and reach liver sinusoids. In the liver sinusoids, sporozoites come to arrest through the interactions of their surface circumsporozoite protein (CSP) with the glycosaminoglycans (GAGs) of stellate cells. According to the gateway hypothesis, the sporozoite glides along the sinusoid wall to locate a Kupffer cell, traverse it and subsequently invade the underlying hepatocyte (Frevert et al., 2005). Recent findings revealed that a heavily glycosylated protein CD68, present exclusively on the surface of the Kupffer cells, acts

like a candidate receptor for sporozoite entry. The sporozoites are also reported to remain enclosed within the CD68 endosome that protects them against lysosomal attack within the Kupffer cell. Although the ligand for CD68 receptor is unknown; however, the Kupffer cells of CD68 knockout mouse imposed a barrier for sporozoite invasion (Cha et al., 2015). These findings are important for developing vaccines to block liver stages (LSs) of sporozoites development.

Interestingly, the $CD68^{+}$ Kupffer cells are known to exhibit both phagocytic activity and ROS production capacity (Kinoshita et al., 2010). Thus, the survival of traversing sporozoites must be dependent on the modulation of immune pathways in these macrophages. Studies found that the sporozoite CSP interaction with heparan sulfate proteoglycans (HSPGs) and the low-density lipoprotein receptor-related protein LRP-1 on the surface of these Kupffer cells activates adenylyl cyclase (AC) enzyme. This enzyme, in turn, upregulates cAMP activity and inhibits NADPH oxidase-mediated generation of ROS by the Kupffer cells (Usynin et al., 2007; Kinoshita et al., 2010; Tavares et al., 2013; Cha et al., 2015). These mechanisms create an anti-inflammatory environment that protects sporozoites from the immune attacks in the liver. It is also observed that Kupffer cells exhibit the sign of apoptosis during transformation of sporozoites into early exo-erythrocytic forms (Usynin et al., 2007; Klotz and Frevert, 2008). In conclusion, the sporozoite not only induces desensitization of Kupffer cells to pro-inflammatory stimuli, it also forces their programmed cell death for its own survival.

Plasmodium Manipulates Host Immunity and Apoptosis of Hepatocytes

The intracellular sporozoites are protected from the host immune responses; however, the infected hepatocytes have other mechanisms to eliminate them. Studies have shown that infected hepatocytes undergo apoptosis without external triggers. When sporozoites traverse the hepatocytes, they deform the hepatocyte morphology that results in wounding. This wounding is the trigger for induction of apoptosis in hepatocytes. The apoptosis in infected hepatocytes has been confirmed by previous studies that observed the uptake of fluorescein isothiocyanate (FITC) labeled dextran and propidium iodide by these cells (Mota et al., 2001; Kaushansky et al., 2013). Blocking the apoptosis process in hepatocytes increases LS parasite burden in mice. This suggests that apoptosis of hepatocytes has negative effect on *Plasmodium* progression through hepatocytic stages (Kaushansky et al., 2013). In other words, apoptosis of infected hepatocytes is beneficial for vertebrate host but detrimental for the sporozoites. It is of note that the apoptosis of *Plasmodium* infected hepatocytes exposes the parasitic antigens to initiate protective immune responses in the host (Leiriao et al., 2005b). Therefore, for a successful completion of the LSs, sporozoites must be able to modulate the host immunity and the process of cell death in hepatocytes.

Sporozoites induce the release of hepatocyte growth factor (HGF) from the traversed cells that inhibits tumour necrosis factor (TNF)-mediated apoptosis of hepatocytes. This process helps in successful establishment of the liver infection (Carrolo et al., 2003; Huh et al., 2004; Leiriao et al., 2005a). In addition, sporozoites also inhibit the translocation of PS into the outer leaflet of the cell membrane to block the 'eat me' signal displayed by infected hepatocytes to the Kupffer cells. In fact, the swapping of PS into the outer leaflet is driven by the increased cytosolic Ca^{2+}. The hepatic stages of parasite sequester this intracellular Ca^{2+} and ultimately inhibit the cellular autophage in hepatocytes (Sturm et al., 2006). A similar mechanism is also displayed by intra-erythrocytic parasites as discussed later.

Plasmodium sporozoites are also equipped with other specific mechanisms to manipulate the apoptosis of infected hepatocytes and completing the LSs. Sporozoites mostly invade those hepatocytes that express high levels of EphA2, a transmembrane receptor tyrosine kinase (RTK). EphA2 is generally expressed in the majority of epithelial cells and the interaction of this receptor with its ligand ephrin leads to the contact-dependent cell–cell communication (Park et al., 2013). Interestingly, in hepatocytes the sporozoite remains enclosed inside a parasitophorous vacuole (PV) that is the part of hepatocyte plasma membrane. Recent studies revealed that the interaction of sporozoite surface protein P36 with hepatocyte EphA2 determines the formation of PV. The formation of PV is a viable process to establish a tolerant environment for the replication of sporozoite in the intracellular compartment. In the absence of PV formation, the sporozoites infected hepatocytes suffer extensive cell death and reduce the burden of LSs (Mueller et al., 2005; Kaushansky et al., 2015). Experimental evidences also revealed that the LS infection of *Plasmodium* was largely decreased in $EphA2^{-/-}$ mice in comparison to wild-type mice. This indicated that EphA2 receptor mediated invasion is helpful in the survival of sporozoites and manipulation of apoptosis in the hepatocytes (Kaushansky et al., 2015). Furthermore, studies carried with *P. berghei*-hepatocyte infection stages revealed that the parasite surface protein P36p, the member of P48/45 family of proteins, is responsible for delaying the apoptosis in hepatocytes. It is of note that P36p-deficient *P. berghei* sporozoites are capable of infecting HepG2 cells in culture as well as mouse liver cells *in vivo*. However, the mutant parasites are eliminated faster due to an increased apoptosis in the infected hepatocytes in comparison to the wild type parasite infection. In addition, the mutant parasites also developed the protective immunity *in vivo* (Van Dijk et al., 2005). On the other hand, sporozoite secreted cysteine protease inhibitor also blocks the cysteine proteases of the invaded hepatocytes and suppress their apoptosis (Rennenberg et al., 2010). These findings might conclude that it is a must for the sporozoites to either delay or block the apoptosis of the infected hepatocytes. If the infected liver cell undergoes apoptosis, it will help the antigen presenting cells to display parasite antigens to the acquired immune system. These findings provide newer ways to develop protective immunity against the LSs of *Plasmodium* through manipulation of apoptosis in hepatocytes and demands further exploring this area in details.

Apoptosis in Erythrocytes Has Dual Effects on Malaria Progression

The enucleated RBCs exhibit programmed cell death known as eryptosis. This phenomenon is similar to apoptosis and

characterized by the cell shrinkage, membrane bebbling, and exposure of PS at the cell surface. A major signal of eryptosis is an increased cytosolic Ca^{2+} that involves the cell membrane scrambling in a way similar to the liver cells as discussed before (Föller et al., 2008; Lang et al., 2008). In fact, *Plasmodium* induces oxidative burst that, in turn, activates ion channels in the infected RBC for the uptake of nutrients, Na^{+} and Ca^{2+} ions and excretion of waste product. This Ca^{2+} entry is the major signal for inducing eryptosis (Brand et al., 2003; Tanneur et al., 2006). The phenomenon of eryptosis has been observed in *P. yoelii* 17XL and *P. berghei* ANKA infected RBCs *in vivo* as well as in *P. falciparum* culture. Studies carried in case of *P. yoelii* 17XL revealed that in addition to the parasitized RBCs (pRBCs), non-parasitized RBCs (nRBC) also undergo eryptosis in response to a high parasite load (Eda and Sherman, 2002; Koka et al., 2008; Totino et al., 2010, 2013).

Apoptosis of RBCs has dual role in the advancement of malaria disease. In one way, infected cells undergoing apoptosis are removed by splenic phagocytosis and this process controls the parasite load as well as contributes to the anaemic conditions (Lang et al., 2009; Totino et al., 2010). The death of infected RBCs is the sign of risk for *Plasmodium* development. Thus, to avoid splenic phagocytosis, *Plasmodium*-infected RBCs adhere to the microvascular endothelium via PS and chondroitin sulfate A (CSA) and ends up in more complications such as cerebral malaria, blood–brain barrier (BBB) dysfunction and multiple organ failure (Eda and Sherman, 2002; Setty et al., 2002; Hunt and Grau, 2003; Tripathi et al., 2007; Craig et al., 2012). To overcome the eryptosis-associated side effects, *Plasmodium* tends to delay this process by reducing both the intra-erythrocytic Ca^{2+} levels and the activity of erythrocyte Ca^{2+} pump (Duranton et al., 2003; Huber et al., 2005). Thus, *Plasmodium* should manage the apoptosis of RBCs to balance between the progression of disease and death of the vertebrate host. These mechanisms are important to control erythrocytic stages of *Plasmodium* development. Target specific blockers may be synthesized to manipulate the *Plasmodium*-induced channels in RBC membrane. This will open new opportunities in this field. Several research labs are actively engaged in this area to develop these channel blockers to inhibit the progression of erythrocytic cycle in human malaria (Kang et al., 2005).

CELL APOPTOSIS IN GENERAL IS ASSOCIATED WITH SEVERE COMPLICATIONS

Severe complications in malaria are associated with the sequestration of *Plasmodium*-infected red blood cells (pRBCs) in the brain and other organs. Sequestration involves the cytoadherence of pRBCs, which causes over-expression of inflammatory cytokines and target cell apoptosis (Hunt and Grau, 2003; Pino et al., 2003; Wilson et al., 2008). In the severe malaria, vascular integrity is altered as a result of endothelial cell (EC) activation and death, which is caused by the activation of *Plasmodium* apoptosis-linked pathogenicity factors (PALPF), PALPF-2, PALPF-5, and PF11_0521 (N'Dilimabaka et al., 2014). Some findings also suggested that glycosylphosphatidylinositol (GPI) of *P. falciparum* is responsible for the cardiomyocyte apoptosis that reveals malaria associated severity (Wennicke et al., 2008). Acute organ failure (for example, kidney or liver) during complicated malaria is associated with the sequestration and adhesion of pRBCs to the ECs in target organs. In *P. berghei* ANKA infected BALB/c mice high expression of ICAM-1 was found on renal tissues, which is responsible for the interaction of pRBCs with them. This cytoadherence causes change in vascular permeability and recruits inflammatory cells, which contributes to the increased rate of cell apoptosis (Elias et al., 2012). Hence, switching from uncomplicated to severe malaria is possibly caused by the activation of cell apoptosis in different organs. Thus, the target-specific strategies against these major factors that are associated with the disease 'severity' might be helpful to control complications during malaria infections.

APOPTOSIS IN *Plasmodium* IS CRUCIAL FOR HOST SURVIVAL

Plasmodium demonstrates stress responses in vertebrate host and this may result in inducing its own apoptosis. The progression of *Plasmodium* merozoites through intraerythrocytic development includes high fever where temperature of the host body raises up to 41°C. The rise in body temperature is associated with erythrocyte rupture and release of new merozoites. This high temperature can be a natural stimulus for *Plasmodium* to undergo programmed cell death process. *In vitro* studies demonstrated that the DNA fragmentation takes place in *P. falciparum* strain 3D7 after 2 h at 41°C (Oakley et al., 2007). We believe that *Plasmodium* must be equipped by the mechanism that can provide temperature tolerance for its survival.

If the parasite numbers increase beyond an optimal level, it may cause stress and death of the host. In this situation, parasite should adopt a strategy to regulate its own number. The induction of self-apoptosis by the *Plasmodium* may be one of the ways to achieve this goal. *In vitro Plasmodium* culture studies revealed that a growth arrested phenomenon through apoptosis occurs when the parasites undergo a certain percentage of parasitaemia. For example, highly synchronous culture of *P. falciparum* strains Dd2 and 3D7 exhibit cell apoptosis at >11% parasitaemia. Interestingly, these parasites continued their life cycle in the ring stages and failed to progress to the trophozoites and schizonts stages (Mutai and Waitumbi, 2010). This phenomenon of arresting self-development may also reveal a spontaneous judgment by the parasite to protect the host. Host environment also plays an important role in the regulation of *Plasmodium* development. The heme degraded product bilirubin is reported to induce apoptosis in *Plasmodium*. *In vitro* studies demonstrated that the addition of bilirubin to *P. falciparum* culture increased mRNA expression of apoptosis-related genes in the parasites (Kumar et al., 2008). This further suggests that regulating the phenomenon of self-apoptosis in *Plasmodium* might be a good way to control the disease

progression in human host. This proposal demands further research in this area.

CONCLUSION

The interaction of *Plasmodium* with vertebrate and invertebrate systems is very complex. At different stages of malaria infection, the *Plasmodium* number is regulated by the host immune system or sometime it is a self-directed decision by the parasite. In addition, the program cell death in both the hosts also contributes to this phenomenon; however, *Plasmodium* is capable of suppressing this event to confer its successful development. Thus, the manipulation of apoptosis in *Plasmodium* or host cell might be a promising strategy to control the disease.

AUTHOR CONTRIBUTIONS

PK and SS carried the literature survey and contributed to the initial draft. LG and SK wrote the manuscript with input from others. All authors read and approved the manuscript.

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REFERENCES

Abraham, E. G., Pinto, S. B., Ghosh, A., Vanlandingham, D. L., Budd, A., Higgs, S., et al. (2005). An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proc. Natl. Acad. Sci. U.S.A.* 102, 16327–16332. doi: 10.1073/pnas.0508335102

Ahmed, A. M., Baggott, S. L., Maingon, R., and Hurd, H. (2002). The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. *Oikos* 97, 371–377. doi: 10.1034/j.1600-0706.2002.970307

Ahmed, A. M., and Hurd, H. (2006). Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. *Microbes Infect.* 8, 308–315. doi: 10.1016/j.micinf.2005.06.026

Ali, M., Al-Olayan, E. M., Lewis, S., Matthews, H., and Hurd, H. (2010). Naturally occurring triggers that induce apoptosis-like programmed cell death in *Plasmodium berghei* ookinetes. *PLoS ONE* 5:e12634. doi: 10.1371/journal.pone.0012634

Al-Olayan, E. M., Williams, G. T., and Hurd, H. (2002). Apoptosis in the malaria protozoan, *Plasmodium berghei*: a possible mechanism for limiting intensity of infection in the mosquito. *Int. J. Parasitol.* 32, 1133–1143. doi: 10.1016/S0020-7519(02)00087-5

Atkinson, S. C., Armistead, J. S., Mathias, D. K., Sandeu, M. M., Tao, D., Borhani-Dizaji, N., et al. (2015). The *Anopheles*-midgut APN1 structure reveals a new malaria transmission-blocking vaccine epitope. *Nat. Struct. Mol. Biol.* 22, 532–539. doi: 10.1038/nsmb.3048

Baton, L. A., and Ranford-Cartwright, L. C. (2004). *Plasmodium falciparum* ookinete invasion of the midgut epithelium of *Anopheles stephensi* is consistent with the Time Bomb model. *Parasitology* 129, 663–676. doi: 10.1017/S0031182004005979

Baton, L. A., and Ranford-Cartwright, L. C. (2005). How do malaria ookinetes cross the mosquito midgut wall? *Trends Parasitol.* 21, 22–28. doi: 10.1016/j.pt.2004.11.001

Beier, J. C. (1998). Malaria parasite development in mosquitoes. *Annu. Rev. Entomol.* 43, 519–543. doi: 10.1146/annurev.ento.43.1.519

Blandin, S., Shiao, S. H., Moita, L. F., Janse, C. J., Waters, A. P., Kafatos, F. C., et al. (2004). Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* 116, 661–670. doi: 10.1016/S0092-8674(04)00173-4

Blumberg, B. J., Trop, S., Das, S., and Dimopoulos, G. (2013). Bacteria- and IMD pathway-independent immune defenses against *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS ONE* 8:e72130. doi: 10.1371/journal.pone.0072130

Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., et al. (2012). Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog.* 8:e1002742. doi: 10.1371/journal.ppat.1002742

Brand, V. B., Sandu, C. D., Duranton, C., Tanneur, V., Lang, K. S., Huber, S. M., et al. (2003). Dependence of *Plasmodium falciparum* in vitro growth on the cation permeability of the human host erythrocyte. *Cell. Physiol. Biochem.* 13, 347–356. doi: 10.1159/000075122

Carrolo, M., Giordano, S., Cabrita-Santos, L., Corso, S., Vigario, A. M., Silva, S., et al. (2003). Hepatocyte growth factor and its receptor are required for malaria infection. *Nat. Med.* 9, 1363–1369. doi: 10.1038/nm947

Cha, S. J., Park, K., Srinivasan, P., Schindler, C. W., van Rooijen, N., Stins, M., et al. (2015). CD68 acts as a major gateway for malaria sporozoite liver infection. *J. Exp. Med.* 212, 1391–1403. doi: 10.1084/jem.20110575

Chose, O., Sarde, C. O., Gerbod, D., Viscogliosi, E., and Roseto, A. (2003). Programmed cell death in parasitic protozoans that lack mitochondria. *Trends Parasitol.* 19, 559–564. doi: 10.1016/j.pt.2003.09.016

Cliffe, L. J., Humphreys, N. E., Lane, T. E., Potten, C. S., Booth, C., and Grencis, R. K. (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* 308, 1463–1465. doi: 10.1126/science.1108661

Cowman, A. F., Berry, D., and Baum, J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J. Cell Biol.* 198, 961–971. doi: 10.1083/jcb.201206112

Craig, A. G., Khairul, M. F., and Patil, P. R. (2012). Cytoadherence and severe malaria. *Malays. J. Med. Sci.* 19, 5–18.

Crampton, A., and Luckhart, S. (2001). The role of As60A, a TGF-beta homolog, in *Anopheles stephensi* innate immunity and defense against *Plasmodium* infection. *Infect. Genet. Evol.* 1, 131–141. doi: 10.1016/S1567-1348(01)00017

De Almeida Oliveira, G., Lieberman, J., and Barillas-Mury, C. (2012). Epithelial nitration by a Peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. *Science* 335, 856–859. doi: 10.1126/science.1209678

Duranton, C., Huber, S., Tanneur, V., Lang, K., Brand, V., Sandu, C., et al. (2003). Electrophysiological properties of the *Plasmodium falciparum*-induced cation conductance of human erythrocytes. *Cell. Physiol. Biochem.* 13, 189–198. doi: 10.1159/000072421

Eda, S., and Sherman, I. W. (2002). Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell. Physiol. Biochem.* 12, 373–384. doi: 10.1159/000067908

Elias, R. M., Correa-Costa, M., Barreto, C. R., Silva, R. C., Hayashida, C. Y., Castoldi, Â, et al. (2012). Oxidative stress and modification of renal vascular permeability are associated with acute kidney injury during *P. berghei* ANKA infection. *PLoS ONE* 7:e44004. doi: 10.1371/journal.pone.0044004

Foley, D. A. (1978). "Innate cellular defense by mosquito hemocytes," in *Comparative Pathobiology: Invertebrate Models for Biomedical Research*, ed. L. A. Bulla Jr. (New York, NY: Plenum Press), 113–144.

Föller, M., Huber, S. M., and Lang, F. (2008). Erythrocyte programmed cell death. *IUBMB Life* 60, 661–668. doi: 10.1002/iub.106

Frevert, U. (2004). Sneaking in through the back entrance: the biology of malaria liver stages. *Trends Parasitol.* 20, 417–424. doi: 10.1016/j.pt.2004.07.007

Frevert, U., Engelmann, S., Zougbédé, S., Stange, J., Ng, B., Matuschewski, K., et al. (2005). Intravital observation of *Plasmodium berghei* sporozoite infection of the liver. *PLoS Biol.* 3:e192. doi: 10.1371/journal.pbio.0030192

Garver, L. S., Oliveira, G. A., and Barillas-Mury, C. (2013). The JNK pathway is a key mediator of *Anopheles gambiae* antiplasmodial immunity. *PLoS Pathog.* 9:e1003622. doi: 10.1371/journal.ppat.1003622

Ghosh, A. K., and Jacobs-Lorena, M. (2009). *Plasmodium* sporozoite invasion of the mosquito salivary gland. *Curr. Opin. Microbiol.* 12, 394–400. doi: 10.1016/j.mib.2009.06.010

Gupta, L., Kumar, S., Han, Y. S., Pimenta, P. F. P., and Barillas-Mury, C. (2005). Midgut epithelial responses of different mosquito–*Plasmodium* combinations: the actin cone zipper repair mechanism in *Aedes aegypti*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4010–4015. doi: 10.1073/pnas.0409642102

Gupta, L., Molina-Cruz, A., Kumar, S., Rodrigues, J., Dixit, R., Zamora, R. E., et al. (2009). The STAT pathway mediates late phase immunity against *Plasmodium* in the mosquito *Anopheles gambiae*. *Cell Host Microbe* 5, 498–507. doi: 10.1016/j.chom.2009.04.003

Han, Y. S., Thompson, J., Kafatos, F. C., and Barillas-Mury, C. (2000). Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J.* 19, 6030–6040. doi: 10.1093/emboj/19.22.6030

Hernández-Martínez, S., Lanz, H., Rodríguez, M. H., González-Ceron, L., and Tsutsumi, V. (2002). Cellular-mediated reactions to foreign organisms inoculated into the hemocoel of *Anopheles albimanus* (Diptera: Culicidae). *J. Med. Entomol.* 39, 61–69. doi: 10.1603/0022-2585-39.1.61

Hillyer, J. F., Barreau, C., and Vernick, K. D. (2007). Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito hemocoel. *Int. J. Parasitol.* 37, 673–681. doi: 10.1016/j.ijpara.2006.12.00

Hillyer, J. F., Schmidt, S. L., and Christensen, B. M. (2003). Rapid phagocytosis and melanization of bacteria and *Plasmodium* sporozoites by hemocytes of the mosquito *Aedes aegypti*. *J. Parasitol.* 89, 62–69. doi: 10.1645/0022-3395(2003)089[0062:RPAMOB]2.0.CO;2

Hopwood, J. A., Ahmed, A. M., Polwart, A., Williams, G. T., and Hurd, H. (2001). Malaria-induced apoptosis in mosquito ovaries: a mechanism to control vector egg production. *J. Exp. Biol.* 204, 2773–2780.

Huber, S. M., Duranton, C., and Lang, F. (2005). Patch-clamp analysis of the "new permeability pathways" in malaria-infected erythrocytes. *Int. Rev. Cytol.* 246, 59–134. doi: 10.1016/S0074-7696(05)46003-9

Huh, C. G., Factor, V. M., Sanchez, A., Uchida, K., Conner, E. A., and Thorgeirsson, S. S. (2004). Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4477–4482. doi: 10.1073/pnas.0306068101

Hunt, N. H., and Grau, G. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol.* 24, 491–499. doi: 10.1016/S1471-4906(03)00229-1

Hurd, H. (2003). Manipulation of medically important insect vectors by their parasites. *Annu. Rev. Entomol.* 48, 141–161. doi: 10.1146/annurev.ento.48.091801.112722

Kajla, M., Gupta, K., Gupta, L., and Kumar, S. (2015a). A fine-tuned management between physiology and immunity maintains the gut microbiota in insects. *Biochem. Physiol.* 4, 182–190. doi: 10.4172/2168-9652.1000182

Kajla, M., Gupta, K., Kakani, P., Dhawan, R., Choudhury, T. P., Gupta, L., et al. (2015b). Identification of an *Anopheles* lineage-specific unique Heme Peroxidase HPX15: a plausible candidate for arresting malaria parasite development. *J. Phylogenetics Evol. Biol.* 3, 160. doi: 10.4172/2329-9002.1000160

Kang, M., Lisk, G., Hollingworth, S., Baylor, S. M., and Desai, S. A. (2005). Malaria parasites are rapidly killed by dantrolene derivatives specific for the plasmodial surface anion channel. *Mol. Pharmacol.* 68, 34–40.

Kaushansky, A., Douglass, A. N., Arang, N., Vigdorovich, V., Dambrauskas, N., Kain, H. S., et al. (2015). Malaria parasites target the hepatocyte receptor EphA2 for successful host infection. *Science* 350, 1089–1092. doi: 10.1126/science.aad3318

Kaushansky, A., Metzger, P. G., Douglass, A. N., Mikolajczak, S. A., Lakshmanan, V., Kain, H. S., et al. (2013). Malaria parasite liver stages render host hepatocytes susceptible to mitochondria-initiated apoptosis. *Cell Death Dis.* 4, e762. doi: 10.1038/cddis.2013.286

Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26, 239–257. doi: 10.1038/bjc.1972.33

Kim, J. M., Eckmann, L., Savidge, T. C., Lowe, D. C., Witthöft, T., and Kagnoff, M. F. (1998). Apoptosis of human intestinal epithelial cells after bacterial invasion. *J. Clin. Invest.* 102, 1815–1823. doi: 10.1172/JCI2466

Kinoshita, M., Uchida, T., Sato, A., Nakashima, M., Nakashima, H., Shono, S., et al. (2010). Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *J. Hepatol.* 53, 903–910. doi: 10.1016/j.jhep.2010.04.037

Klotz, C., and Frevert, U. (2008). *Plasmodium yoelii* sporozoites modulate cytokine profile and induce apoptosis in murine Kupffer cells. *Int. J. Parasitol.* 38, 1639–1650. doi: 10.1016/j.ijpara.2008.05.018

Koka, S., Lang, C., Boini, K. M., Bobbala, D., Huber, S. M., and Lang, F. (2008). Influence of chlorpromazine on eryptosis, parasitemia and survival of *Plasmodium berghei* infected mice. *Cell. Physiol. Biochem.* 22, 261–268. doi: 10.1159/000149804

Kumar, S., and Barillas-Mury, C. (2005). Cell biology of *Plasmodium falciparum* midgut invasion of Anopheline mosquitoes. *Am. J. Trop. Med. Hyg.* 73, 153.

Kumar, S., Christophides, G. K., Cantera, R., Charles, B., Han, Y. S., Meister, S., et al. (2003). The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 14139–14144. doi: 10.1073/pnas.2036262100

Kumar, S., Guha, M., Choubey, V., Maity, P., Srivastava, K., Puri, S. K., et al. (2008). Bilirubin inhibits *Plasmodium falciparum* growth through the generation of reactive oxygen species. *Free Radic. Biol. Med.* 44, 602–613. doi: 10.1016/j.freeradbiomed.2007.10.057

Kumar, S., Gupta, L., Han, Y. S., and Barillas-Mury, C. (2004). Inducible peroxidases mediate nitration of *Anopheles* midgut cells undergoing apoptosis in response to *Plasmodium* invasion. *J. Biol. Chem.* 279, 53475–53482. doi: 10.1074/jbc.M409905200

Kumar, S., Molina-Cruz, A., Gupta, L., Rodrigues, J., and Barillas-Mury, C. (2010). A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. *Science* 327, 1644–1648. doi: 10.1126/science.1184008

Lang, F., Gulbins, E., Lerche, H., Huber, S. M., Kempe, D. S., and Foller, M. (2008). Eryptosis, a window to systemic disease. *Cell. Physiol. Biochem.* 22, 373–380. doi: 10.1159/000185448

Lang, P. A., Kasinathan, R. S., Brand, V. B., Duranton, C., Lang, C., Koka, S., et al. (2009). Accelerated clearance of *Plasmodium*-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. *Cell. Physiol. Biochem.* 24, 415–428. doi: 10.1159/000257529

Lavazec, C., Boudin, C., Lacroix, R., Bonnet, S., Diop, A., Thiberge, S., et al. (2007). Carboxypeptidases B of *Anopheles gambiae* as targets for a *Plasmodium falciparum* transmission-blocking vaccine. *Infect. Immun.* 75, 1635–1642. doi: 10.1128/IAI.00864-06

Leiriao, P., Albuquerque, S. S., Corso, S., van Gemert, G. J., Sauerwein, R. W., Rodriguez, A., et al. (2005a). HGF/MET signalling protects *Plasmodium*-infected host cells from apoptosis. *Cell. Microbiol.* 7, 603–609. doi: 10.1111/j.1462-5822.2004.00490.x

Leiriao, P., Mota, M. M., and Rodriguez, A. (2005b). Apoptotic *Plasmodium*-infected hepatocytes provide antigens to liver dendritic cells. *J. Infect. Dis.* 191, 1576–1581. doi: 10.1086/429635

Lensen, A., Mulder, L., Tchuinkam, T., Willemsen, L., Eling, W., and Sauerwein, R. (1998). Mechanisms that reduce transmission of *Plasmodium falciparum* malaria in semi immune and non immune persons. *J. Infect. Dis.* 177, 1358–1363. doi: 10.1086/515263

Lensen, A. H., Bolmer-Van de Vegte, M., van Gemert, G. J., Eling, W. M., and Sauerwein, R. W. (1997). Leukocytes in a *Plasmodium falciparum*-infected blood meal reduce transmission of malaria to *Anopheles* mosquitoes. *Infect. Immun.* 65, 3834–3837.

Luckhart, S., Crampton, A. L., Zamora, R., Lieber, M. J., Dos Santos, P. C., Peterson, T. M. L., et al. (2003). Mammalian transforming growth factor β1 activated after ingestion by *Anopheles stephensi* modulates mosquito immunity. *Infect. Immun.* 71, 3000–3009. doi: 10.1128/IAI.71.6.3000-3009.2003

Luckhart, S., Vodovotz, Y., Cui, L., and Rosenberg, R. (1998). The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5700–5705. doi: 10.1073/pnas.95.10.5700

Michel, K., and Kafatos, F. C. (2005). Mosquito immunity against *Plasmodium*. *Insect Biochem. Mol. Biol.* 35, 677–689. doi: 10.1016/j.ibmb.2005.02.009

Minard, G., Mavingui, P., and Moro, C. V. (2013). Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasit. Vectors* 6, 146. doi: 10.1186/1756-3305-6-146

Moss, S. F., Calam, J., Agarwal, B., Wang, S., and Holt, P. R. (1996). Induction of gastric epithelial apoptosis by *Helicobacter pylori*. *Gut* 38, 498–501. doi: 10.1136/gut.38.4.498

Mota, M. M., Pradel, G., Vanderberg, J. P., Hafalla, J. C., Frevert, U., Nussenzweig, R. S., et al. (2001). Migration of *Plasmodium* sporozoites through cells before infection. *Science* 291, 141–144. doi: 10.1126/science.291.5501.141

Mueller, A.-K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., et al. (2005). *Plasmodium* liver stage developmental arrest by depletion of a protein at the parasite–host interface. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3022–3027. doi: 10.1073/pnas.0408442102

Mueller, A.-K., Kohlhepp, F., Hammerschmidt, C., and Michel, K. (2010). Invasion of mosquito salivary glands by malaria parasites: prerequisites and defense strategies. *Int. J. Parasitol.* 40, 1229–1235. doi: 10.1016/j.ijpara.2010.05.005

Muniz-Junqueira, M. I., dos Santos-Neto, L. L., and Tosta, C. E. (2001). Influence of tumor necrosis factor-alpha on the ability of monocytes and lymphocytes to destroy intraerythrocytic *Plasmodium falciparum* in vitro. *Cell. Immunol.* 208, 73–79. doi: 10.1006/cimm.2001.1770

Mutai, B. K., and Waitumbi, J. N. (2010). Apoptosis stalks *Plasmodium falciparum* maintained in continuous culture condition. *Malar. J.* 9, S6. doi: 10.1186/1475-2875-9-S3-S6

Naotunne, T. S., Karunaweera, N. D., Mendis, K. N., and Carter, R. (1993). Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates. *Immunology* 78, 555–562.

N'Dilimabaka, N., Taoufiq, Z., Zougbédé, S., Bonnefoy, S., Lorthiois, A., Couraud, P. O., et al. (2014). *P. falciparum* isolate-specific distinct patterns of induced apoptosis in pulmonary and brain endothelial Cells. *PLoS ONE.* 9:e90692. doi: 10.1371/journal.pone.0090692

Oakley, M. S. M., Kumar, S., Anantharaman, V., Zheng, H., Mahajan, B., Haynes, J. D., et al. (2007). Molecular factors and biochemical pathways induced by febrile temperature in intraerythrocytic *Plasmodium falciparum* parasites. *Infect. Immun.* 75, 2012–2025. doi: 10.1128/IAI.01236-06

Park, J. E., Son, A. I., and Zhou, R. (2013). Roles of EphA2 in development and disease. *Genes* 4, 334–357. doi: 10.3390/genes4030334

Pino, P., Vouldoukis, I., Kolb, J. P., Mahmoudi, N., Desportes-Livage, I., Bricaire, F., et al. (2003). *Plasmodium falciparum*-infected erythrocyte adhesion induces caspase activation and apoptosis in human endothelial cells. *J. Infect. Dis.* 187, 1283–1290. doi: 10.1086/373992

Pinto, S. B., Kafatos, F. C., and Michel, K. (2008). The parasite invasion marker SRPN6 reduces sporozoite numbers in salivary glands of *Anopheles gambiae*. *Cell. Microbiol.* 10, 891–898. doi: 10.1111/j.1462-5822.2007.01091.x

Prudêncio, M., Mota, M. M., and Mendes, A. M. (2011). A toolbox to study liver stage malaria. *Trends Parasitol.* 27, 565–574. doi: 10.1016/j.pt.2011.09.004

Ramiro, R. S., Alpedrinha, J., Carter, L., Gardner, A., and Reece, S. E. (2011). Sex and death: the effects of innate immune factors on the sexual reproduction of malaria parasites. *PLoS Pathog.* 7:e1001309. doi: 10.1371/journal.ppat.1001309

Ramphul, U. N., Garver, L. S., Molina-Cruz, A., Canepa, G. E., and Barillas-Mury, C. (2015). *Plasmodium falciparum* evades mosquito immunity by disrupting JNK-mediated apoptosis of invaded midgut cells. *Proc. Natl. Acad. Sci. U.S.A.* 112, 1273–1280. doi: 10.1073/pnas.1423586112

Rennenberg, A., Lehmann, C., Heitmann, A., Witt, T., Hansen, G., Nagarajan, K., et al. (2010). Exoerythrocytic *Plasmodium* parasites secrete a cysteine protease inhibitor involved in sporozoite invasion and capable of blocking cell death of host hepatocytes. *PLoS Pathog.* 6:e1000825. doi: 10.1371/journal.ppat.1000825

Rosinski-Chupin, I., Briolay, J., Brouilly, P., Perrot, S., Gomez, S. M., Chertemps, T., et al. (2007). SAGE analysis of mosquito salivary gland transcriptomes during *Plasmodium* invasion. *Cell. Microbiol.* 9, 708–724. doi: 10.1111/j.1462-5822.2006.00822.x

Sakurai, T., Itoh, K., Liu, Y., Higashitsuji, H., Sumitomo, Y., Sakamaki, K., et al. (2005). Low temperature protects mammalian cells from apoptosis initiated by various stimuli in vitro. *Exp. Cell Res.* 309, 264–272. doi: 10.1016/j.yexcr.2005.06.002

Setty, B. N. Y., Kulkarni, S., and Stuart, M. J. (2002). Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. *Blood* 99, 1564–1571. doi: 10.1182/blood.V99.5.1564

Shaha, C. (2006). Apoptosis in *Leishmania* species and its relevance to disease pathogenesis. *Indian J. Med. Res.* 123, 233–244.

Simon, N., Lasonder, E., Scheuermayer, M., Kuehn, A., Tews, S., Fischer, R., et al. (2013). Malaria parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito midgut. *Cell Host Microbe* 13, 29–41. doi: 10.1016/j.chom.2012.11.013

Sinai, A. P., Payne, T. M., Carmen, J. C., Hardi, L., Watson, S. J., and Molestina, R. E. (2004). Mechanisms underlying the manipulation of host apoptotic pathways by *Toxoplasma gondii*. *Int. J. Parasitol.* 34, 381–391. doi: 10.1016/j.ijpara.2003.11.009

Sinden, R. E. (1999). *Plasmodium* differentiation in the mosquito. *Parassitologia* 41, 139–148.

Sinden, R. E. (2002). Molecular interactions between *Plasmodium* and its insect vectors. *Cell. Microbiol.* 4, 713–724. doi: 10.1046/j.1462-5822.2002.00229.x

Sinden, R. E., and Billingsley, P. F. (2001). *Plasmodium* invasion of mosquito cells: hawk or dove? *Trends Parasitol.* 17, 209–211. doi: 10.1016/S1471-4922(01)01928-6

Smith, R. C., Barillas-Mury, C., and Jacobs-Lorena, M. (2015). Hemocyte differentiation mediates the mosquito late-phase immune response against *Plasmodium* in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3412–3420. doi: 10.1073/pnas.1420078112

Smith, R. C., Eappen, A. G., Radtke, A. J., and Jacobs-Lorena, M. (2012). Regulation of anti-*Plasmodium* immunity by a LITAF-like transcription factor in the malaria vector *Anopheles gambiae*. *PLoS Pathog.* 8:e1002965. doi: 10.1371/journal.ppat.1002965

Smith, R. C., Vega-Rodríguez, J., and Jacobs-Lorena, M. (2014). The *Plasmodium* bottleneck: malaria parasite losses in the mosquito vector. *Mem. Inst. Oswaldo Cruz* 109, 644–661. doi: 10.1590/0074-0276130597

Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., et al. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* 313, 1287–1290. doi: 10.1126/science.1129720

Tanneur, V., Duranton, C., Brand, V. B., Sandu, C. D., Akkaya, C., Kasinathan, R. S., et al. (2006). Purinoceptors are involved in the induction of an osmolyte permeability in malaria-infected and oxidized human erythrocytes. *FASEB J.* 20, 133–135. doi: 10.1096/fj.04-3371fje

Tavares, J., Formaglio, P., Thiberge, S., Mordelet, E., Van Rooijen, N., Medvinsky, A., et al. (2013). Role of host cell traversal by the malaria sporozoite during liver infection. *J. Exp. Med.* 210, 905–915. doi: 10.1084/jem.20121130

Totino, P. R., Magalhães, A. D., Silva, L. A., Banic, D. M., Daniel-Ribeiro, C. T., Ferreira-da-Cruz, M., et al. (2010). Apoptosis of non-parasitized red blood cells in malaria: a putative mechanism involved in the pathogenesis of anaemia. *Malar. J.* 9, 350. doi: 10.1186/1475-2875-9-350

Totino, P. R. R., Pinna, R. A., De-Oliveira, A. C. A. X., Banic, D. M., Daniel-Ribeiro, C. T., Ferreira-da-Cruz, M., et al. (2013). Apoptosis of non-parasitised red blood cells in *Plasmodium yoelii* malaria. *Mem. Inst. Oswaldo Cruz* 108, 686–690. doi: 10.1590/0074-0276108062013003

Tripathi, A. K., Sullivan, D. J., and Stins, M. F. (2007). *Plasmodium falciparum*-infected erythrocytes decrease the integrity of human blood-brain barrier endothelial cell monolayers. *J. Infect. Dis.* 195, 942–950. doi: 10.1086/512083

Usynin, I., Klotz, C., and Frevert, U. (2007). Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells. *Cell. Microbiol.* 9, 2610–2628. doi: 10.1111/j.1462-5822.2007.00982

Van Dijk, M. R., Douradinha, B., Franke-Fayard, B., Heussler, V., van Dooren, M. W., van Schaijk, B., et al. (2005). Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12194–12199. doi: 10.1073/pnas.0500925102

Van Zandbergen, G., Bollinger, A., Wenzel, A., Kamhawi, S., Voll, R., Klinger, M., et al. (2006). *Leishmania* disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13837–13842. doi: 10.1073/pnas.0600843103

Wennicke, K., Debierre-Grockiego, F., Wichmann, D., Brattig, N. W., Pankuweit, S., Maisch, B., et al. (2008). Glycosylphosphatidylinositol-induced cardiac myocyte death might contribute to the fatal outcome of *Plasmodium*

falciparum malaria. *Apoptosis* 13, 857–866. doi: 10.1007/s10495-008-0217-6

Williams, G. T. (1994). Programmed cell death: a fundamental protective response to pathogens. *Trends Microbiol.* 2, 463–464. doi: 10.1016/0966-842X(94)90648-3

Wilson, N. O., Huang, M.-B., Anderson, W., Bond, V., Powell, M., Thompson, W. E., et al. (2008). Soluble factors from *Plasmodium falciparum*-infected erythrocytes induce apoptosis in human brain vascular endothelial and neuroglia cells. *Mol. Biochem. Parasitol.* 162, 172–176. doi: 10.1016/j.molbiopara.2008.09.003

World Health Organization [WHO] (2014). "*World Malaria Report: 2014*," *in WHO Global Malaria Programme*. Geneva: World Health Organization.

Zheng, L. (1997). Molecular approaches to mosquito parasite interactions. *Arch. Insect Biochem. Physiol.* 34, 1–18. doi: 10.1002/(SICI)1520-6327(1997)34:1<1::AID-ARCH1>3.3.CO;2-R

Zieler, H., and Dvorak, J. A. (2000). Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11516–11521. doi: 10.1073/pnas.97.21.11516

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MINI REVIEW
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Role of *Trypanosoma cruzi* *Trans*-sialidase on the Escape from Host Immune Surveillance

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Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi*, affecting millions of people throughout Latin America. The parasite dampens host immune response causing modifications in diverse lymphoid compartments, including the thymus. *T. cruzi trans*-sialidase (TS) seems to play a fundamental role in such immunopathological events. This unusual enzyme catalyses the transference of sialic acid molecules from host glycoconjugates to acceptor molecules placed on the parasite surface. TS activity mediates several biological effects leading to the subversion of host immune system, hence favoring both parasite survival and the establishment of chronic infection. This review summarizes current findings on the roles of TS in the immune response during *T. cruzi* infection.

Keywords: *Trypanosoma cruzi*, *trans*-sialidase, parasites, immune evasion, glycoimmunology

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INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) is the causative agent of Chagas disease. During their life cycle, *T. cruzi* acquires different morphologies, alternating between an insect vector and the vertebrate host (Tarleton et al., 2007; Ribeiro et al., 2012). Vector-borne transmission occurs when infected bugs take a blood meal releasing simultaneously contaminated feces with infective metacyclic trypomastigotes near the site of the mammal bite wound (Tyler and Engman, 2001). Once inside the host, parasites differentiate to bloodstream trypomastigotes, which can infect host cells where they differentiate into intracellular amastigotes.

Besides vectorial transmission, other forms of infection exist, i.e., organ transplantation, blood transfusion as well as vertical and oral transmission. Chagas disease progresses from an initial acute phase characterized by a large number of circulating parasites and a broad range of symptoms (same individuals can develop fever, muscle pain, lymphadenopathy or an inflammatory reaction at the biting site known as chagoma), to a chronic and asymptomatic phase where the parasite load is nearly undetectable (Devera et al., 2003; Tarleton et al., 2007). Such latent stage could persist for the lifetime of individuals. However, nearly 30% of chronically infected individuals progress to a symptomatic disease, with the development of cardiomyopathy, megacolon, or megaesophagus (Coura and Borges-Pereira, 2010).

The parasite has evolved diverse mechanisms to subvert or escape from the host innate and adaptative immune system. One of them is the induction of an immunosuppressive state, which was described both during the acute phase of experimental and human *T. cruzi* infection (Oladiran and Belosevic, 2012).This condition is characterized by anergy or clonal deletion of T lymphocytes as well as polyclonal activation of B cells with production of low affinity antibodies against

T. cruzi (Ortiz-Ortiz et al., 1980; Maleckar and Kierszenbaum, 1983). Thus, the inhibition of host immunity observed during the acute phase is an essential way for parasite persistence and the consequent establishment of chronic disease.

Sialic acids (SAcs) are a family of nine-carbon monosaccharides present on the surface of all mammalian cells, conferring diverse biologically activities to glycoproteins and glycolipids, like the promotion of cell–cell interactions or masking recognition sites due to its negative charge (Frasch, 2000). SAcs act as recognition receptors for diverse pathogens including viruses, bacteria and parasites (Varki, 1997; Esko and Sharon, 2009). Moreover, pathogenic bacteria like *Escherichia coli K1* and *N. meningitidis* synthesize SAcs and use it to decorate their surfaces to evade the immune system in their mammalian hosts (Vimr and Lichtensteiger, 2002). Unlike these microorganisms, *T. cruzi* is unable to synthesize SAcs *de novo*. Since *T. cruzi* requires SAcs to survive in the mammalian *milieu*, the parasite exploites the presence of SAcs on host cells, transferring the monosaccharide from host sialyl-glycoconjugates to terminal β-galactoses of acceptor molecules located on its own surface. This enzymatic activity is carried out by an unusual parasite enzyme known as *trans*-sialidase (TS), a modified sialidase (Previato et al., 1985; Freire-de-Lima et al., 2015).

TS displays a diversity of biological properties (many of them independent of their enzymatic activity), which promote the evasion of the innate and adaptative immune responses, acting as an important *T. cruzi* virulence factor (Burleigh and Andrews, 1995). The comprehension of mechanisms involving TS in the abrogation of immunity against *T. cruzi* infection is crucial for the developing and establishment of effective therapeutic approaches.

Trypanosoma cruzi TRANS-SIALIDASE: A GLANCE ON ITS CHARACTERIZATION AND STRUCTURE

The first description of the presence of SAcs residues on *T. cruzi* surface came from studies performed in the eighties (Pereira et al., 1980). Later, it was demonstrated that SAcs found on the parasite surface were previously transferred from the extracellular *milieu*, since no conventional precursors were found on parasites (Schauer et al., 1983). Furthermore, it was determined that trypomastigotes also displayed neuraminidase activity, because they hydrolyzed SAcs residues from erythrocytes and plasma glycoproteins (Pereira, 1983). Finally, Previato et al. (1985) demonstrated that *T. cruzi* performs the enzymatic transference of SAcs by an alternative route involving a *trans*-glycosylation reaction. Subsequent studies demonstrated that such *trans*-glycosylase activity is specific for alpha(α)2,3-SAcs (Schenkman et al., 1991). Further, genetic studies performed to characterize *T. cruzi*-TSs genes showed that *trans*-sialidase and neuraminidase activities were associated to the same parasite enzyme (Pereira et al., 1991; Parodi et al., 1992; Uemura et al., 1992).

Trypanosoma cruzi-TS is part of the TS-superfamily, encoded by 1430 genes. There have been characterized ∼15 genes for enzymatically active-TS, and more than 700 for the inactive-TS which lack the catalytic domain; while the rest are pseudogenes (Atwood et al., 2005; El-Sayed et al., 2005). Moreover, TS-superfamily was recently classified by genomic analysis in eight groups. Active-TS members belong to the group-I, while inactive-TS members belong to groups-II to VIII (Freitas et al., 2011).

The general TS protein structure derived from metacyclic and bloodstream trypomastigote forms shows two major regions. As seen in **Figure 1**, one region is a N-terminal catalytic region whereas the other one consist of a C-terminal region displaying repeats of 12 amino acids in tandem, called SAPA (by Shed Acute Phase Antigen) (Pollevick et al., 1991). The hydrophobic catalytic pocket, responsible for the interaction of transferred SAcs with the terminal β-galactose acceptor, is a three-dimensional structure rich in aromatic residues (Buschiazzo et al., 2002). Inactive-TS members belonging to the TS group II (Freitas et al., 2011) differ from the active ones in their N-terminal region by the presence of mutations in catalytic residues causing the lack of enzymatic activity (Tyr^{342} by His^{342} is the commonest mutation) (**Figure 1B**). These inactive-TSs are lectin-like proteins since they maintain the capacity of binding SAcs and β-galactose residues (Cremona et al., 1995, 1999; Oppezzo et al., 2011), and are involved in host cell attachment and invasion (Freitas et al., 2011). Furthermore, both TSs are surface glycophosphatidylinositol (GPI)-anchored molecules, which can be released into the bloodstream by the action of phosphatidylinositol phospholipase C on GPI-anchors (Schenkman et al., 1992).

THE *Trypanosoma cruzi TRANS*-SIALIDASE IS ABLE TO OVERCOME THE FIRST LINE OF THE IMMUNE DEFENSES

As sialic acid residues can be found on the surface of all mammalian cells, exerting crucial roles in regulating both innate and adaptative host immunity (Amon et al., 2014), it is not surprising that *T. cruzi* takes advantage of such host cell sialoglycophenotype. In this sense, in addition to transfering SAcs to the parasite surface, the TS can also transfer SAcs between host cell glycoconjugates, allowing the parasite to affect the host immune response (**Figure 2**).

The subversion of immune response by *T. cruzi* depends at first on their early action upon innate compounds. The host *milieu* is plenty of potential SAcs donors, allowing the parasite to acquire a negatively charged cover surface right after entering into the host, through the reaction catalyzed by TS. The fact of acquiring such negatively charged mask enables *T. cruzi* to circumvent the effects of some serum compounds (Vimr and Lichtensteiger, 2002). The elimination of this protective cover by sialidase treatment, make trypomastigotes more susceptible to the complement-mediated lysis (Kipnis et al., 1981). In addition, GPI-anchored surface GP160/CRP and T-DAF proteins, putative

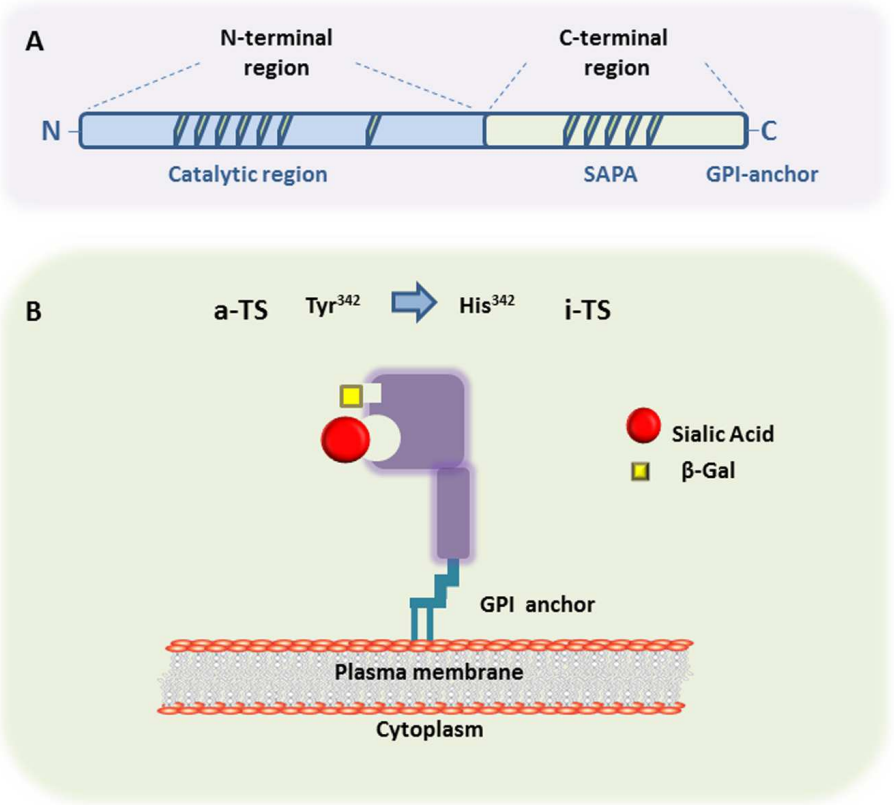

FIGURE 1 | *Trans*-sialidase structure. (A) Representative scheme of the primary structure of metacyclic- and a bloodstream trypomastigote-TS members belonging to either Group I (active-TS) or Group II (inactive-TS), showing the N-terminal and C-terminal domains. **(B)** Representative 3D structure of active (a-TS) and inactive (i-TS) forms of *Trypanosoma cruzi*-TS. *T. cruzi*-TS proteins are bound to the parasite surface through GPI-anchors. Active-TS sialylates parasite's mucin-like molecules, as well as host cell surface glycoconjugates. Inactive-TS in turn acts as a parasite adhesin and it is differentiated from a-TS by a single mutation in the catalytic residue of Tyrosine at the position 342, which is commonly changed by a Histidine.

members of inactive-TS family, conferred protection against the complement-mediating killing by avoiding C3 convertase assembly (Tomlinson et al., 1994; Norris, 1998) (**Figure 2A**). Furthermore, sialylglycoconjugates present on the parasite surface due to TS activity, can bind SAcs binding Ig-type lectins (Siglec-E) on dendritic cells, suppressing the production of the proinflammatory cytokine IL-12, thus impairing the central branch connecting the innate and adaptative immune responses and favoring immunosuppression (Erdmann et al., 2009) (**Figure 2B**).

Trypanosoma cruzi can infect any nucleated cell, including phagocytic cells. To facilitate the adhesion and invasion of mammalian host cells, *T. cruzi* parasites use different molecules, including TS (Barrias et al., 2013). The reduced ability of *T. cruzi* to invade SAcs-deficient cells compared to wild type cells, clearly show that key steps of parasitism, such as the adhesion and invasion of host cells require the participation of TS (Ming et al., 1993; Schenkman et al., 1993). Moreover, both active- and inactive-TS seem to participate in these steps (Barrias et al., 2013). After cellular binding, *T. cruzi* invades non-phagocytic host cells by diverse processes that alternatively require fusion of lysosomes at the site of entry, participation of plasma membrane components or fusion with endosome compartments at the site of invasion (de Souza et al., 2010; Barrias et al., 2013). Regardless of the mechanisms of infection, the parasite will be later located in a vesicle called parasitophorous vacuole (Tardieux et al., 1992; de Souza et al., 2010). The internal membrane of this organelle is enriched with highly sialylated LAMP proteins (Albertti et al., 2010).The acidic pH of the parasitophorous vacuole favors the activity of TS in transferring SAcs from LAMP proteins to the parasite surface. The desialyzation process renders the vesicle more prone to lysis by the action of Tc-Tox (a parasite-derived protein with membrane pore-forming activity), enabling the parasite to escape into the cytoplasm (Andrews and Whitlow, 1989; Hall et al., 1992; Rubin-de-Celis et al., 2006; Albertti et al., 2010) (**Figure 2B**).

Trypanosoma cruzi parasites can be also internalized via phagocytosis by macrophages, a process that triggers an oxidative burst to kill parasites (Alvarez et al., 2011). However, the parasite can avoid macrophage-induced cytotoxicity using an antioxidant complex system to ensure their escape into the cytoplasm in order to establish a productive infection (Piacenza et al., 2009; Nagajyothi et al., 2012).

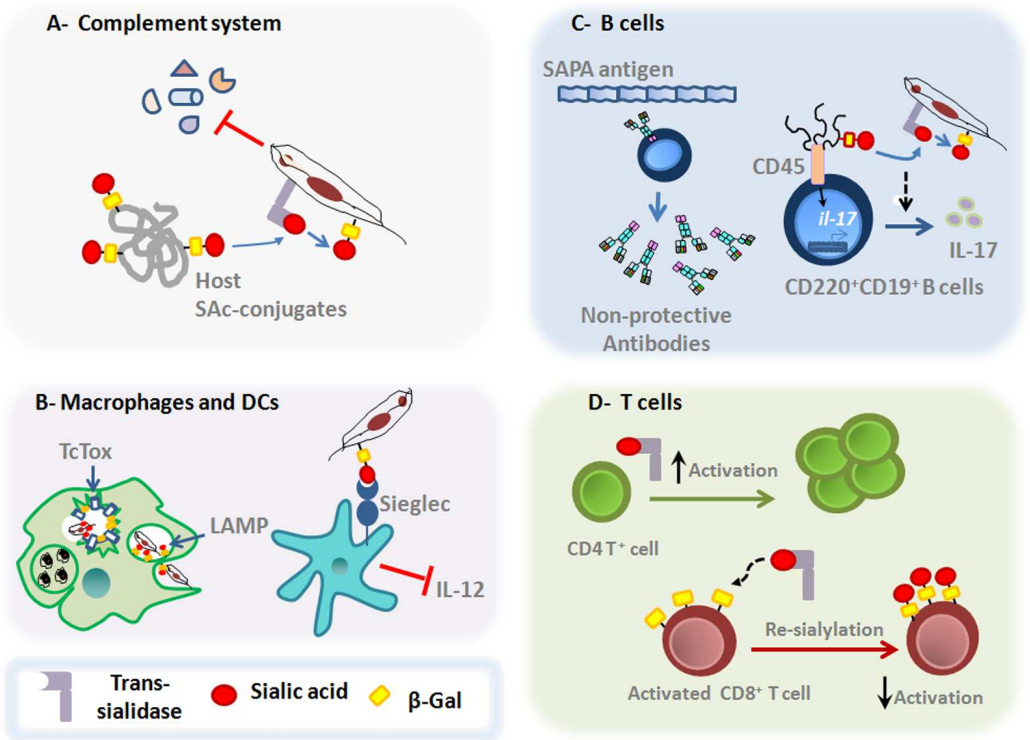

FIGURE 2 | Main roles of *T. cruzi* TS on the host immune responses. (A) Upon entry in the host *milieu*, which is rich in SAcs donors, the parasite surface gets a negatively charged barrier due to a reaction catalyzed by TS, conferring protection against the complement-mediated killing. **(B)** TS favors *T. cruzi* escape from the parasitophorous vacuole, process in which LAMP and Tc-Tox proteins are also shown to be involved. Moreover, TS is able to interfere with IL-12 secretion by dendritic cells through the interaction between sialylated molecules on *T. cruzi* surface and Siglecs on dendritic cell surface. **(C)** SAPA-antigen induces the production of non-protective antibodies. Moreover, recent findings have show that TS acts on both mature follicular and marginal zone CD220^{+}CD19^{+}B cells to induce the expression of IL-17 by a non-canonical signaling pathway. These signaling events promote changes in the glycosylation profile of CD45 that result in their phosphatase activity and subsequent activation of downstream signals leading to the *Il-17* gene expression. **(D)** TS can also induce the activation of CD4^{+}T cells *in vivo*. In addition, TS can also promote changes in the sialylation pattern of activated CD8 $^{+}$T cells, dampening the protective functions of MHC class I-dependent lymphocyte killing responses against *T. cruzi* parasites.

Moreover, TS can also remodel the surface of surrounding host cells. As mentioned earlier, TS can be shed into the bloodstream, causing removal of SAcs located on platelet surface, an event that increases their clearance thus favoring the occurrence of thrombocytopenia (Tribulatti et al., 2005).

Trypanosoma cruzi TRANS-SIALIDASE COMPROMISES BOTH B AND T CELL RESPONSES

During the acute phase, it has been shown the involvement of TS in the polyclonal B-lymphocyte activation (Gao and Pereira, 2001). Probably, the B cell polyclonal activation may restrict the niche size required for an optimal development of specific and protective lymphocytes, by increasing the competition for activation and survival signals in the lymphoid organs (Freitas and Rocha, 2000; Montaudouin et al., 2013). Interestingly, the presence of immunodominant epitopes derived from shed acute phase antigens (SAPAs) shared by TS, drives the production of non-inhibitory antibodies against regions close to the catalytic site of the enzyme (Alvarez et al., 2001) (**Figure 2C**). These antibodies are produced in a T cell-independent manner, delaying the acquisition of inhibitory antibodies (Gao and Pereira, 2001; Gao et al., 2002). Importantly, these inhibitory antibodies are mainly neutralizing IgG immunoglobulins directed against the catalytic site of TS, and their production is strongly correlated with an IFN-γ enriched *milieu* (Pereira-Chioccola et al., 1994; Ribeirão et al., 2000; Risso et al., 2007). It is possible that the SAPA antigens have evolved to avoid the production of protective antibodies directed against the N-terminal catalytic region, since they are absent in the epimastigote forms. Additionally, these antigens are responsible to increase the half-life of TS shed into the bloodstream (Ribeirão et al., 1997; Buscaglia et al., 1999). Furthermore, the trypomastigote surface is rich in terminal α-galactosylmucins, which are also targeted by lytic antibodies. Nevertheless, the *T. cruzi* parasites can avoid the killing induced by human anti-α-galactosyl antibodies by the highly sialylated negatively charged surface (Chioccola et al., 2000; Buscaglia et al., 2006).

Interestingly, recent data demonstrate that during *T. cruzi* infection, B220^{+}CD19^{+}B cells can produce IL-17, a cytokine

involved in the protective response against the parasite (ToselloBoari et al., 2012; Bermejo et al., 2013; Erdmann et al., 2013) (**Figure 2C**). Increased levels of IL-17 in the infection seems to be driven by active-TS acting on CD45 mucins located on B220$^+$CD19$^+$B cell surface, in a ROR-γt/AhR-independent manner (Bermejo et al., 2013). Despite that this type of B cells could be considered as innate cells, such results point out that B cells and different CD45 isoforms are also targets of *T. cruzi*-TS activity (Freire-de-Lima et al., 2010; Muia et al., 2010).

Differentiation, homeostasis and activation processes of T cells greatly depend on the sialylation process. Thus, *T. cruzi*-TS-mediated modifications of the T cell sialophenotype indeed influence the host immune response.

During acute phase of *T. cruzi* infection, T cells become anergic and undergo clonal deletion. Moreover, T cells exhibit low IL-2 expression and are more prone to suffer apoptosis after activation (Nunes et al., 1998; Alcaide and Fresno, 2004). Interestingly, the induction of CD4$^+$Th1 and CD8$^+$ cytotoxic T-cell protective responses is partly elicited by TS (Rodrigues et al., 1999). Both active- and inactive-TS forms engage the CD43 sialomucin in CD4$^+$T cells, favoring their activation (**Figure 2D**). Such mechanism is shown to be able to rescue T cells from activation-induced cell death, intensifying the mitogenic capacity of these lymphocytes (Todeschini et al., 2002). Furthermore, the antigen-specific CD8$^+$T cell responses are regulated by a distinct sialylation process (Moody et al., 2001; Pappu and Shrikant, 2004). During T cell activation, the expression of SAcs on lymphocyte surface is downregulated, leading to the exposure of desialylated glycans (Priatel et al., 2000). Such decreased sialylation process is important to augment the reactivity of T cells for their cognate peptide in the context of MHC class I molecules (Kao et al., 2005). In this context, recent studies showed that TS is able to re-sialylate the surface of CD8$^+$T lymphocytes, limiting the acquisition of antigen-specific CD8$^+$T cell responses (**Figure 2D**). These findings suggest that the re-sialylation ability of TS over activated T cells is an important parasite evasion strategy that directly influences the *T. cruzi* half-life time inside the host by preventing the immune control elicited by CD8$^+$T cells (Freire-de-Lima et al., 2010). In fact, a large number of epitopes recognized by CD8$^+$T cells in both experimental *T. cruzi* models and human disease is encoded by the TS family of genes, although the studies reveal that the epitope immunodominance of TS members varies according to the parasite strain (Martin et al., 2006).

ROLE OF *Trypanosoma cruzi TRANS*-SIALIDASE IN THE *T. cruzi*-DRIVEN THYMIC ATROPHY AND DEVELOPMENT OF UNCONVENTIONAL EXTRATHYMIC T CELL SUBSETS IN CHAGAS DISEASE

The thymus is the primary lymphoid organ where bone marrow-derived T cell precursors undergo differentiation. As a result of this process, positively CD4$^+$ and CD8$^+$ selected thymocytes migrate as mature T cells to T-cell areas of peripheral lymphoid organs (Savino and Dardenne, 2000). Several infectious pathologies, including Chagas' disease, promote disturbances of the intrathymic compartment (Savino, 2006). During the acute phase of murine *T. cruzi* infection is commonly observed

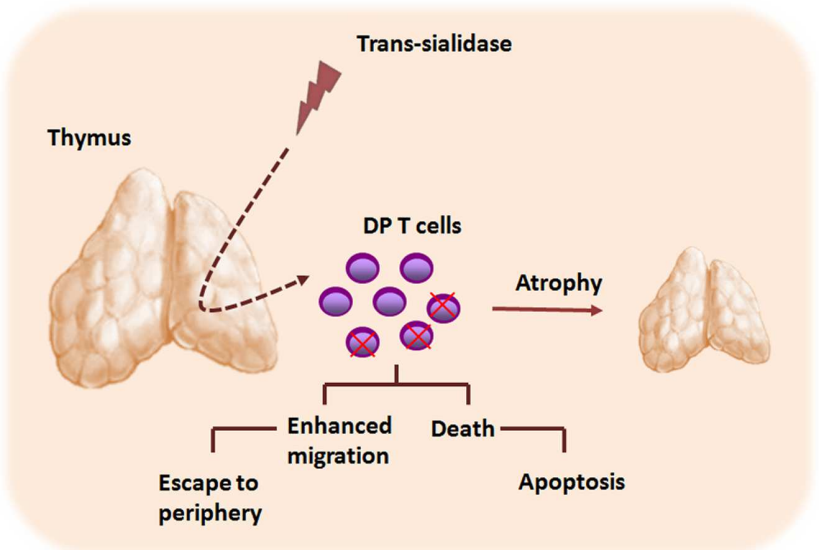

FIGURE 3 | *Trypanosoma cruzi* TS contributes to the thymic atrophy seen during acute phase of Chagas disease. The thymic atrophy caused by *T. cruzi* parasite is a complex phenomenon and involves several host–parasite molecule interactions, including those elicited by TS. TS promotes CD4$^+$CD8$^+$ double positive (DP) cells apoptosis within the thymic nurse cell complex, contributing to the thymic atrophy process. Moreover, TS also modulates the migratory properties of thymocytes, and may contribute to the abnormal release of DP thymocytes to the periphery.

an intense thymic atrophy, mainly caused by the depletion of immature $CD4^+CD8^+$ double-positive (DP) thymocytes. Such phenomenon involves not only thymocyte death but also their abnormal proliferation and migration responses (Leite de Moraes et al., 1991; Roggero et al., 2002; Henriques-Pons et al., 2004).

Interestingly, changes in the cell surface sialylation caused by TS seem also to play an important role in *T. cruzi*-induced morphological and phenotypic thymic alterations (**Figure 3**). When TS is artificially shedding to circulation, the parasite enzyme is able to induce apoptosis of thymocytes. In addition, even when TS doses became undetectable, thymic apoptosis could also be observed (Leguizamón et al., 1999). Moreover, TS-treated animals displayed enhanced thymocyte apoptosis within the thymic nurse cell complexes, findings resembling thymic alterations in infected animals. Additionally, the TS treatment was able to promote a decrease of thymocyte proliferative ratios after Concanavalin A stimulation, in a similar manner to those observed in the experimental models of *T. cruzi* infection. The use of TS-neutralizing antibodies apparently rescued the normal thymocyte proliferation indexes (Mucci et al., 2002). Importantly, the thymocyte apoptosis induced by TS depends on androgens, since TS administration in both female and androgen-depleted mice did not result in the increase of thymocyte death. Furthermore, lactitol, a competitive inhibitor of TS that blocks the transference of sialyl residues by TS, was able to prevent the thymic cell depletion induced by *T. cruzi* (Mucci et al., 2005, 2006). Further studies showed that a rise in the glucocorticoid levels, as consequence of infective stress, is also involved in the *T. cruzi*-driven thymic atrophy (Roggero et al., 2006; Pérez et al., 2007; Lepletier et al., 2013). Interestingly, it has been reported that glucocorticoids can change the expression of sialylated and nonsialylated Lewis (a) epitopes of adhesion molecules involved in leukocyte migration processes (Delbrouck et al., 2002). Whether similar results occur during *T. cruzi*-driven thymic atrophy it remains to be determined.

Finally, recently studies from our group revealed that TS influences the thymocyte differentiation process via activation of MAPK signaling pathways, increasing thymocyte migratory activity by inducing actin filament mobilization. Such effects were also related to the ability of TS to modulate the adhesive properties of thymocytes to thymic epithelial cells. Moreover, we found increased frequencies of activated DPT cells in the blood of chronic chagasic patients with heart disease, in association to elevated titters of anti-TS antibodies as compared to healthy individuals (Nardy et al., 2013). These findings suggest a probably role for TS in the intrathymic maturation disturbances and subsequent abnormal thymic exit of immature thymocytes seen in *T. cruzi* infection.

CONCLUDING REMARKS

A successful establishment of an infection relies on efficient strategies adopted by the infectious agents to evade early their detection by the host immune system. Considering that protozoans are the most ancient members of the animal kingdom, it is conceivable to think that they evolved sophisticated mechanisms to ensure their survival as intracellular parasites. *T. cruzi* is part a diverse group of unicellular organisms that can modify host cells to their own benefits (Jackson, 2015). In this review, we highlighted important aspects of *T. cruzi* immune evasion involving TS, a major parasite virulence factor. The diverse functions displayed by this molecule enable the parasite to interfere in many crucial host biological processes, including those responsible for protective immune responses. Despite several years of research directed toward understanding the role of *T. cruzi*-derived TS on the host-parasite interplay, there are still some points to be uncovered, especially those involving the distribution of genes encoding TS in different parasite strains. This question becomes even more important if we consider the coexistence of different *T. cruzi* strains in their natural reservoirs (Noireau et al., 2009). The existence of antigen variation within the parasite population, may lead the expression of competing T cell epitopes with different affinities to MHC molecules that could influence the acquisition of protective adaptive immune responses. Given the importance of TS for the establishment of an efficient infection, this molecule has gained potential attention as a drug target for disease therapies. Thus, efforts to understand the biology of TS would strengthen the use of TS inhibitors in therapeutic approaches for treatment of Chagas disease.

AUTHOR CONTRIBUTIONS

AN, CF, and AP wrote the paper. AM made substantial contributions to the conception of the work. All authors read and approved the final version of the manuscript.

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REFERENCES

Albertti, L. A., Macedo, A. M., Chiari, E., Andrews, N. W., and Andrade, L. O. (2010). Role of host lysosomal associated membrane protein (LAMP) in *Trypanosoma cruzi* invasion and intracellular development. *Microbes Infect.* 12, 784–789. doi: 10.1016/j.micinf.2010.05.015

Alcaide, P., and Fresno, M. (2004). The *Trypanosoma cruzi* membrane mucin AgC10 inhibits T cell activation and IL-2 transcription through L-selectin. *Int. Immunol.* 16, 1365–1375. doi: 10.1093/intimm/dxh138

Alvarez, M. N., Peluffo, G., Piacenza, L., and Radi, R. (2011). Intraphagosomal peroxynitrite as a macrophage derived cytotoxin against internalized *Trypanosoma cruzi*: consequences for oxidative killing and role of microbial peroxiredoxins in infectivity. *J. Biol. Chem.* 286, 6627–6640. doi: 10.1074/jbc.M110.167247

Alvarez, P., Leguizamon, M. S., Buscaglia, C. A., Pitcovsky, T. A., and Campetella, O. (2001). Multiple overlapping epitopes in the repetitive unit of the shed acute-phase antigen from *Trypanosoma cruzi* enhance its immunogenic properties. *Infect. Immun.* 69, 7946–7949. doi: 10.1128/IAI.69.12.7946-7949.2001

Amon, R., Reuven, E. M., Ben-Arye, S. L., and Padler-Karavani, V. (2014). Glycans in immune recognition and response. *Carbohydr. Res.* 389, 115–122. doi: 10.1016/j.carres.2014.02.004

Andrews, N. W., and Whitlow, M. B. (1989). Secretion of *Trypanosoma cruzi* of a hemolysin active at low pH. *Mol. Biochem. Parasitol.* 33, 249–256. doi: 10.1016/0166-6851(89)90086-8

Atwood, J. A., Weatherly, D. B., Minning, T. A., Bundy, B., Cavola, C., Opperdoes, F. R., et al. (2005). The *Trypanosoma cruzi* proteome. *Science* 309, 473–476. doi: 10.1126/science.1110289

Barrias, E. S., de Carvalho, T. M., and De Souza, W. (2013). *Trypanosoma cruzi*: entry into mammalian host cells and parasitophorous vacuole formation. *Front. Immunol.* 4:186. doi: 10.3389/fimmu.2013.00186

Bermejo, D. A., Jackson, S. W., Gorosito-Serran, M., Acosta-Rodriguez, E. V., Amezcua-Vesely, M. C., Sather, B. D., et al. (2013). *Trypanosoma cruzi* trans-sialidase initiates a program independent of the transcription factors RORγt and Ahr that leads to IL-17 production by activated B cells. *Nat. Immunol.* 14, 514–522. doi: 10.1038/ni.2569

Burleigh, B. A., and Andrews, N. W. (1995). The mechanisms of *Trypanosoma cruzi* invasion of mammalian cells. *Ann. Rev. Microbiol.* 49, 175–200. doi: 10.1146/annurev.mi.49.100195.001135

Buscaglia, C. A., Alfonso, J., Campetella, O., and Frasch, A. C. (1999). Tandem amino acid repeats from *Trypanosoma cruzi* shed antigens increase the half-life of proteins in blood. *Blood* 93, 2025–2032.

Buscaglia, C. A., Campo, V. A., Frasch, A. C., and Di Noia, J. M. (2006). *Trypanosoma cruzi* surface mucins: host-dependent coat diversity. *Nat. Rev. Microbiol.* 4, 229–236. doi: 10.1038/nrmicro1351

Buschiazzo, A., Amaya, M. F., Cremona, M. L., Frasch, A. C., and Alzari, P. M. (2002). The crystal structure and mode of action of trans-sialidase, a key enzyme in *Trypanosoma cruzi* pathogenesis. *Mol. Cell* 10, 757–768. doi: 10.1016/S1097-2765(02)00680-9

Chioccola, V. L. P., Acosta-Serrano, A., Almeida, I. C., Ferguson, M. A. J., Souto-Padron, T., Rodrigues, M. M., et al. (2000). Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies. *J. Cell Sci.* 113, 1299–1307.

Coura, J. R., and Borges-Pereira, J. (2010). Chagas disease: 100 years after its discovery. A systemic review. *Acta Trop.* 115, 5–13. doi: 10.1016/j.actatropica.2010.03.008

Cremona, M. L., Campetell, A. O., Sánchez, D. O., and Frasch, A. C. (1999). Enzymically inactive members of the trans-sialidase family from *Trypanosoma cruzi* display beta-galactose binding activity. *Glycobiology* 9, 581–587. doi: 10.1093/glycob/9.6.581

Cremona, M. L., Sánchez, D. O., Frasch, A. C., and Campetella, O. (1995). A single tyrosine differentiates active and inactive *Trypanosoma cruzi* trans-sialidases. *Gene* 160, 123–128. doi: 10.1016/0378-1119(95)00175-6

de Souza, W., de Carvalho, T. M., and Barrias, E. S. (2010). Review on *Trypanosoma cruzi*: host cell interaction. *Int. J. Cell Biol.* 2010, 295394. doi: 10.1155/2010/295394

Delbrouck, C., Kaltner, H., Danguy, A., Nifant'ev, N. E., Bovin, N. V., and Vandenhoven, G. (2002). Glucocorticoid-induced differential expression of the sialylated and nonsialylated Lewis(a) epitopes and respective binding sites in human nasal polyps maintained under ex vivo tissue culture conditions. *Ann. Otol. Rhinol. Laryngol.* 111, 1097–1107. doi: 10.1177/000348940211101207

Devera, R., Fernandes, O., and Coura, J. R. (2003). Should *Trypanosoma cruzi* be called "cruzi" complex? A review of the parasite diversity and the potential of selecting population after in vitro culturing and mice infection. *Mem. Inst. Oswaldo Cruz* 98, 1–12. doi: 10.1590/S0074-02762003000100001

El-Sayed, N. M., Myler, P. J., Bartholomeu, D. C., Nilsson, D., Aggarwal, G., Tran, A. N., et al. (2005). The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* 309, 409–415. doi: 10.1126/science.1112631

Erdmann, H., Roßnagel, C., Böhme, J., Iwakura, Y., Jacobs, T., Schaible, U. E., et al. (2013). IL-17A promotes macrophage effector mechanisms against *Trypanosoma cruzi* by trapping parasites in the endolysosomal compartment. *Immunobiology* 218, 910–923. doi: 10.1016/j.imbio.2012.10.005

Erdmann, H., Steeg, C., Koch-Nolte, F., Fleischer, B., and Jacobs, T. (2009). Sialylated ligands on pathogenic *Trypanosoma cruzi* interact with Siglec-E (sialic acid binding Ig-like lectin-E). *Cell. Microbiol.* 11, 1600–1611. doi: 10.1111/j.1462-5822.2009.01350.x

Esko, J., and Sharon, N. (2009). "Microbial lectins: hemagglutinins, adhesins, and toxins," in *Essentials of Glycobiology*, 2nd Edn, eds A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, et al. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Frasch, A. C. (2000). Functional diversity in the trans-sialidase and mucin families in *Trypanosoma cruzi*. *Parasitol. Today* 16, 282–286. doi: 10.1016/S0169-4758(00)01698-7

Freire-de-Lima, L., Alisson-Silva, F., Carvalho, S. T., Takiya, C. M., Rodrigues, M. M., DosReis, G. A., et al. (2010). *Trypanosoma cruzi* subverts host cell sialylation and may compromise antigen-specific CD8+ T cell responses. *J. Biol. Chem.* 285, 13388–13396. doi: 10.1074/jbc.M109.096305

Freire-de-Lima, L., Fonseca, L. M., Oeltmann, T., Mendonça-Previato, L., and Previato, J. O. (2015). The trans-sialidase, the major *Trypanosoma cruzi* virulence factor: three decades of studies. *Glycobiology* 25, 1142–1149. doi: 10.1093/glycob/cwv057

Freitas, A. A., and Rocha, B. (2000). Population biology of lymphocytes: the fight for survival. *Annu. Rev. Immunol.* 18, 83–111. doi: 10.1146/annurev.immunol.18.1.83

Freitas, L. M., dos Santos, S. L., Rodrigues-Luiz, G. F., Mendes, T. A. O., Rodrigues, T. S., Gazzinelli, R. T., et al. (2011). Genomic analyses, gene expression and antigenic profile of the trans-sialidase superfamily of *Trypanosoma cruzi* reveal an undetected level of complexity. *PLoS ONE* 6:e25914. doi: 10.1371/journal.pone.0025914

Gao, W., and Pereira, M. A. (2001). *Trypanosoma cruzi* trans-sialidase potentiates T cell activation through antigen presenting cells: role of IL-6 and Bruton's tyrosine kinase. *Eur. J. Immunol.* 31, 1503–1512. doi: 10.1002/1521-4141(200105)31:5<1503::AID-IMMU1503>3.0.CO;2-W

Gao, W., Wortis, H. H., and Pereira, M. A. (2002). The *Trypanosoma cruzi* trans-sialidase is a T cell-independent B cell mitogen and an inducer of non-specific Ig secretion. *Int. Immunol.* 14, 299–308. doi: 10.1093/intimm/14.3.299

Hall, B. F., Webster, P., Ma, A. K., Joiner, K. A., and Andrews, N. W. (1992). Desialylation of lysosomal membrane glycoproteins by *Trypanosoma cruzi*: a role for the surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. *J. Exp. Med.* 176, 313–325. doi: 10.1084/jem.176.2.313

Henriques-Pons, A., De Meis, J., Cotta-De-Almeida, V., Savino, W., and Araujo-Jorge, T. C. (2004). Fas and perforin are not required for thymus atrophy induced by *Trypanosoma cruzi* infection. *Exp. Parasitol.* 107, 1–4. doi: 10.1016/j.exppara.2004.04.010

Jackson, A. P. (2015). The evolution of parasite genomes and the origins of parasitism. *Parasitology* 142(Suppl. 1), S1–S5. doi: 10.1017/S0031182014001516

Kao, C., Daniels, M. A., and Jameson, S. C. (2005). Loss of CD8 and TCR binding to Class I MHC ligands following T cell activation. *Int. Immunol.* 17, 1607–1617. doi: 10.1093/intimm/dxh340

Kipnis, T. L., David, J. R., Alper, C. A., Sher, A., and da Silva, W. D. (1981). Enzymatic treatment transforms trypomastigotes of *Trypanosoma cruzi* into activators of alternative complement pathway and potentiates their uptake by macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 78, 602–605. doi: 10.1073/pnas.78.1.602

Leguizamón, M. S., Mocetti, E., GarcíaRivello, H., Argibay, P., and Campetella, O. (1999). Trans-sialidase from *Trypanosoma cruzi* induces apoptosis in cells from the immune system in vivo. *J. Infect. Dis.* 180, 1398–1402. doi: 10.1086/315001

Leite de Moraes, M. C., Hontebeyrie-Joskowicz, M., Leboulenger, F., Savino, W., Dardenne, M., and Lepault, F. (1991). Studies on the thymus in Chagas' disease. II. Thymocyte subset fluctuations in *Trypanosoma cruzi*-infected mice: relationship to stress. *Scand. J. Immunol.* 33, 267–275. doi: 10.1111/j.1365-3083.1991.tb01772.x

Lepletier, A., de Carvalho, V. F., Rodrigues e Silva, P. M., Villar, S., Pérez, A. R., Savino, W., et al. (2013). *Trypanosoma cruzi* disrupts thymic homeostasis by altering intrathymic and systemic stress-related endocrine circuitries. *PLoS Negl. Trop. Dis.* 7:e2470. doi: 10.1371/journal.pntd.0002470

Maleckar, J. R., and Kierszenbaum, F. (1983). Inhibition of mitogen-induced proliferation of mouse T and B lymphocytes by bloodstream forms of *Trypanosoma cruzi*. *J. Immunol.* 130, 908–911.

Martin, D. L., Weatherly, D. B., Laucella, S. A., Cabinian, M. A., Crim, M. T., Sullivan, S., et al. (2006). CD8+ T-Cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. *PLoS Pathog.* 2:e77. doi: 10.1371/journal.ppat.0020077

Ming, M., Chuenkova, M., Ortega-Barria, E., and Pereira, M. E. (1993). Mediation of *Trypanosoma cruzi* invasion by sialic acid on the host cell and trans-sialidase on the trypanosome. *Mol. Biochem. Parasitol.* 59, 243–252.

Montaudouin, C., Anson, M., and Hao, Y. (2013). Quorum sensing contributes to activated IgM-secreting B cell homeostasis. *J. Immunol.* 190, 106–114. doi: 10.4049/jimmunol.1200907

Moody, A. M., Chui, D., Reche, P. A., Priatel, J. J., Marth, J. D., and Reinherz, E. L. (2001). Developmentally regulated glycosylation of the CD8αβ coreceptor stalk modulates ligand binding. *Cell* 107, 501–512. doi: 10.1016/S0092-8674(01)00577-3

Mucci, J., Hidalgo, A., Mocetti, E., Argibay, P. F., Leguizamon, M. S., and Campetella, O. (2002). Thymocyte depletion in *Trypanosoma cruzi* infection is mediated by trans sialidase-induced apoptosis onnurse cells complex. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3896–3901. doi: 10.1073/pnas.052496399

Mucci, J., Mocetti, E., Leguizamón, M. S., and Campetella, O. (2005). A sexual dimorphism in intrathymic sialylation survey is revealed by the trans-sialidase from *Trypanosoma cruzi*. *J. Immunol.* 174, 4545–4550. doi: 10.4049/jimmunol.174.8.4545

Mucci, J., Risso, M. G., Leguizamón, M. S., Frasch, A. C., and Campetella, O. (2006). The trans-sialidase from *T. cruzi* triggers apoptosis by target cell sialylation. *Cell. Microbiol.* 8, 1086–1095. doi: 10.1111/j.1462-5822.2006.00689.x

Muia, R. P., Yu, H., Prescher, J. A., Hellman, U., Chen, X., Bertozzi, C. R., et al. (2010). Identification of glycoproteins targeted by *Trypanosoma cruzi* trans-sialidase, a virulence factor that disturbs lymphocyte glycosylation. *Glycobiology* 20, 833–842. doi: 10.1093/glycob/cwq037

Nagajyothi, F., Machado, F. S., Burleigh, B. A., Jelicks, L. A., Scherer, P. E., Mukherjee, S., et al. (2012). Mechanisms of *Trypanosoma cruzi* persistence in Chagas disease. *Cell. Microbiol.* 14, 634–643. doi: 10.1111/j.1462-5822.2012.01764.x

Nardy, A. F. F., da Silva Filho, J. L., Pérez, A. R., de Meis, J., Farias-de-Oliveira, D. A., Penha, L., et al. (2013). Trans-sialidase from *Trypanosoma cruzi* enhances the adhesion properties and fibronectin-driven migration of thymocytes. *Microbes Infect.* 15, 365–374. doi: 10.1016/j.micinf.2013.02.003

Noireau, F., Diosque, P., and Jansen, A. M. (2009). *Trypanosoma cruzi*: adaptation to its vectors and its hosts. *Vet. Res.* 40, 26. doi: 10.1051/vetres/2009009

Norris, K. A. (1998). Stable transfection of *Trypanosoma cruzi* epimastigotes with the trypomastigote-specific complement regulatory protein cDNA confers complement resistance. *Infect. Immun.* 66, 2460–2465.

Nunes, M. P., Andrade, R. M., Lopes, M. F., and DosReis, G. A. (1998). Activation-induced T cell death exacerbates *Trypanosoma cruzi* replication in macrophages cocultured with CD4+ T lymphocytes from infected hosts. *J. Immunol.* 160, 1313–1319.

Oladiran, A., and Belosevic, M. (2012). Immune evasion strategies of trypanosomes: a review. *J. Parasitol.* 98, 284–292. doi: 10.1645/GE-2925.1

Oppezzo, P., Obal, G., Baraibar, M. A., Pritsch, O., Alzari, P. M., and Buschiazzo, A. (2011). Crystal structure of an enzymatically inactive trans-sialidase-like lectin from *Trypanosoma cruzi*: the carbohydrate binding mechanism involves residual sialidase activity. *Biochim. Biophys. Acta* 1814, 1154–1161. doi: 10.1016/j.bbapap.2011.04.012

Ortiz-Ortiz, L., Parks, D. E., Rodriguez, M., and Weigle, W. O. (1980). Polyclonal B lymphocyte activation during *Trypanosoma cruzi* infection. *J. Immunol.* 124, 121–126.

Pappu, B. P., and Shrikant, P. A. (2004). Alteration of cell surface sialylation regulates antigen-induced naive CD8+ T cell responses. *J. Immunol.* 173, 275–284. doi: 10.4049/jimmunol.173.1.275

Parodi, A. J., Pollevick, G. D., Mautner, M., Buschiazzo, A., Sanchez, D. O., and Frasch, A. C. (1992). Identification of the gene(s) coding for the trans-sialidase of *Trypanosoma cruzi*. *EMBO J.* 11, 1705–1710.

Pereira, M. E. (1983). A developmentally regulated neuraminidase activity in *Trypanosoma cruzi*. *Science* 219, 1444–1446. doi: 10.1126/science.6338592

Pereira, M. E., Loures, M. A., Villalta, F., and Andrade, A. F. (1980). Lectin receptors as markers for *Trypanosoma cruzi*. Developmental stages and a study of the interaction of wheat germ agglutinin with sialic acid residues on epimastigote cells. *J. Exp. Med.* 152, 1375–1392. doi: 10.1084/jem.152.5.1375

Pereira, M. E., Mejia, J. S., Ortega-Barria, E., Matzilevich, D., and Prioli, R. P. (1991). The *Trypanosoma cruzi* neuraminidase contains sequences similar to bacterial neuraminidases, YWTD repeats of the low density lipoprotein receptor, and type III modules offibronectin. *J. Exp. Med.* 174, 179–191. doi: 10.1084/jem.174.1.179

Pereira-Chioccola, V. L., Schenkman, S., and Kloetzel, J. (1994). Sera from chronic Chagasic patients and animals infected with *Trypanosoma cruzi* inhibit trans-sialidase by recognizing its catalyticdomain. *Infect. Immun.* 62, 2973–2978.

Pérez, A. R., Roggero, E., Nicora, A., Palazzi, J., Besedovsky, H. O., del Rey, A., et al. (2007). Thymus atrophy during *Trypanosoma cruzi* infection is caused by an immuno-endocrine imbalance. *Brain Behav. Immun.* 21, 890–900. doi: 10.1016/j.bbi.2007.02.004

Piacenza, L., Zago, M. P., Peluffo, G., Alvarez, M. N., Basombrio, M. A., and Radi, R. (2009). Enzymes of the antioxidant network as novel determiners of *Trypanosoma cruzi* virulence. *Int. J. Parasitol.* 39, 1455–1464. doi: 10.1016/j.ijpara.2009.05.010

Pollevick, G. D., Affranchino, J. L., Frasch, A. C. C., and Sanchez, D. O. (1991). The complete sequence of a shed acute-phase antigen of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* 47, 247–250. doi: 10.1016/0166-6851(91)90185-9

Previato, J. O., Andrade, A. F., Pessolani, M. C., and Mendonça-Previato, L. (1985). Incorporation of sialic acid into *Trypanosoma cruzi* macromolecules. A proposal for a new metabolic route. *Mol. Biochem. Parasitol.* 16, 85–96. doi: 10.1016/0166-6851(85)90051-9

Priatel, J. J., Chui, D., Hiraoka, N., Simmons, C. J., Richardson, K. B., Page, D. M., et al. (2000). The ST3Gal-I sialyltransferase controls CD8+ T lymphocyte homeostasis by modulating O-glycan biosynthesis. *Immunity* 12, 273–283. doi: 10.1016/S1074-7613(00)80180-6

Ribeirão, M., Pereira-Chioccola, V. L., Eichinger, D., Rodrigues, M. M., and Schenkman, S. (1997). Temperature differences for trans-glycosylation and hydrolysis reaction reveal an acceptor binding site in the catalytic mechanism of *Trypanosoma cruzi* trans-sialidase. *Glycobiology* 7, 1237–1246. doi: 10.1093/glycob/7.8.1237

Ribeirão, M., Pereira-Chioccola, V. L., Renia, L., Augusto Fragata Filho, A., Schenkman, S., and Rodrigues, M. M. (2000). Chagasic patients develop a type 1 immune response to *Trypanosoma cruzi* trans-sialidase. *Parasite Immunol.* 22, 49–53. doi: 10.1046/j.1365-3024.2000.00260.x

Ribeiro, A. L., Nunes, M. P., Teixeira, M. M., and Rocha, M. O. (2012). Diagnosis and management of Chagas disease and cardiomyopathy. *Nat. Rev. Cardiol.* 9, 576–589. doi: 10.1038/nrcardio.2012.109

Risso, M. G., Pitcovsky, T. A., Caccuri, R. L., Campetella, O., and Leguizamon, M. S. (2007). Immune system pathogenesis is prevented by the neutralization of the systemic trans-sialidase from *Trypanosoma cruzi* during severe infections. *Parasitology* 134, 503–510. doi: 10.1017/S0031182006001752

Roggero, E., Pérez, A. R., Tamae-Kakazu, M., Piazzon, I., Nepomnaschy, I., Besedovsky, H. O., et al. (2006). Endogenous glucocorticoids cause thymus atrophy but are protective during acute *Trypanosoma cruzi* infection. *J. Endocrinol.* 190, 495–503. doi: 10.1677/joe.1.06642

Rodrigues, M. M., Ribeirão, M., Pereira-Chioccola, V. L., Renia, L., and Costa, F. (1999). Predominance of CD4 Th1 and CD8 TC1 cells revealed by characterization of the cellular immune response generated by immunization with a DNA vaccine containing a *Trypanosoma cruzi* gene. *Infect. Immun.* 67, 3855–3863.

Roggero, E., Perez, A., Tamae-Kakazu, M., Piazzon, I., Nepomnaschy, I., Wietzerbin, J., et al. (2002). Differential susceptibility to acute *Trypanosoma cruzi* infection in BALB/c and C57BL/6 mice is not associated with a distinct parasite load but cytokine abnormalities. *Clin. Exp. Immunol.* 128, 421–428. doi: 10.1046/j.1365-2249.2002.01874.x

Rubin-de-Celis, S. S., Uemura, H., Yoshida, N., and Schenkman, S. (2006). Expression of trypomastigote trans-sialidase in metacyclic forms of *Trypanosoma cruzi* increases parasite escape from its parasitophorous vacuole. *Cell. Microbiol.* 8, 1888–1898. doi: 10.1111/j.1462-5822.2006.00755.x

Savino, W. (2006). The thymus is a common target organ in infectious diseases. *PLoS Pathog.* 2:e62. doi: 10.1371/journal.ppat.0020062

Savino, W., and Dardenne, M. (2000). Neuroendocrine control of thymus physiology 1. *Endocr. Rev.* 21, 412–443. doi: 10.1210/edrv.21.4.0402

Schauer, R., Reuter, G., Muhlpfordt, H., Andrade, A. F., and Pereira, M. E. (1983). The occurrence of N-acetyl- and N-glycoloylneuraminic acid in *Trypanosoma cruzi*. *Hoppe Seylers Z. Physiol. Chem.* 364, 1053–1057. doi: 10.1515/bchm2.1983.364.2.1053

Schenkman, R. P., Vandekerckhove, F., and Schenkman, S. (1993). Mammalian cell sialic acid enhances invasion by *Trypanosoma cruzi*. *Infect. Immun.* 61, 898–902.

Schenkman, S., Jiang, M. S., Hart, G. W., and Nussenzweig, V. (1991). A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. *Cell* 65, 1117–1125. doi: 10.1016/0092-8674(91)90008-M

Schenkman, S., Pontes de Carvalho, L., and Nussenzweig, V. (1992). *Trypanosoma cruzi* trans-sialidase and neuraminidase activities can be mediated by the same enzymes. *J. Exp. Med.* 175, 567–575. doi: 10.1084/jem.175.2.567

Tardieux, I., Webster, P., Ravesloot, J., Boron, W., Lunn, J. A., Heuser, J. E., et al. (1992). Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. *Cell* 71, 1117–1130. doi: 10.1016/S0092-8674(05)80061-3

Tarleton, R. L., Reithinger, R., Urbina, J. A., Kitron, U., and Gurtler, R. E. (2007). The challenges of Chagas disease: grim outlook or glimmer of hope? *PLoS Med.* 4:e332. doi: 10.1371/journal.pmed.0040332

Todeschini, A. R., Nunes, M. P., Pires, R. S., Lopes, M. F., Previato, J. O., Mendonça-Previato, L., et al. (2002). Costimulation of host T lymphocytes by a trypanosomal trans-sialidase: involvement of CD43 signaling. *J. Immunol.* 168, 5192–5198. doi: 10.4049/jimmunol.168.10.5192

Tomlinson, S., Pontes de Carvalho, L. C., Vandekerckhove, F., and Nussenzweig, V. (1994). Role of sialic acid in the resistance of *Trypanosoma cruzi* trypomastigotes to complement. *J. Immunol.* 153, 3141–3147.

ToselloBoari, J., AmezcuaVesely, M. C., Bermejo, D. A., Ramello, M. C., Montes, C. L., Cejas, H., et al. (2012). IL-17RA signaling reduces inflammation and mortality during *Trypanosoma cruzi* infection by recruiting suppressive IL-10-producing neutrophils. *PLoS Pathog.* 8:e1002658. doi: 10.1371/journal.ppat.1002658

Tribulatti, M. V., Mucci, J., Van Rooijen, N., Leguizamon, M. S., and Campetella, O. (2005). The trans-sialidase from *Trypanosoma cruzi* induces thrombocytopenia during acute Chagas' disease by reducing the platelet sialic acid contents. *Infect. Immun.* 73, 201–207. doi: 10.1128/IAI.73.1.201-207.2005

Tyler, K. M., and Engman, D. M. (2001). The life cycle of *Trypanosoma cruzi* revisited. *Int. J. Parasitol.* 31, 472–481. doi: 10.1016/S0020-7519(01)00153-9

Uemura, H., Schenkman, S., Nussenzweig, V., and Eichinger, D. (1992). Only some members of a gene family in *Trypanosoma cruzi* encode proteins that express both trans-sialidase and neuraminidase activities. *EMBO J.* 11, 3837–3844.

Varki, A. (1997). Sialic acids as ligands in recognition phenomena. *FASEB J.* 11, 248–255.

Vimr, E., and Lichtensteiger, C. (2002). To sialylate, or not to sialylate: that is the question. *Trends Microbiol.* 10, 254–257. doi: 10.1016/S0966-842X(02)02361-2

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Role of Small RNAs in Trypanosomatid Infections

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Trypanosomatid parasites survive and replicate in the host by using mechanisms that aim to establish a successful infection and ensure parasite survival. Evidence points to microRNAs as new players in the host-parasite interplay. MicroRNAs are small non-coding RNAs that control proteins levels via post-transcriptional gene down-regulation, either within the cells where they were produced or in other cells via intercellular transfer. These microRNAs can be modulated in host cells during infection and are among the growing group of small regulatory RNAs, for which many classes have been described, including the transfer RNA-derived small RNAs. Parasites can either manipulate microRNAs to evade host-driven damage and/or transfer small RNAs to host cells. In this mini-review, we present evidence for the involvement of small RNAs, such as microRNAs, in trypanosomatid infections which lack RNA interference. We highlight both microRNA profile alterations in host cells during those infections and the horizontal transfer of small RNAs and proteins from parasites to the host by membrane-derived extracellular vesicles in a cell communication mechanism.

Keywords: small RNAs, microRNAs, host-parasite interaction, trypanosomatid infections, extracellular vesicles, cell communication

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INTRODUCTION

Trypanosomatid parasites comprise the African trypanosomes (*Trypanosoma brucei*), South american trypanosomes (*Trypanosoma cruzi*) and Leishmania, that profoundly affect mankind and substantially impact world public health (Coura and Viñas, 2010; Alvar et al., 2012). The diseases caused by these parasites predominantly affect the populations of developing regions of Africa, Asia and the Americas; however, population movement creates a new epidemiological challenge with worldwide spreading (Coura and Viñas, 2010; Alvar et al., 2012). Through different mechanisms these parasites establish a successful infection. Among these mechanisms, the small RNAs emerge as new players in the host-parasite interplay.

Small non-coding RNAs play an essential regulatory role in complex biological systems without protein translation (Aalto and Pasquinelli, 2012). Of these RNAs, the short-length RNA molecules (ranging from 20 to 30 nucleotides), such as microRNAs, small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs), stand out. Through sequence complementarity, these RNAs guide recognition of target genes within the ribonucleoprotein (RNP) complex and typically reduce the expression of a specific gene through the process of RNA interference (RNAi). Despite having the same mechanism of action, these different classes of regulatory RNAs differ in their association with Argonaute (AGO) protein family members to form the RNP complex, in their biogenesis, in their gene regulation pathways and in their biological functions (Ghildiyal and Zamore, 2009). Furthermore, the world of small non-coding RNAs is expanding, with new classes continuing to

be discovered, even in organisms that were not previously thought to express small RNA-mediated pathways, such as *T. cruzi* and some *Leishmania* species (Ullu et al., 2004; Ghildiyal and Zamore, 2009; Garcia-Silva et al., 2010a,b; Lye et al., 2010).

RNA-mediated silencing is an evolutionarily conserved mechanism that may have evolved together with parasite infection, as parasites developed strategies to interfere with host microRNA populations, thus recognizing the RNAi pathway as a new means of reshaping their environment to evade host immune surveillance and establish a successful infection (Cerutti and Casas-Mollano, 2006; Hakimi and Cannella, 2011). Obviously, changes in microRNA profiles might also be a defense mechanism of the infected cell. Nevertheless, the alteration of host microRNA levels after parasitic infection has been demonstrated (Geraci et al., 2015; Linhares-Lacerda et al., 2015), with some data revealing the intricate connection between the parasite and the RNAi machinery of the host organism (Ghosh et al., 2013). Moreover, the identification of predictive microRNA signatures associated with each specific parasitic infection could aid in the development of tools for diagnosis, prognosis, monitoring therapy and improving patient stratifications (Manzano-román and Siles-lucas, 2012).

In this mini-review, we briefly discuss current knowledge about the involvement of small RNAs in host-parasite interactions on trypanosomatid parasites that lack the AGO and Dicer genes and as a consequence do not have functional RNAi machinery. These parasites include *T. cruzi* (the etiologic agent of Chagas disease), *Leishmania major* and *Leishmania donovani* (which cause cutaneous leishmaniasis and visceral leishmaniasis, respectively). We focus on the microRNA profile alterations that occur in host cells due to infection with those parasites and on the trans-kingdom transfer of small RNAs and proteins from parasites to the host by membrane-derived extracellular vesicles (EVs) in a cell communication mechanism that may favor parasite survival.

MicroRNA PROFILE MODULATION DUE TO PARASITIC INFECTION

The elaborate relationship between parasites and their hosts aims to establish a successful parasite infection/infestation and promote survival, with parasites manipulating the host cellular machinery to avoid and regulate the host immune effector response (Manzano-román and Siles-lucas, 2012). In this context, gene expression modulation by microRNAs may be an ideal tool for parasites because microRNAs can function as master switches of many biological functions, fine tuning protein production (Zheng et al., 2013). It is reasonable to propose that cellular infection will be counteracted by cellular microRNAs that target crucial host factors as a defense mechanism; however, parasites subvert microRNA-directed functions as a means of altering gene expression in host cells (Hakimi and Cannella, 2011).

MicroRNAs are Related to Cardiac Alterations and Thymic Atrophy in Chagas Disease

The alteration of host microRNA levels after *T. cruzi* infection has been demonstrated in a murine model (Linhares-Lacerda et al., 2015; Navarro et al., 2015) and in Chagas disease patients (Ferreira et al., 2014). Chagas disease is a neglected tropical illness that is endemic to Latin America (Coura and Borges-pereira, 2012) and has an acute phase characterized by bloodstream circulating parasites and tissue parasitism, in addition to an intense immune response and hormonal imbalance (Pérez et al., 2011). Immune effector responses control *T. cruzi* numbers in the blood, and individuals enter the chronic phase of the disease with low parasites levels in several tissues (de Meis et al., 2013). Moreover, chronic infection can persist undetected, but ∼30% of patients develop severe complications, such as abnormal heart rhythm, heart failure, and digestive problems (Clayton, 2010; World Health Organization, 2010a).

The hearts of mice with experimental acute *T. cruzi* infection present an intense inflammatory cell infiltrate with myocarditis, arrhythmia and parasite nests in addition to a modified microRNA expression profile. Upon infection, 113 of 641 microRNAs were dysregulated; moreover, some microRNAs correlated with the maximal heart rate-corrected QT interval, which is a cardiac alteration (Navarro et al., 2015). Resembling the experimental model, chronic Chagas disease patients who develop cardiomyopathy can also present alterations in heart microRNAs and heart arrhythmia, among other cardiac complications. miR-1, miR-133a-2, miR-133b, miR-208a, and miR-208b were significantly downregulated in Chagas disease patients in comparison to uninfected patients (heart transplant donors). Moreover, in a comparison between two cardiomyopathy groups (chronic Chagas disease patients and dilated cardiomyopathy patients), miR-1, miR-133a-2, and miR-208b expression was reduced in infected patients (Ferreira et al., 2014). These microRNAs are highly enriched in the heart, where they regulate heart development and myocyte differentiation (Lagos-Quintana et al., 2002; Chen et al., 2006). In addition, atypical expression of these microRNAs has been linked to cardiovascular diseases (Carè et al., 2007; Ikeda et al., 2013). Therefore, variations of host microRNAs in experimental models of acute Chagas disease could shed light on the mechanism that triggers heart clinical alterations, with possible relevance for chronic Chagas disease patients with cardiomyopathy, as the downregulation of miR-208 was detected in both patients and mice infected by *T. cruzi* (Ferreira et al., 2014; Navarro et al., 2015). It is noteworthy that cardiac damage releases miR-208 and other factors into the bloodstream, and the levels of these factors exhibit distinctive patterns that correlate with different cardiovascular diseases, showing great potential for use as biomarkers for cardiac illness (Gupta et al., 2010). However, no data are available concerning circulating microRNAs in chronic Chagas disease patients who develop cardiomyopathy.

In addition to heart manifestations, mice with acute *T. cruzi* infection also present a severe thymic atrophy, primarily due to the apoptosis of $CD4^+CD8^+$ double-positive immature T cells

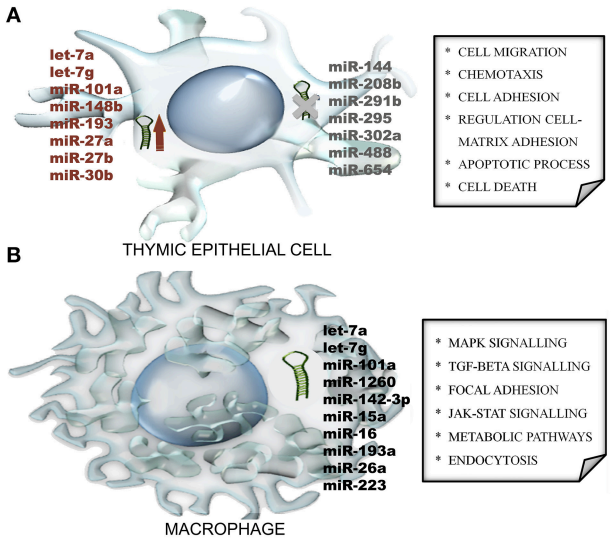

FIGURE 1 | MicroRNA modulation during parasitic infection: clues from Gene Ontology (GO) enrichment analysis of microRNA *in silico*-predict targets. (A) Mice with acute *Trypanosoma cruzi* infection modulate microRNA expression in thymic epithelial cells, with some microRNAs upregulated (red) and others undetectable (gray). The regulated microRNAs were used to identify biological processes (depicted within the square) that were over-represented among the list of microRNA target genes. **(B)** The examination of mature microRNA expression patterns in human macrophages revealed unique microRNA profiles in response to the *Leishmania major* and *L. donovani* parasites. In addition, biological pathway enrichment was performed for the dysregulated microRNAs to identify signaling pathways (depicted within the square) that might be involved.

and also due to migratory abnormalities that release potential autoreactive T cells to secondary lymphoid organs, which may play a role in the chronic phase of the disease (Savino, 2006). The development of increased T cell migration may be a consequence of signals delivered by thymic epithelial cells (TECs) that enhance the deposition of extracellular matrix proteins upon infection (Cotta-de-Almeida et al., 2003; Pérez et al., 2012). These signals might be under the control of microRNAs that are modulated in TECs from infected mice before the induction of thymic atrophy. Interestingly, microRNAs were primarily upregulated (29 out of 85 microRNAs), even if the TECs that were sorted from the thymus exhibited a cortical or medullary phenotype. Furthermore, Gene Ontology (GO) enrichment analysis of microRNA targets was used to identify biological processes that were over-represented among the list of target genes (**Figure 1A**). Indeed, the theoretical relationships of these microRNAs with their putative RNA targets revealed transforming growth factor-β (TGF-β) as a molecular node of infection, as the gene encoding its receptor (the Tgfbr1 gene) appears in the middle of our microRNA network, with 8 different microRNAs (let-7a, let-7g, miR-101a, miR-148b, miR-193, miR-27a, miR-27b, and miR-30b) regulating this gene (Linhares-Lacerda et al., 2015).

Taken together, these reports highlight the importance of microRNA alterations in Chagas disease. Furthermore, additional studies are needed to define microRNA biomarkers of *T. cruzi* infection. In this context, it is reasonable to hypothesize that miR-208 is a potential biomarker for *T. cruzi* infection because this microRNA was downregulated in both the human and mouse heart and undetectable in TECs from *T. cruzi*-infected samples, revealing a possible common regulation pattern in response to *T. cruzi* infection.

Macrophages Change their microRNA Profiles in Response to *Leishmania*

Sophisticated strategies for surviving and establishing a successful infection, such as antigen presentation inhibition, were developed by *Leishmania* parasites, which cause cutaneous or visceral diseases and are among the neglected diseases (World Health Organization, 2010b). *Leishmania* is an obligate intracellular pathogen in mammalian hosts and primarily infects macrophages, where it avoids anti-parasitic responses and subverts host innate immunity. The parasite modifies both microRNAs and mRNAs from the host, leading to altered expression of lipid metabolic genes, among other genes, resulting in reduced cholesterol synthesis, the disruption of membrane lipid rafts and the inhibition of antigen presentation to T cells (Ghosh et al., 2013; Chakraborty et al., 2015).

Upon infection with *L. major,* human primary macrophages change the microRNA-levels of 64 of 365 microRNAs, as assessed via a quantitative PCR time course. These dysregulated microRNAs virtually targeted several transcripts with critical cellular functions, such as cellular movement, secretion, communication, enzyme production, activity in the extracellular space, signal transduction, and gene expression naturally induced by an abiotic stimulus, which were all evaluated via a GO enrichment analysis followed by a pathway analysis (Lemaire et al., 2013). Additional examination of mature microRNA expression patterns in *L. major*- and *L. donovani*-infected human primary dendritic cells and macrophages using next-generation sequencing revealed unique mature microRNA expression profiles in response to both parasite species in different human host cell types. Indeed, *L. donovani*-infected cells exhibited higher expression of the identified microRNAs than *L. major*-infected cells. The biological pathway enrichment was performed again with predicted targets of the dysregulated microRNAs and identified the mitogen-activated protein kinase (MAPK) signaling pathway, among others (**Figure 1B**), regardless cell type or the infecting *Leishmania* species (Geraci et al., 2015).

In general, those studies revealed the remarkable capacity of *Leishmania* to modify microRNA expression in the host; nevertheless, the biological significance of the dysregulated microRNAs requires further investigation. For this purpose, the use of microRNA mimics and inhibitors is an excellent tool. For example, the transfection of the mmu-miR-210-5p inhibitor into *L. major*-infected murine macrophages significantly decreased the infection rates of these cells, suggesting a role of miR-210 in anti-parasitic activity (Frank et al., 2015). Moreover, the RNA targets obtained via *in silico* prediction require experimental evidence with further functional analysis to determine the role of each microRNA/mRNA-target in the specific pathways in which it participates. In the future, the knowledge gained from

those investigations will assist in the discovery of new targets for diagnostics or therapeutic approaches.

TRANS-KINGDOM TRANSFER OF EXTRACELLULAR VESICLES

Cells exchange information with their environments, influencing the behavior of other cells, and themselves. Cells can communicate through a variety of chemical, mechanical, and biological signals that trigger cell signaling and allow the cells to process information from the outside to support survival. Intercellular communication through biological signals involves the transfer of many different molecules, such as hormones, cytokines, and small RNAs, primarily via membrane vesicle trafficking (Barteneva et al., 2013). Taking advantage of cellular communication through the transfer of membrane-derived extracellular vesicle (EV) cargo to host cells, parasites manipulate host functions to establish a successful infection (Marcilla et al., 2014). In this section, we review the trans-kingdom transfer of EVs from *T. cruzi* and *L. donovani* to host cells.

Trypanosomatid Parasites Deliver Small RNAs through Extracellular Vesicles to Host Cells

EVs are key players in cell-to-cell communication. Like other pathogens, *T. cruzi* releases proteins associated with vesicles into the extracellular milieu to enable pathogen survival and replication within the host (Marcilla et al., 2014). *T. cruzi*'s protein vesicle content was defined in a proteomic study that among other classes, identified a relatively high proportion of RNA-binding proteins, suggesting a possible role in intercellular communication and gene expression regulation (Bayer-Santos et al., 2013). On the other hand, short transcriptome analysis using unbiased and genome-wide deep sequencing indicated an abundance of small RNAs derived from non-coding RNAs, of which tRNA-derived small RNAs (tsRNAs) derived from the 3′end with a median length of 38 nt were the most frequently detected type. Moreover, a comparison between certain tRNA isoacceptors from which tsRNAs were derived revealed that tsRNAs are differentially expressed and may be actively produced rather than being random degradation products from tRNA turnover (Franzén et al., 2011). Quite strikingly, *T. cruzi* lacks functional RNAi machinery but does express a unique open reading frame for an AGO/PIWI protein with the conserved domain architecture of a canonical AGO in all stages of its life cycle (Garcia-Silva et al., 2010b). Interestingly, tsRNA colocalizes with this distinctive trypanosomatid AGO protein (TcPIWI-tryp) in EVs that are transferred to surrounding parasites and to susceptible mammalian host cells, where the protein changes gene expression profiles (Garcia-Silva et al., 2010a, 2014a,b; **Figure 2**).

Recently, evidences demonstrated that Leishmania parasites release exosomes containing RNA sequences and that this exosomos and their cargo can be internalized by host cells. This is true for two different species of Leishmania, namely *L. donovani* and *L. braziliensis*, suggesting that the packing of specific RNA sequences into exosomes may be a conserved phenomenon in Leishmania (Lambertz et al., 2015). Like *T. cruzi* the authors found a richness of tRNA fragments originating from a small subset of tRNA isoacceptors, the tsRNAs, in both species, moreover in *L. braziliensis* which is RNAi-competent organism, they also found sequences derived from siRNA-coding regions in both sense and anti-sense suggesting that they appear as double-stranded RNAs in exosomes (Lambertz et al., 2015) (**Figure 2**). Although these finds, the *in vivo* biological effect of EVs carrying tsRNAs remains obscure, and more studies are required to assess the molecular mechanisms associated with these non-coding RNAs.

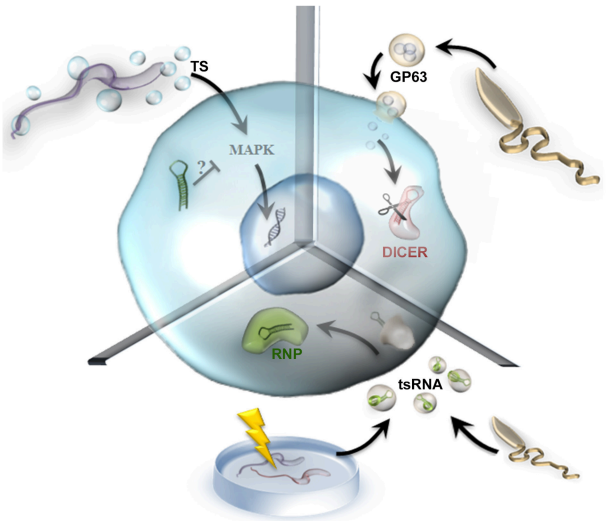

FIGURE 2 | Transfer of parasitic extracellular vesicle cargo to host cells. In this figure, we used a generic host cell to exemplify three different molecules delivered by parasitic extracellular vesicles. In the **upper left part** of the diagram, *Trypanosoma cruzi* releases EVs containing TS (trans-sialidase, *blue circles*) that can trigger gene modulation through the MAPK signaling pathway, which may be regulated by microRNAs. In the **upper right part** of the diagram, *Leishmania donovani* releases GP63 vesicles (*orange circles*) that cleave DICER (*red*), impairing microRNA maturation. Finally, in the **bottom part** of the diagram, EVs containing tsRNA (tRNA-derived small RNAs, in *green*) from Leishmania and from stressed *Trypanosoma cruzi* parasites modulate gene expression and might form RNP complexes (*green*).

T. cruzi also releases EVs containing members of the trans-sialidase glycoprotein superfamily. One member of this family led to the aggravation of experimental Chagas disease, with a severe inflammatory reaction of the heart and an increased number of amastigote nests in animals that received these EVs prior to *T. cruzi* infection (Trocoli Torrecilhas et al., 2009). The *T. cruzi* trans-sialidase transfers host sialic acid to parasite surface glycoconjugates, a process that supports host–cell recognition, infectivity, and parasite survival. Indeed, this trans-sialidase activity can remodel parasite glycomolecules, altering host immune responses against the parasite and playing a role as a virulence factor (Freire-de-Lima et al., 2012).

The presence of the trans-sialidase was confirmed in the peripheral blood of chronic Chagas disease patients, where the antibody titre against the trans-sialidase increased with

the frequency of peripheral double-positive immature T cells, potentially contributing to the clinical manifestations observed in the chronic phase of the disease. On the other hand, the thymus of *T. cruzi*-infected mice presents trans-sialidase depots near the parasite nests, which play a role in thymic atrophy and the premature release of double-positive $CD4^+CD8^+$ immature T cells. In contrast, intrathymic trans-sialidase injection increased the splenic double-positive immature T cell population and activated the MAPK/JNK signaling pathway in immature T cells (Nardy et al., 2013). Despite great advances toward understanding the effects of the *T. cruzi* trans-sialidase (Alves and Colli, 2010), the components involved in this signaling process remain a mystery. This process appears to be cell type-dependent, with MAPK/ERK-1/2 induction in naive splenic CD4 T cells (Todeschini et al., 2015), MAPK/JNK induction in immature T cells (Freire-de-Lima et al., 2012) and NF–kB induction in endothelial cells (Dias et al., 2008), but no microRNAs have been described to date. We suggest miR-199a as a good candidate for future studies of the *T. cruzi* trans-sialidase pathway. miR-199a regulates the PI3K/Akt and ERK/MAPK signaling pathways (Santhakumar et al., 2010) and targets a sialyltransferase (ST6 β-galactosamide α-2,6-sialyltransferase 1, ST6GAL1) (Minami et al., 2013). In addition, miR-199a is upregulated during human hypertrophy-related heart failure (van Rooij et al., 2006); however, we do not know how the expression pattern of miR-199a changes in the hearts of Chagas disease patients (**Figure 2**).

Taken together, the EVs from *T. cruzi* could be an additional strategy for modulating host cells via pathogen-to-host communication through the delivery of tsRNAs and virulence factors. However, the involvement of small RNAs is a recent discovery, and more studies are needed to elucidate this issue.

Exosome Cargo Impairs microRNA Maturation during *Leishmania donovani* Infection

During intercellular signaling and communication, EVs are used as a mechanism to actively regulate protein release from the cell. In *Leishmania,* changes in parasite culture temperature (26/37°C) lead to protein-specific enrichment in vesicles, affecting the cargo of the released exosomes. This exosome-based protein secretion mechanism delivers cargo to macrophages and triggers biological effects, such as the induction of interleukin-8 secretion (Silverman et al., 2010). Thus, EVs serve as an excellent pathogen-to-host communication process that could deliver effector molecules, such as proteins, and may also release RNAs into the host cytosol.

In this context, the delivery of the *Leishmania* surface protein metalloprotease GP63, which is a membrane-bound glycosylphosphatidylinositol (GPI)-anchored glycoprotein and a known virulence factor (Brittingham et al., 1995), participates in the parasite's strategy to evade immune surveillance. *L. donovani* extracts membrane cholesterol from macrophages, preventing T cell stimulation and causing hypocholesterolaemia that leads to protection against this infection, with an inverse correlation between serum cholesterol levels and parasite load in infected mice. In fact, the delivery of exosomes containing GP63 produced by Kupffer cell-resident parasites to hepatocytes impairs miR-122 activity by cleaving DICER1, which is a primary target of GP63 (Ghosh et al., 2012, 2013; **Figure 2**). DICER1 processes pre-microRNAs into mature microRNAs and transfers those microRNAs to AGO, forming the RNP complex. In the presence of GP63, hepatocytes accumulated pre-miR-122 and failed to form the RNP-miR-122 complex, possibly leading to the downregulation of cholesterol synthesis because miR-122 is responsible for lipid metabolism and liver homeostasis (Girard et al., 2008; Ghosh et al., 2013). Interestingly, the restoration of Dicer1 expression in parasite-infected livers increased miR-122 expression and restored serum cholesterol levels, with a drastic reduction in liver parasite load. Therefore, this process is a sophisticated example of how parasites evolved strategies to combat regulatory RNA functions in host cells, leading to an important metabolic change that promotes pathogenesis.

CONCLUDING REMARKS

Although few publications are available on this topic, current knowledge emphasizes the alteration of microRNA profiles during infection and EV cargo delivery during host interactions with the parasites *T. cruzi*, *L. major,* and *L. donovani*, which lack functional RNAi machinery. In this mini review, we highlighted some interesting findings in these fields and raised questions for further investigation, such as the status of miR-208 as a potential biomarker for *T. cruzi* infection, the presence of tsRNA in *Leishmania* EVs and the involvement of microRNAs in trans-sialidase triggered pathways during *T. cruzi* infection.

The main open question is to determine the role of each microRNA/mRNA-target in specific pathways through functional analysis and to investigate the importance of these factors in pathogenesis. The knowledge acquired from these futures studies will be useful for aiding the discovery of new targets for diagnosis or therapeutic approaches.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Aalto, A. P., and Pasquinelli, A. E. (2012). Small non-coding RNAs mount a silent revolution in gene expression. *Curr. Opin. Cell Biol.* 24, 333–340. doi: 10.1016/j.ceb.2012.03.006

Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE* 7:e35671. doi: 10.1371/journal.pone.0035671

Alves, M. J. M., and Colli, W. (2010). Signaling pathways in *Trypanosoma cruzi* that modulate host cell interaction. *Open Parasitol. J.* 4, 77–83. doi: 10.2174/1874421401004010077

Barteneva, N. S., Maltsev, N., and Vorobjev, I. A. (2013). Microvesicles and intercellular communication in the context of parasitism. *Front. Cell. Infect. Microbiol.* 3:49. doi: 10.3389/fcimb.2013.00049

Bayer-Santos, E., Aguilar-Bonavides, C., Rodrigues, S. P., Cordero, E. M., Marques, A. F., Varela-Ramirez, A., et al. (2013). Proteomic analysis of *Trypanosoma cruzi* secretome: characterization of two populations of extracellular vesicles and soluble proteins. *J. Proteome Res.* 12, 883–897. doi: 10.1021/pr300947g

Brittingham, A., Morrison, C., McMaster, W., McGwire, B., Chang, K., and Mosser, D. (1995). Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *J. Immunol.* 155, 3102–3111. doi: 10.1016/0169-4758(95)80054-9

Carè, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., et al. (2007). *MicroRNA-133* controls cardiac hypertrophy. *Nat. Med.* 13, 613–618. doi: 10.1038/nm1582

Cerutti, H. D., and Casas-Mollano, J. A. (2006). On the origin and functions of RNA-mediated silencing: from protists to man. *Curr. Genet.* 50, 81–99. doi: 10.1007/s00294-006-0078-x

Chakraborty, D., Banerjee, S., Sen, A., Banerjee, K., Das, P., and Roy, S. (2015). *Leishmania donovani* affects antigen presentation of macrophages by disrupting lipid rafts. *J. Immunol.* 175, 3214–3224. doi: 10.4049/jimmunol.175.5.3214

Chen, J., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., et al. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* 38, 228–233. doi: 10.1038/ng1725

Clayton, J. (2010). Chagas disease 101. *Nature* 465, S4–S5. doi: 10.1038/nature09220

Cotta-de-Almeida, V., Bonomo, A., Mendes-da-Cruz, D. A., Riederer, I., De Meis, J., Lima-Quaresma, K. R. F., et al. (2003). *Trypanosoma cruzi* infection modulates intrathymic contents of extracellular matrix ligands and receptors and alters thymocyte migration. *Eur. J. Immunol.* 33, 2439–2448. doi: 10.1002/eji.200323860

Coura, J. R., and Borges-pereira, J. (2012). Review article / Artigo de Revisão Chagas disease. What is known and what should be improved: a systemic review Doença de Chagas . O que é conhecido e o que deve ser melhorado: uma visão sistêmica. *Rev. Soc. Bras. Med. Trop.* 45, 286–296. doi: 10.1590/S0037-86822012000300002

Coura, J. R., and Viñas, P. A. (2010). Chagas disease: a new worldwide challenge. *Nature* 465, S6–S7. doi: 10.1038/nature09221

de Meis, J., Barreto de Albuquerque, J., Silva Dos Santos, D., Farias-de-Oliveira, D. A., Berbert, L. R., Cotta-de-Almeida, V., et al. (2013). *Trypanosoma cruzi* entrance through systemic or mucosal infection sites differentially modulates regional immune response following acute infection in mice. *Front. Immunol.* 4:216. doi: 10.3389/fimmu.2013.00216

Dias, W. B., Fajardo, F. D., Graça-Souza, A. V., Freire-de-Lima, L., Vieira, F., Girard, M. F., et al. (2008). Endothelial cell signalling induced by trans-sialidase from *Trypanosoma cruzi*. *Cell Microbiol.* 10, 88–99. doi: 10.1111/j.1462-5822.2007.01017.x

Ferreira, L. R., Frade, A., Santos, R. H., et al. (2014). MicroRNAs miR-1, miR-133a, miR-133b, miR-208a and miR-208b are dysregulated in chronic chagas disease cardiomyopathy. *Int. J. Cardiol.* 175, 409–417. doi: 10.1016/j.ijcard.2014.05.019

Frank, B., Marcu, A., de Oliveira Almeida Petersen, A., Weber, H., Stigloher, C., Mottram, J. C., et al. (2015). Autophagic digestion of Leishmania major by host macrophages is associated with differential expression of BNIP3, CTSE, and the miRNAs miR-101c, miR-129, and miR-210. *Parasit. Vectors* 8, 1–31. doi: 10.1186/s13071-015-0974-3

Franzén, O., Arner, E., Ferella, M., Nilsson, D., Respuela, P., Carninci, P., et al. (2011). The short non-coding transcriptome of the protozoan parasite *Trypanosoma cruzi*. *PLoS Negl. Trop. Dis.* 5:e1283. doi: 10.1371/journal.pntd.0001283

Freire-de-Lima, L., Oliveira, I. A., Neves, J. L., Penha, L. L., Alisson-Silva, F., Dias, W. B., et al. (2012). Sialic acid: a sweet swing between mammalian host and *Trypanosoma cruzi*. *Front. Immunol.* 3:356. doi: 10.3389/fimmu.2012.00356

Garcia-Silva, M. R., Cabrera-Cabrera, F., das Neves, R. F., Souto-Padrón, T., Souza, W., and De Cayota, A. (2014b). Gene expression changes induced by *Trypanosoma cruzi* shed microvesicles in mammalian host cells: relevance of tRNA-derived halves. *Biomed. Res. Int.* 2014:305239. doi: 10.1155/2014/305239

Garcia-Silva, M. R., das Neves, R. F. C., Cabrera-Cabrera, F., et al. (2014a). Extracellular vesicles shed by *Trypanosoma cruzi* are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. *Parasitol. Res.* 113, 285–304. doi: 10.1007/s00436-013-3655-1

Garcia-Silva, M. R., Frugier, M., Tosar, J. P., Correa-Dominguez, A., Ronalte-Alves, L., Parodi-Talice, A., et al. (2010a). A population of tRNA-derived small RNAs is actively produced in *Trypanosoma cruzi* and recruited to specific cytoplasmic granules. *Mol. Biochem. Parasitol.* 171, 64–73. doi: 10.1016/j.molbiopara.2010.02.003

Garcia-Silva, M. R., Tosar, J. P., Frugier, M., Pantano, S., Bonilla, B., Esteban, L., et al. (2010b). Cloning, characterization and subcellular localization of a *Trypanosoma cruzi* argonaute protein defining a new subfamily distinctive of trypanosomatids. *Gene* 466, 26–35. doi: 10.1016/j.gene.2010.06.012

Geraci, N. S., Tan, J. C., and Mcdowell, M. A. (2015). Characterization of microRNA expression profiles in Leishmania - infected human phagocytes. *Parasite Immunol.* 37, 43–51. doi: 10.1111/pim.12156

Ghildiyal, M., and Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* 10, 94–108. doi: 10.1038/nrg2504

Ghosh, J., Bose, M., Roy, S., and Bhattacharyya, S. N. (2013). *Leishmania donovani* targets Dicer1 to downregulate Mir-122, lower serum cholesterol, and facilitate murine liver infection. *Cell Host Microbe* 13, 277–288. doi: 10.1016/j.chom.2013.02.005

Ghosh, J., Das, S., Guha, R., Ghosh, D., Naskar, K., Das, A., et al. (2012). Hyperlipidemia offers protection against *Leishmania donovani* infection: role of membrane cholesterol. *J. Lipid Res.* 53, 2560–2572. doi: 10.1194/jlr.M026914

Girard, M., Jacquemin, E., Munnich, A., Lyonnet, S., and Henrion-Caude, A. (2008). miR-122, a paradigm for the role of microRNAs in the liver. *J. Hepatol.* 48, 648–656. doi: 10.1016/j.jhep.2008.01.019

Gupta, S. K., Bang, C., and Thum, T. (2010). Circulating MicroRNAs as biomarkers and potential paracrine mediators of cardiovascular disease. *Circ. Cardiovasc. Genet.* 3, 484–488. doi: 10.1161/CIRCGENETICS.110.958363

Hakimi, M., and Cannella, D. (2011). Apicomplexan parasites and subversion of the host cell microRNA pathway. *Trends Parasitol.* 27, 481–486. doi: 10.1016/j.pt.2011.07.001

Ikeda, S., Kong, S. W., Lu, J., Bisping, E., Zhang, H., Allen, P. D., et al. (2013). Altered microRNA expression in human heart disease. *Physiol. Genomics* 31, 367–373. doi: 10.1152/physiolgenomics.00144.2007

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific MicroRNAs from mouse. *Curr. Biol.* 12, 735–739. doi: 10.1016/S0960-9822(02)00809-6

Lambertz, U., Ovando, M. E. O., Vasconcelos, E. J. R., Unrau, P. J., Myler, P. J., and Reiner, N. E. (2015). Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world Leishmania providing evidence for conserved exosomal RNA Packaging. *BMC Genomics* 16:151. doi: 10.1186/s12864-015-1260-7

Lemaire, J., Mkannez, G., Guerfali, F. Z., et al. (2013). MicroRNA Expression profile in human macrophages in response to leishmania major infection. *PLoS Negl. Trop. Dis.* 7:e2478. doi: 10.1371/journal.pntd.0002478

Linhares-Lacerda, L., Palu, C. C., Ribeiro-Alves, M., Paredes, B. D., Morrot, A., Garcia-Silva, M. R., et al. (2015). Differential expression of microRNAs in thymic epithelial cells from *Trypanosoma cruzi* acutely infected mice: putative role in thymic atrophy. *Front. Immunol.* 6:428. doi: 10.3389/fimmu.2015.00428

Lye, L., Owens, K., Shi, H., Murta, S. M. F., Vieira, A. C., Turco, S. J., et al. (2010). Retention and Loss of RNA Interference pathways in trypanosomatid protozoans. *PLoS Pathog.* 6:e1001161. doi: 10.1371/journal.ppat.1001161

Manzano-román, R., and Siles-lucas, M. (2012). MicroRNAs in parasitic diseases: potential for diagnosis and targeting. *Mol. Biochem. Parasitol.* 186, 81–86. doi: 10.1016/j.molbiopara.2012.10.001

Marcilla, A., Martin-Jaular, L., Trelis, M., de Menezes-Neto, A., Osuna, A., Bernal, D., et al. (2014). Extracellular vesicles in parasitic diseases. *J. Extracell. Vesicles* 3:25040. doi: 10.3402/jev.v3.25040

Minami, A., Shimono, Y., Mizutani, K., Nobutani, K., Momose, K., Azuma, T., et al. (2013). Reduction of the ST6 beta-Galactosamide alpha-2,6-Sialyltransferase 1 (ST6GAL1) -catalyzed sialylation of nectin-like molecule 2/cell adhesion molecule 1 and enhancement of ErbB2/ErbB3 signaling by microRNA-199a. *J. Biol. Chem.* 288, 11845–11853. doi: 10.1074/jbc.M112.405993

Nardy, A. F., Luiz da Silva Filho, J., Pérez, A. R., et al. (2013). Trans-sialidase from *Trypanosoma cruzi* enhances the adhesion properties and fibronectin-driven migration of thymocytes. *Microbes Infect.* 15, 365–374. doi: 10.1016/j.micinf.2013.02.003

Navarro, I. C., Ferreira, F. M., Nakaya, H. I., et al. (2015). MicroRNA Transcriptome profiling in heart of *Trypanosoma cruzi* -infected mice: parasitological and cardiological outcomes. *PLoS Negl. Trop. Dis.* 9:e0003828. doi: 10.1371/journal.pntd.0003828

Pérez, A. R., Berbert, L. R., Lepletier, A., Revelli, S., Bottasso, O., Silva-Barbosa, S. D., et al. (2012). TNF-α is involved in the abnormal thymocyte migration during experimental *Trypanosoma cruzi* infection and favors the export of immature cells. *PLoS ONE* 7:e34360. doi: 10.1371/journal.pone.0034360

Pérez, A. R., Silva-Barbosa, S. D., Berbert, L. R., Revelli, S., Beloscar, J., Savino, W., et al. (2011). Immunoneuroendocrine alterations in patients with progressive forms of chronic Chagas disease. *J. Neuroimmunol.* 235, 84–90. doi: 10.1016/j.jneuroim.2011.03.010

Santhakumar, D., Forster, T., Laqtom, N. N., et al. (2010). Combined agonist – antagonist genome-wide functional screening identifies broadly active antiviral microRNAs. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3–8. doi: 10.1073/pnas.1008861107

Savino, W. (2006). The thymus is a common target organ in infectious diseases. *PLoS Pathog.* 2:e62. doi: 10.1371/journal.ppat.0020062

Silverman, J. M., Clos, J., Oliveira, C. C., De Shirvani, O., Fang, Y., Wang, C., et al. (2010). An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. *J. Cell Sci.* 123, 842–852. doi: 10.1242/jcs.056465

Todeschini, A. R., Nunes, M. P., Pires, R. S., Lopes, M. F., Previato, J. O., Medonça-Previato, L., et al. (2015). Costimulation of host T lymphocytes by a trypanosomal trans-sialidase: involvement of CD43 signaling. *J. Immunol.* 168, 5192–5198. doi: 10.4049/jimmunol.168.10.5192

Trocoli Torrecilhas, A., Tonelli, R., Pavanelli, W., da Silva, J., Schumacher, R., de Souza, W., et al. (2009). *Trypanosoma cruzi*: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. *Microbes Infect.* 11, 29–39. doi: 10.1016/j.micinf.2008.10.003

Ullu, E., Tschudi, C., and Chakraborty, T. (2004). RNA interference in protozoan parasites. *Cell Microbiol.* 6, 509–519. doi: 10.1111/j.1462-5822.2004.00399.x

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., Mcanally, J., Gerard, R. D., et al. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. U.S.A.* 103, 18255–18260. doi: 10.1073/pnas.0608791103

World Health Organization (2010a). *Working to Overcome the Global Impact of Neglected Tropical Diseases* (Geneva).

World Health Organization (2010b). *Control of the Leishmaniasis: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases*, Geneva, 22–26.

Zheng, Y., Cai, X., and Bradley, J. E. (2013). MicroRNAs in parasites and parasite infection. *RNA Biol.* 10, 371–379. doi: 10.4161/rna.23716

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REVIEW
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Cutaneous Leishmaniasis Vaccination: A Matter of Quality

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There have been exhaustive efforts to develop an efficient vaccine against leishmaniasis. Factors like host and parasite genetic characteristics, virulence, epidemiological scenarios, and, mainly, diverse immune responses triggered by *Leishmania* species make the achievement of this aim a complex task. It is already clear that the induction of a Th1, pro-inflammatory response, is important in the protection against *Leishmania* infection. However, many questions must still be answered to fully understand *Leishmania* immunopathology, especially regarding *Leishmania*-specific Th1 response induction, regulation, and persistence. A large number of *Leishmania* antigens able to induce pro-inflammatory response have been selected so far, but none of them demonstrated efficiency in protection assays. A possible explanation is that CD4 T cells display marked heterogeneity at a single-cell level especially regarding the production of Th1-defining cytokines and multifunctionality. It has been established in the literature that Th1 cells undergo a differentiation process, which can generate cells with diverse phenotypes and survival capabilities. Despite that, only a few studies evaluate this heterogenic response and the amount of multifunctional CD4 T cells induced by *Leishmania* vaccine candidates, missing what can be a crucial point in defining a correlate of protection after vaccination. Moreover, most of the knowledge involving the development of cutaneous leishmaniasis (CL) vaccines comes from the mouse model of infection with *Leishmania major*, which cannot be fully applied to New World Leishmaniasis. For this reason, the immune response triggered by infection with New World *Leishmania* species, as well as vaccine candidates, need further studies. In this review, we will reinforce the importance of evaluating the quality of immune response against *Leishmania*, using a multiparametric analysis in order to understand better this complex host-parasite interaction, discussing the differences in the responses triggered by different New World *Leishmania* species, as well as the impact on the development of an effective vaccine against CL.

Keywords: vaccines, multifunctionality, T cell response, *Leishmania amazonensis*, *Leishmania braziliensis*

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INTRODUCTION

World Health Organization (WHO) has classified Leishmaniasis among the tropical neglected, emerging, and uncontrolled diseases that affect mainly poor regions around the Globe. The disease is endemic in 88 countries (72 are developing countries) with approximately 350 million individuals at risk of contracting the disease and an annual incidence of 1.5–2 million new cases (1). Its prevention has been based on control of vectors and animal reservoirs in countries where the disease has a

zoonotic transmission, combined with chemotherapy of infected individuals where the disease possesses anthroponotic features. However, control of reservoir hosts and vectors is difficult due to operational issues, making the development of an effective and affordable vaccine against Leishmaniasis a highly desirable task.

The history of *Leishmania* vaccination dated from twentieth century, in which live, virulent parasites were inoculated in healthy individuals in a process called "Leishmanization." The practice was banned because of safety concerns due to development of non-healing lesions and immunosuppression (2). First generation vaccines using whole-killed *Leishmania* promastigotes replaced Leishmanization and were tested as vaccines against cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) (3, 4). Second and third generation vaccines were also developed, based on the defined synthetic or recombinant subunits and DNA, respectively. Despite many years of efforts in identifying a great number of antigens (5) and advances in vaccine technologies, there does not yet appear to be a vaccine candidate capable of delivering the level of protection needed for disease control.

The localized form of CL, specially the one caused by the Old World specie *Leishmania major*, is a self-healing disease, usually characterized by a state of at least partial immunity against reinfection, demonstrating that prevention through prophylactic vaccination is feasible. On the other hand, although recovery from infection with the New World specie *Leishmania braziliensis* gives firm resistance to homologous challenge, *Leishmania amazonensis* infection does not provide protection against a subsequent challenge with *L. braziliensis,* or other *Leishmania* species from the subgenus *Viannia* (6, 7). Until now, there has been no consistent data, particularly in humans, indicating that recovery from a primary infection with *L. amazonensis* gives complete resistance to a homologous challenge.

The fact that there is not yet an efficient vaccine against Leishmaniasis, especially one that could protect against different species simultaneously, leads us to consider that a better understanding of immune response in *Leishmania* pathogenesis is still needed, taking into consideration the various species that cause different clinical manifestations of the disease. Among the reasons that can be pointed out to explain our failure in developing a vaccine against CL, particularly against American cutaneous leishmaniasis, is the fact that we are still far from fully understand the mechanisms of healing and of memory responses generated after *Leishmania* infections as well as how to evaluate this responses. Far from giving the answers, this review focuses on the current advances in T cell memory knowledge and the differences observed between the immune responses induced after infection with different *Leishmania* species, particularly between *L. braziliensis* and *L. amazonensis*.

AMERICAN TEGUMENTARY LEISHMANIASIS: BEYOND THE Th1/Th2 PARADIGM

American tegumentary leishmaniasis (ATL) is endemic in Latin America and the most common species involved are: *L. braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis* (all from the genus *Viannia*), *L. amazonensis,* and *Leishmania mexicana* (both from the subgenus *Leishmania*). Unlike Old World CL, usually characterized by subclinical or self-healing cutaneous lesions, the infection by ATL causing species can lead to uncontrolled parasite replication, producing non-healing cutaneous, mucosal, or even visceral disease (1) (**Table 1**).

Human infection with *L. braziliensis* leads to a broad spectrum of clinical, immunological, and histopathological manifestations, varying from self-healing cutaneous lesions to the severe and destructive clinical form named mucocutaneous leishmaniasis (ML) (8–10). The localized cutaneous form (LCL) usually manifests as one or a few ulcers with elevated borders and sharp craters that increase rapidly in size and heal slowly without treatment (11). *L. braziliensis* can also cause disseminated leishmaniasis, in which up to hundreds of lesions erupt as a result of hematogenous spread of parasite (12, 13). *L. amazonensis* has also been isolated from patients with diverse clinical forms, such as simple CL lesions to diffuse cutaneous leishmaniasis (DCL) (14) and was also implicated in borderline disseminated CL, an intermediate form of disease (15). Patients with DCL are often resistant to chemotherapy, have negative leishmanin skin test (LST), and low or negative responses after *Leishmania* antigen-specific stimulation *in vitro* but remain responsive for other unrelated antigens, such as tuberculin (8).

For many years, murine CL models have been used to elucidate the cell types, cytokines, signal transduction cascades, and mechanisms needed for parasite control and clinical resolution of the disease. Since *Leishmania* is an obligate intracellular parasite, the protective immunity is associated with a cell-mediated immune response. Indeed, studies in the murine model have been helping to elucidate the immunological pathways that are

TABLE 1 | Major human American cutaneous leishmaniasis causing species and their clinical manifestations.

Species	Subgenus	Clinical forms	Leishmanin skin test	Key cytokines involved in pathology
L. braziliensis	*Viannia*	LCL	++	IFN-γ+++, IL-10++
		Disseminated CL	+++	IFN-γ+, IL-10++
		ML	++++	IFN-γ++++, IL-10+
L. guyanensis	*Viannia*	LCL	++	IFN-γ++, IL-10++
L. panamensis	*Viannia*	LCL	++	IFN-γ++, IL-10++
		ML	++++	IFN-γ++++, IL-10+
L. amazonensis	*Leishmania*	LCL	±	IFN-γ+, IL-10+
		Borderline CL	–	IFN-γ++, IL-10++
		DCL	–	IFN-γ±, IL-10++++
L. mexicana	*Leishmania*	LCL	±	IFN-γ++, IL-10++
		DCL	–	IFN-γ±, IL-10++++

LCL, localized cutaneous leishmaniasis; ML, mucosal leishmaniasis; DCL, diffuse cutaneous leishmaniaisis.

–, Absence.

+, Presence; +, low; ++, moderate; +++, high; ++++, very high.

responsible for resistance or susceptibility to *Leishmania* and were responsible for the description of the CD4 T cells Th1/Th2 dichotomy. It is well accepted that protective immunity against *Leishmania* parasites is mediated by a type 1, pro-inflammatory response, and most of the early studies, particularly on *L. major* infection, largely defined the Th1/Th2 paradigm of resistance/susceptibility to infection and the role of interleukin 12 (IL-12) and IL-4, respectively, in driving Th1 and Th2 cell development (16, 17). On the other hand, in human *L braziliensis* infection, some evidences suggest that higher percentage of activated IFNγ+ producing T CD4+ lymphocytes are associated with larger lesions (18), and an exacerbated Th1 response is observed in ML (19). The polarized CD4 lymphocyte response detected in the murine *L. major* model is not so evident in the human Leishmanasis, and the importance of IL-4 as a primary mediator of susceptibility to *Leishmania* infection is not corroborated by clinical trials (19). Indeed, in DCL (the most severe form of human ATL), the main cytokine associated with immunosuppression and pathology it is not IL-4 but IL-10 (20–22).

The vast majority of experimental CL studies come from the murine model of infection with *L. major*, although the disease outcome in inbred strains of mice differs among *Leishmania* species. While C57BL/6 and C3H mice are resistant to infection with *L. major*, they develop chronic lesions when infected with *L. amazonensis* while BALB/c mice are highly susceptible to *L. major* and *L. amazonensis* infection, but develop self-limited lesions when infected with *L. braziliensis* (16). Little information has been generated in the murine model regarding ATL causing *Leishmania* species. Although some data have been published with *L. braziliensis* and *L. amazonensis* infection, the protocols are heterogeneous with respect to the stage of parasite used (stationary phase or metacyclic promastigotes) and the inoculation route (subcutaneous or intradermal), making it difficult to compare the results obtained (23–26).

Even though we still lack a reliable, largely accepted, and utilized murine model for ATL, much progress was made in understanding the mechanisms involved in human pathology. However, many questions are still unanswered, especially those related to the immunological mechanisms leading to lesions healing and natural resistance to infection and cross-protection, as well as to the induction, regulation, and persistence of *Leishmania*-specific T cell response.

CD4 IMMUNE RESPONSE AND MEMORY

The goal of vaccination is the development of immunological memory, classically defined as the ability of the immune system to respond more effectively and faster to a pathogen previously encountered. In the late twentieth century, memory T cells were divided into central memory (TCM) and effector memory cell (TEM) populations, based on the expression of different cell surface markers (27). TCM cells constitutively express CCR7 and CD62L and are found in T cell areas of secondary lymphoid organs where they are able to proliferate and differentiate into effector cells in response to antigenic stimulation. TEM cells downregulate the expression of CCR7, have heterogeneous expression of CD62L, and are able to migrate to inflamed tissues, and have immediate effector functions (27, 28). One study in murine *L. major* infection demonstrated the importance of two populations of memory CD4 T cells in the protection against reinfection. While effector CD4+ T cells are lost in the absence of parasites, the central memory CD4+ T cells are kept and become tissue-homing effector T cells to mediate protection, suggesting that central memory T cells should be the targets for vaccines against *Leishmania* (29). The same group recently identified the presence of skin tissue *Leishmania*-specific resident memory T cells, and indicated the necessity of these cells, together with circulating memory T cells, for the success of a vaccine (30).

The induction of memory T cells was also evaluated in patients with CL. In patients healed form *L. major* infection both TEM IFN-γ producing cells (CD4+CD45RO+CD45RA−CCR7−) and *Leishmania*-reactive IL-2 producing TCM cells (CD4+CD 45RO+CD45RA−CCR7+) were observed after "*in vitro*" stimulation with *Leishmania* soluble antigen (SLA), suggesting that both populations might play a role in protective recall immune response against reinfection (31). On the other hand, the majority of *L. braziliensis*-healed CL and ML patients did not produce IFN-γ "*in vitro*" after SLA stimulation, but are still responsive "*in vivo*" to LST. A positive LST was found in 87.5% of CL and 100% of ML cured individuals who did not produce IFN-γ, and in the individuals that maintains SLA-specific IFN-γ production, the main source of the cytokine was effector memory CD4+ T cell (32).

Usually, *L. braziliensis* patients healed from CL lesions should be monitored for approximately 5 years to rule out of the possibility of relapses or the development of metastatic mucosal lesions (33, 34). In one study where healed *L. braziliensis* CL patients were grouped according to the time elapsed since the end of therapy, a regulated leishmanial-specific response appeared to emerge only about 2 years after initial contact with the parasite. *Ex vivo* analyses showed a contraction for both CD4 and CD8 TEM compartments in patients with long-time elapsed after clinical cure (2–5 years). However, after "*in vitro*" SLA stimulation, they exhibit a recall response with expansion of TEM cells (35).

CD4 T cells also present different capacities to develop into memory cells based on their cytokine production (36), and the quality of a Th1 immune response has been related with a differentiation spectrum based on the production of three cytokines: IFN-γ, IL-2, and TNF-α (37). Cells that enter this differentiation processes are, at first, single producers of IL-2 or double producers of IL-2/TNF-α, but are negative for IFN-γ. They can be classified as central memory cells, since are long-lasting cells able to respond quickly to a second antigen encounter. Te other pole of this spectrum is IFN-γ single-positives cells that are short-lived, terminal effector cells (36, 37). From one pole to the other, a variety of phenotypes can be found, including multifunctional CD4 T that are triple positives for IFN-γ, IL-2, and TNF-α (37). Interestingly, the amount of IFN-γ produced by multifunctional cells is much higher than the amount produced by double- or single-positive cells (38, 39). The IL-2 produced by those cells together with the high production of IFN-γ and TNF-α give to multifunctional CD4 T cells the remarkable capacity to possess optimal effector functions and proliferation.

CD4+ T cells not always go through each possible stage of differentiation and after antigen recognition, an IL-2 single-positive cell can go straight to the IFN-γ single-positive effector phenotype, particularly if the stimulus is strong (36). Thus, it is possible that a vaccine candidate can elicit an immune response predominantly composed by effector cells, and fail to induce long, last protection against infection. A promising vaccine candidate should be able to induce multifunctional T cells that are able to proliferate and generate memory and effector cells.

In past years, the majority of studies designed to evaluate possible immunogens against *Leishmania* infection utilized the production of IFN-γ by antigen-specific T cells as the main factor to predict protection. However, it is clear that the quality and the magnitude of a T-cell response measured by a single parameter do not reflect its full functional potential which may be the reason why vaccines that reached phase III trials failed to protect against *Leishmania* infection (40–42). In 2007, the first compelling evidence for the importance of multifunctional Th1 cells in mediating protection against Leishmaniasis revealed, after immunization with various vaccine formulations encoding specific *L. major* antigens, a strong correlation between the generation of multifunctional CD4 T cells and the degree of protection observed after a subsequent challenge (38). Intriguingly, the best degree of protection and the higher percentage of multifunctional T cells were observed in animals that healed primary lesions and were reinfected ("live vaccination"). Afterward, this approach started to be utilized by many other research groups to characterize immune correlates of protection after infection or after immunization against CL and VL (**Table 2**) (39, 43–51), but only two concerned ATL causing species (39, 51). In all of them, protection was demonstrated to be associated with the induction of multifunctional T cells among other double producers or with TNF-α producing cells (either TNF-α single-positives or TNF-α/IL-2 and TNF-α/IFN-γ double positive cells) (45–47, 51).

TABLE 2 | Multifunctional T cells analysis on *Leishmania* infection and vaccination.

Specie	Model	CD4 T cell phenotype associated with cure or protection	Reference
L. major	C57BL/6	IFN+TNF+IL-2+	Darrah et al. (38)
L. major	C57BL/6 and human	IFN+TNF+IL-2+	Darrah et al. (50)
L. major	Balb/c	IFN+TNF+L-2+, IFN+NF+, and TNF+	Sánchez-Sampedro et al. (45)
L. major	Balb/c	IFN+TNF+ and IFN+IL-2+	Hugentobler et al. (49)
L. amazonensis and *L. braziliensis*	Human	IFN+TNF+IL-2+	Macedo et al. (39)
L. donovani	Balb/c	IFN+TNF+L-2+, IFN+TNF+, and IFN+IL-2+	Dey et al. (48)
L. donovani	Balb/c	TNF+IL-2+ and IFN+TNF+	Guha et al. (46)
L. donovani	Balb/c	IFN+TNF+IL-2+, IFN+TNF+, and IFN+IL-2+	Guha et al. (47)
L. amazonensis	Balb/c	TNF+IL-2+ and TNF+	Nico et al. (51)
L. major	Balb/c and C57BL/6	IFN+TNF+IL-2+	Matos et al. (43)
L. major	Human	IFN+TNF+IL-2+	Lakhal-Naouar et al. (44)

L. AMAZONENSIS VERSUS *L. BRAZILIENSIS*: DIFFERENCES ON QUALITY OF IMMUNE RESPONSE

It has already been reported that patients infected with parasites from the subgenus *Viannia* (as *L. braziliensis*) display higher T cell responses (evaluated by proliferation and IFN-γ production) to *Leishmania* crude antigens than *L. amazonnsis*-infected patients, and that *L. amazonensis*-infected patients also have stronger responses to *L. braziliensis* than to *L. amazonensis* antigens *in vitro*, before and after therapy (52).

Vaccine candidates formulated with *L. braziliensis* total extract have been tested against Canine VL (LBSap and LBSApSal) with promising results in phase I and II trials (53–55). LBSap induced both humoral and cellular immune responses against *Leishmania infantum*, with high levels of total IgG and its subtypes (IgG1 and IgG2), expansion of circulating CD5+, CD4+, and CD8+ T lymphocytes as well as reduction of splenic parasite load (55).

One previous study designed to evaluate the quality of the Th1 response induced by *L. amazonensis* and *L. braziliensis* promastigotes extracts in PBMC from healed CL patients demonstrated that *L. amazonensis* response is associated with a low contribution of multifunctional T cells and a high number of IFN-γ single-positive effector cells, while *L. braziliensis* induces a Th1 response with high proportion of multifunctional T cells and low proportion of IFN-γ single-positive cells (39). As IFN-γ single-positive CD4+ T cells are short-lived, this can offer a possible explanation for the contrasting results observed in prophylaxis and immunotherapy studies with *L. amazonensis* whole-cell extract vaccine (Leishvacin®) (40, 56–58). The substantial amount of IFN-γ single-positive effector CD4+ T cells induced by this antigen may not be sufficient to induce long-term and good-quality protection against infection, but could be effective when a rapid and transient Th1 response is needed, as in the case of immunotherapeutic interventions. In addition, the capacity of *L. amazonensis* promastigotes extract to induce IL-10 secretion (59, 60), together with the generation of short-lived IFN-γ producing CD4+ T cells, could result in equilibrium between inflammatory and anti-inflammatory responses, allowing parasite killing and lesion resolution, as observed in the immunotherapeutic protocols tested so far.

If we combine the information that mice healed from a primary infection with *L. major* present the highest proportion of multifunctional CD4+ T cells and protection after a homologous challenge (38), together with the results obtained in healed CL patients after stimulation with *L. braziliensis* and *L. amazonensis* promastigotes extracts (39), we can consider the possibility that patients healed from *L. braziliensis* infection should display better protection to reinfection than *L. amazonensis* healed patients.

It has never been reported that individuals that were infected with *L. braziliensis* or any other *Leishmania* specie are more susceptible to infection with *L. amazonensis*, but *L. amazonensis* infection does not give protection against a subsequent

challenge with *L. braziliensis* or other *Leishmania* specie from the subgenus *Viannia*. On the other hand, recovery from *L. braziliensis* infection confers resistance to homologous challenge as well as to infection with *L. amazonensis* or *L. mexicana* parasites (6, 7). Interestingly, cells from DCL patients infected with *L. amazonensis* are able to differentiate into multifunctional T cells *in vitro* only after simulation with *L. braziliensis* promastigotes extract, while *L. amazonensis* stimulates high proportions of IFN-γ single-positive, terminal differentiated cells (**Figure 1**). This finding indicates that something intrinsic to *L. amazonensis* parasite antigens is responsible for the weak specific Th1 immune response observed during *L. amazonensis* infection (52, 61).

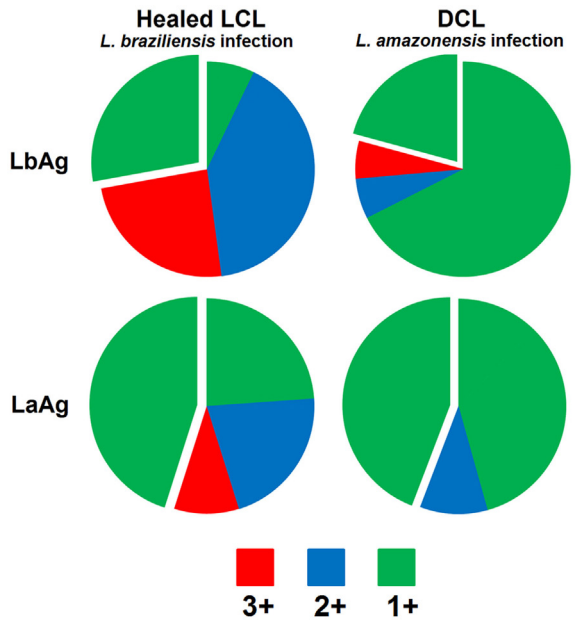

FIGURE 1 | Demonstrative figure of the CD4 T cell response induce in PBMC cultures of one patient healed from localized cutaneous Leishmaniasis caused by *L. braziliensis* (Healed LCL) in comparison to the response induced in PBMC cultures obtained from one patient with diffuse cutaneous Leishmaniasis caused by *L. amazonensis*, during remission of symptoms (DCL). Cells were stimulated "*in vitro*" with total promastigotes extracts form *L. braziliensis* (LbAg) or *L. amazonensis* (LaAg) and stained with monoclonal antibodies to determine the frequency of CD4 T cells expressing IFN-γ, IL-2, and TNF-α by multiparametric flow cytometry. Combination gates were applied to determine the percentage of cells that were able to produce any combination of these three cytokines. To determine the contribution of each phenotype to the total Th1 immune response analyzed the results are represented in the pie charts comprising cells expressing all three cytokines (in red – IFN-γ+TNF-α+IL-2+), any two cytokines (in blue – IFN-γ+TNF-α+IL-2−, IFN-γ+TNF-α−IL-2+, and IFN-γ−TNF-α+IL-2+), or any one cytokine (in green – IFN-γ+TNF-α−IL-2−, IFN-γ−TNF-α+IL-2−, and IFN-γ−TNF-α−IL-2+). Detached is the contribution of the IFN-γ+TNF-α−IL-2− single-positive cells phenotype. Data showed in this figure are part of a published study (39) approved by the National Ethical Clearance Committee of Brazil (CONEP), as well as by the Ethical Committee for Human Research from IPEC/FIOCRUZ, all of which adhere to the principles laid out in the Declaration of Helsinki. Informed consent was obtained from all participants.

Even though parasites from the *Viannia* and *Leishmania* subgenera show highly conserved gene sequences with very few genes restricted to a given species (62–64), these similarities did not prevent different species from evolving some particularities related to the expression of virulence factors and the development of particular evasion mechanisms. Recently, the genome of *L. amazonensis* was sequenced and compared with other human pathogenic *Leishmania* spp. indicating that *L. amazonensis* and *L. mexicana* share groups of amastin surface proteins unique to the genus that could be related to specific disease outcomes. Additionally, a hypothetical interactome model of host protein and secreted *L.* (*L.*) *amazonensis* proteins revealed a possible interaction between an *L. (L.) amazonensis* heat-shock protein and mammalian Toll-like receptor 9 (65).

The low generation of multifunctional T cells induced by *L. amazonensis* can be one more factor, or, and most likely, can be a consequence of many others already described in the literature, implicated with the susceptibility to this *Leishmania* specie (23, 59, 65–73).

CONCLUDING REMARKS

Prophylactic immunization is accepted as the most efficient and low-cost/benefit alternative to control infectious diseases. An ideal vaccine against Leishmaniasis must have several attributes: (1) safety, (2) accessibility for populations at risk, (3) induce long-lasting CD4 and CD8 specific T cell response, (4) be effective against *Leishmania* species responsible for visceral and tegumentary forms, (5) stability at room temperature to be used in the field, and (6) have prophylactic and therapeutic potential (74). Although it is possible to fulfill the attributes related to cost/benefit and safety, the development of a Leishmaniasis vaccine has proven a difficult goal to achieve. Not because of the discovery of candidate molecules, especially after the sequencing of the genome of different species of parasite, but rather because of the difficulties related to the still incomplete knowledge involving pathogenesis, the complex immune response needed for induction of protection, the lack of suitable experimental models, and the still fragmented knowledge about the development of immunological memory mechanisms.

Currently more than 30 *Leishmania* antigens have been or are being tested as candidate vaccines against visceral or tegumentary leishmaniasis. Many of them are very well conserved among different species of the parasite, but were not capable of inducing protection in clinical trials or are unable to protect against all species of the parasite. However, one study has demonstrated that heterologous protection is feasible, and associated with the presence of a "multifunctional Th1 response." BALB/c mice immunized with a non-pathogenic *Leishmania donovani* parasite showed cross-protection against the challenge with *L. major* or *L. braziliensis*, and the immunization induced a long-term immune response characterized by high levels of multifunctional CD4 and CD8 T cells (48). Additionally, other authors observed a reduction in the frequency of parasitism in the bone marrow (54), as well as a reduction in splenic parasite loads (55) in dogs vaccinated against VL with LbSAP (a preparation of killed *L. braziliensis*

promastigotes together with saponin), after *L. infantum* infection, although multifunctionality were not evaluated in those studies.

A point that also needs to be emphasized is that in natural infection, all the *Leishmania* species are co-deposited into the skin together with the vector saliva, and that saliva contains factors able to modulate the immune response (75–77). Studies have demonstrated that pre-exposure of sand fly saliva lead to either disease exacerbation (78, 79) or protection (80–83) upon *Leishmania* infectious challenge. Carregaro et al. (84) demonstrated that different inocula of *Lutzomyia longipalpis* salivary gland extract could modify the cellular immune response, reflecting in the pattern of susceptibility or resistance to *L. braziliensis* infection. It would be interesting to investigate whether a combination of saliva proteins with *Leishmania* proteins or extracts can shape the immune responses against infection, altering the quality of the immune responses by increasing the frequencies of multifunctional T cells. Moreover, the use of components that participate in the initial phase of infection could improve vaccine efficiency at the earlier stages of infection.

Certainly, there is still a long road ahead of us until an ideal Leishmaniasis vaccine be developed, but it is also undoubtable that multiparametric flow cytometry gave us a powerful tool to better evaluate correlates of protection and the development of memory T cell responses after infection and immunization. Since the crude and synthetic antigens tested so far were not able to induce consistent protection against *Leishmania* infections, it may be time to turn away our efforts from finding new candidate molecules, and focus on evaluating new presentation approaches of existing conserved molecules, specially the design of safe new adjuvants, that could direct the T cell-specific response toward long-lasting memory and multifunctional T cell phenotypes.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. World Health Organization. *Leishmaniasis Situation and Trends. Global Health Observatory* (2016). Available from: http://www.who.int/gho/neglected_diseases/leishmaniasis/en/
2. Nadim A, Javadian E, Tahvildar-Biruni G, Ghorbani M. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. *Bull Soc Pathol Exot Filiales* (1983) **76**:377–83.
3. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. *Vaccine* (2008) **26**:6759–67. doi:10.1016/j.vaccine.2008.09.085
4. Mayrink W, Williams P, da Costa CA, Magalhães PA, Melo MN, Dias M, et al. An experimental vaccine against American dermal leishmaniasis: experience in the State of Espírito Santo, Brazil. *Ann Trop Med Parasitol* (1985) **79**:259–69.
5. Nagill R, Kaur S. Vaccine candidates for leishmaniasis: a review. *Int Immunopharmacol* (2011) **11**:1464–88. doi:10.1016/j.intimp.2011.05.008
6. Lainson R, Shaw JJ. Leishmaniasis in Brazil: XII. Observations on cross-immunity in monkeys and man infected with *Leishmania mexicana mexicana*, *L. m. amazonensis*, *L. braziliensis braziliensis*, *L. b. guyanensis* and *L. b. panamensis*. *J Trop Med Hyg* (1977) **80**:29–35.
7. Porrozzi R, Teva A, Amaral VF, Santos da Costa MV, Grimaldi G. Cross-immunity experiments between different species or strains of *Leishmania* in rhesus macaques (*Macaca mulatta*). *Am J Trop Med Hyg* (2004) **71**:297–305.
8. Grimaldi G, Tesh RB. Leishmaniases of the New World: current concepts and implications for future research. *Clin Microbiol Rev* (1993) **6**:230–50.
9. de Oliveira-Neto MP, Mattos MS, Perez MA, Da-Cruz AM, Fernandes O, Moreira J, et al. American tegumentary leishmaniasis (ATL) in Rio de Janeiro State, Brazil: main clinical and epidemiologic characteristics. *Int J Dermatol* (2000) **39**:506–14. doi:10.1046/j.1365-4362.2000.00969.x
10. de Oliveira Guerra JA, Prestes SR, Silveira H, Coelho LI, Gama P, Moura A, et al. Mucosal leishmaniasis caused by *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *guyanensis* in the Brazilian Amazon. *PLoS Negl Trop Dis* (2011) **5**:e980. doi:10.1371/journal.pntd.0000980
11. Bittencourt A, Silva N, Straatmann A, Nunes VLC, Follador I, Badaró R. Post-kala-azar dermal leishmaniasis associated with AIDS. *Braz J Infect Dis* (2003) **7**:229–33. doi:10.1590/S1413-86702003000300009
12. Costa JM, Marsden PD, Llanos-Cuentas EA, Netto EM, Carvalho EM, Barral A, et al. Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. *J Trop Med Hyg* (1986) **89**:319–23.
13. Turetz ML, Machado PR, Ko AI, Alves F, Bittencourt A, Almeida RP, et al. Disseminated leishmaniasis: a new and emerging form of leishmaniasis observed in northeastern Brazil. *J Infect Dis* (2002) **186**:1829–34. doi:10.1086/345772
14. Almeida RP, Barral-Netto M, De Jesus AMR, De Freitas LAR, Carvalho EM, Barral A. Biological behavior of *Leishmania amazonensis* isolated from humans with cutaneous, mucosal, or visceral leishmaniasis in BALB/c mice. *Am J Trop Med Hyg* (1996) **54**:178–84.
15. Silveira FT, Lainson R, Corbett CEP. Further observations on clinical, histopathological, and immunological features of borderline disseminated cutaneous leishmaniasis caused by *Leishmania* (*Leishmania*) *amazonensis*. *Mem Inst Oswaldo Cruz* (2005) **100**:525–34. doi:10.1590/S0074-02762005000500013
16. McMahon-Pratt D, Alexander J. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol Rev* (2004) **201**:206–24. doi:10.1111/j.0105-2896.2004.00190.x
17. Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. *Annu Rev Immunol* (1995) **13**:151–77. doi:10.1146/annurev.iy.13.040195.001055
18. Antonelli LRV, Dutra WO, Almeida RP, Bacellar O, Carvalho EM, Gollob KJ. Activated inflammatory T cells correlate with lesion size in human cutaneous leishmaniasis. *Immunol Lett* (2005) **101**:226–30. doi:10.1016/j.imlet.2005.06.004
19. Oliveira WN, Ribeiro LE, Schrieffer A, Machado P, Carvalho EM, Bacellar O. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of human tegumentary leishmaniasis. *Cytokine* (2014) **66**:127–32. doi:10.1016/j.cyto.2013.12.016
20. Akuffo H, Maasho K, Blostedt M, Hojeberg B, Britton S, Bakhiet M. *Leishmania aethiopica* derived from diffuse leishmaniasis patients preferentially induce mRNA for interleukin-10 while those from localized leishmaniasis patients induce interferon-gamma. *J Infect Dis* (1997) **175**:737–41. doi:10.1093/infdis/175.3.737
21. Gollob KJ, Viana AG, Dutra WO. Immunoregulation in human American leishmaniasis: balancing pathology and protection. *Parasite Immunol* (2014) **36**:367–76. doi:10.1111/pim.12100
22. Soong L, Henard CA, Melby PC. Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis. *Semin Immunopathol* (2012) **34**:735–51. doi:10.1007/s00281-012-0350-8
23. Jones DE, Buxbaum LU, Scott P. IL-4-independent inhibition of IL-12 responsiveness during *Leishmania amazonensis* infection. *J Immunol* (2000) **165**:364–72. doi:10.4049/jimmunol.165.1.364

24. Jones DE, Ackermann MR, Wille U, Hunter CA, Scott P. Early enhanced Th1 response after *Leishmania amazonensis* infection of C57BL/6 interleukin-10-deficient mice does not lead to resolution of infection. *Infect Immun* (2002) **70**:2151–8. doi:10.1128/IAI.70.4.2151-2158.2002
25. de Moura TR, Novais FO, Oliveira F, Clarencio J, Noronha A, Barral A, et al. Toward a novel experimental model of infection to study American cutaneous leishmaniasis caused by *Leishmania braziliensis*. *Infect Immun* (2005) **73**:5827–34. doi:10.1128/IAI.73.9.5827
26. Giudice A, Camada I, Leopoldo PTG, Pereira JMB, Riley LW, Wilson ME, et al. Resistance of *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis* to nitric oxide correlates with disease severity in tegumentary leishmaniasis. *BMC Infect Dis* (2007) **7**:7. doi:10.1186/1471-2334-7-7
27. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
28. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* (2004) **22**:745–63. doi:10.1146/annurev.immunol.22.012703.104702
29. Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. *Nat Med* (2004) **10**:1104–10. doi:10.1038/nm1108
30. Glennie ND, Yeramilli VA, Beiting DP, Volk SW, Weaver CT, Scott P. Skin-resident memory CD4+ T cells enhance protection against *Leishmania major* infection. *J Exp Med* (2015) **212**:1405–14. doi:10.1084/jem.20142101
31. Keshavarz Valian H, Nateghi Rostami M, Tasbihi M, Miramin Mohammadi A, Eskandari SE, Sarrafnejad A, et al. CCR7+ central and CCR7 effector memory CD4+ T cells in human cutaneous leishmaniasis. *J Clin Immunol* (2013) **33**:220–34. doi:10.1007/s10875-012-9788-7
32. Carvalho AM, Magalhães A, Carvalho LP, Bacellar O, Scott P, Carvalho EM. Immunologic response and memory T cells in subjects cured of tegumentary leishmaniasis. *BMC Infect Dis* (2013) **13**:529. doi:10.1186/1471-2334-13-529
33. Zajtchuk JT, Casler JD, Netto EM, Grogl M, Neafie RC, Hessel CR, et al. Mucosal leishmaniasis in Brazil. *Laryngoscope* (1989) **99**:925–39. doi:10.1288/00005537-198909000-00006
34. Netto EM, Marsden PD, Llanos-Cuentas EA, Costa JML, Cuba CC, Barreto AC, et al. Long-term follow-up of patients with *Leishmania (Viannia) braziliensis* infection and treated with Glucantime®. *Trans R Soc Trop Med Hyg* (1990) **84**:367–70. doi:10.1016/0035-9203(90)90321-5
35. Pereira-Carvalho R, Mendes-Aguiar CO, Oliveira-Neto MP, Covas CJF, Bertho ÁL, Da-Cruz AM, et al. *Leishmania braziliensis*-reactive T cells are down-regulated in long-term cured cutaneous leishmaniasis, but the renewal capacity of T effector memory compartments is preserved. *PLoS One* (2013) **8**:e81529. doi:10.1371/journal.pone.0081529
36. Wu C-Y, Kirman JR, Rotte MJ, Davey DF, Perfetto SP, Rhee EG, et al. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat Immunol* (2002) **3**:852–8. doi:10.1038/ni832
37. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* (2008) **8**:247–58. doi:10.1038/nri2355
38. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* (2007) **13**:843–50. doi:10.1038/nm1592
39. Macedo AB, Sánchez-Arcila JC, Schubach AO, Mendonça SC, Marins-Dos-Santos A, de Fatima Madeira M, et al. Multifunctional CD4 +T cells in patients with American cutaneous leishmaniasis. *Clin Exp Immunol* (2012) **167**:505–13. doi:10.1111/j.1365-2249.2011.04536.x
40. Vélez ID, Gilchrist K, Arbelaez MP, Rojas CA, Puerta JA, Antunes CMF, et al. Failure of a killed *Leishmania amazonensis* vaccine against American cutaneous leishmaniasis in Colombia. *Trans R Soc Trop Med Hyg* (2005) **99**:593–8. doi:10.1016/j.trstmh.2005.04.002
41. Armijos RX, Weigel MM, Calvopina M, Hidalgo A, Cevallos W, Correa J. Safety, immunogenecity, and efficacy of an autoclaved *Leishmania amazonensis* vaccine plus BCG adjuvant against New World cutaneous leishmaniasis. *Vaccine* (2004) **22**:1320–6. doi:10.1016/j.vaccine.2003.06.002
42. Marques-Da-Silva EA, Coelho EA, Gomes DC, Vilela MC, Masioli CZ, Tavares CA, et al. Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-γ production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. *Parasitol Res* (2005) **98**:67–74. doi:10.1007/s00436-005-0008-8
43. Matos I, Mizenina O, Lubkin A, Steinman RM, Idoyaga J. Targeting *Leishmania major* antigens to dendritic cells in vivo induces protective immunity. *PLoS One* (2013) **8**:e67453. doi:10.1371/journal.pone.0067453
44. Lakhal-Naouar I, Slike BM, Aronson NE, Marovich MA. The immunology of a healing response in cutaneous leishmaniasis treated with localized heat or systemic antimonial therapy. *PLoS Negl Trop Dis* (2015) **9**:e0004178. doi:10.1371/journal.pntd.0004178
45. Sánchez-Sampedro L, Gómez CE, Mejías-Pérez E, Sorzano CO, Esteban M. High quality long-term CD4 + and CD8 + effector memory populations stimulated by DNA-LACK/MVA-LACK regimen in *Leishmania major* BALB/C model of infection. *PLoS One* (2012) **7**:e38859. doi:10.1371/journal.pone.0038859
46. Guha R, Das S, Ghosh J, Naskar K, Mandala A, Sundar S, et al. Heterologous priming-boosting with DNA and vaccinia virus expressing kinetoplastid membrane protein-11 induces potent cellular immune response and confers protection against infection with antimony resistant and sensitive strains of *Leishmania (Leishmania) donovani*. *Vaccine* (2013) **31**:1905–15. doi:10.1016/j.vaccine.2013.02.025
47. Guha R, Gupta D, Rastogi R, Vikram R, Krishnamurthy G, Bimal S, et al. Vaccination with *Leishmania* hemoglobin receptor-encoding DNA protects against visceral leishmaniasis. *Sci Transl Med* (2013) **5**:202ra121. doi:10.1126/scitranslmed.3006406
48. Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. *J Immunol* (2013) **190**:2138–49. doi:10.4049/jimmunol.1202801
49. Hugentobler F, Yam KK, Gillard J, Mahbuba R, Olivier M, Cousineau B. Immunization against *Leishmania major* infection using LACK- and IL-12-expressing *Lactococcus lactis* induces delay in footpad swelling. *PLoS One* (2012) **7**:e30945. doi:10.1371/journal.pone.0030945
50. Darrah PA, Hegde ST, Patel DT, Lindsay RWB, Chen L, Roederer M, et al. IL-10 production differentially influences the magnitude, quality, and protective capacity of Th1 responses depending on the vaccine platform. *J Exp Med* (2010) **207**:1421–33. doi:10.1084/jem.20092532
51. Nico D, Gomes DC, Palatnik-de-Sousa I, Morrot A, Palatnik M, Palatnik-de-Sousa CB. *Leishmania donovani* nucleoside hydrolase terminal domains in cross-protective immunotherapy against *Leishmania amazonensis* murine infection. *Front Immunol* (2014) **5**:273. doi:10.3389/fimmu.2014.00273
52. Silveira FT, Lainson R, De Castro Gomes CM, Laurenti MD, Corbett CEP. Immunopathogenic competences of *Leishmania (V.) braziliensis* and *L. (L.) amazonensis* in American cutaneous leishmaniasis. *Parasite Immunol* (2009) **31**:423–31. doi:10.1111/j.1365-3024.2009.01116.x
53. Vitoriano-Souza J, Moreira Nd, Menezes-Souza D, Roatt BM, de Oliveira Aguiar-Soares RD, Siqueira-Mathias FA, et al. Dogs immunized with LBSap vaccine displayed high levels of IL-12 and IL-10 cytokines and CCL4, CCL5 and CXCL8 chemokines in the dermis. *Mol Immunol* (2013) **56**:540–8. doi:10.1016/j.molimm.2013.05.231
54. Resende LA, Roatt BM, Aguiar-Soares RD, Viana KF, Mendonça LZ, Lanna MF, et al. Cytokine and nitric oxide patterns in dogs immunized with LBSap vaccine, before and after experimental challenge with *Leishmania chagasi* plus saliva of *Lutzomyia longipalpis*. *Vet Parasitol* (2013) **198**:371–81. doi:10.1016/j.vetpar.2013.09.011
55. Aguiar-Soares R, Roatt B, Ker H, Moreira N, Mathias F, Cardoso J, et al. LBSapSal-vaccinated dogs exhibit increased circulating T-lymphocyte subsets (CD4+ and CD8+) as well as a reduction of parasitism after challenge with *Leishmania infantum* plus salivary gland of *Lutzomyia longipalpis*. *Parasit Vectors* (2014) **7**:61. doi:10.1186/1756-3305-7-61
56. Mayrink W, Cristina A, Botelho DC, Magalhães PA, Batista SM, Lima ADO, et al. Immunotherapy, immunochemotherapy and chemotherapy for American cutaneous leishmaniasis treatment. *Rev Soc Bras Med Trop* (2006) **39**:14–21.
57. Convit J, Ulrich M, Zerpa O, Borges R, Aranzazu N, Valera M, et al. Immunotherapy of American cutaneous leishmaniasis in Venezuela during the period 1990-99. *Trans R Soc Trop Med Hyg* (2003) **97**:469–72. doi:10.1016/S0035-9203(03)90093-9
58. Carneiro MB, de Andrade e Sousa LM, Vaz LG, Dos Santos LM, Vilela L, de Souza CC, et al. Short-term protection conferred by Leishvacin® against experimental *Leishmania amazonensis* infection in C57BL/6 mice. *Parasitol Int* (2014) **63**:826–34. doi:10.1016/j.parint.2014.07.010

59. Veras PST, Welby-Borges M, de Santana CD, Nihei J, Cardillo F, de Freitas LAR. *Leishmania amazonensis*: participation of regulatory T and B cells in the in vitro priming (PIV) of CBA/J spleen cells susceptible response. *Exp Parasitol* (2006) **113**:201–5. doi:10.1016/j.exppara.2006.01.008
60. Telino E, De Luca PM, Matos DC, Azeredo-Coutinho RB, Meirelles MN, Conceição-Silva F, et al. In vitro responses of human peripheral blood mononuclear cells to whole-cell, particulate and soluble extracts of *Leishmania* promastigotes. *Clin Exp Immunol* (2006) **143**:338–44. doi:10.1111/j.1365-2249.2006.02995.x
61. Afonso LC, Scott DF. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect Immun* (1993) **61**:2952–9. doi:10.1007/s00436-004-1193-6
62. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, et al. Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nat Genet* (2007) **39**:839–47. doi:10.1038/ng2053
63. Raymond F, Boisvert S, Roy G, Ritt J-F, Legare D, Isnard A, et al. Genome sequencing of the lizard parasite *Leishmania tarentolae* reveals loss of genes associated to the intracellular stage of human pathogenic species. *Nucleic Acids Res* (2012) **40**:1131–47. doi:10.1093/nar/gkr834
64. Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, Cotton JA, et al. Whole genome sequencing of multiple *Leishmania donovani* clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome Res* (2011) **21**:2143–56. doi:10.1101/gr.123430.111
65. Real F, Vidal RO, Carazzolle MF, Mondego JMC, Costa GGL, Herai RH, et al. The genome sequence of *Leishmania (Leishmania) amazonensis*: functional annotation and extended analysis of gene models. *DNA Res* (2013) **20**:567–81. doi:10.1093/dnares/dst031
66. Maioli TU, Takane E, Arantes RME, Fietto JLR, Afonso LCC. Immune response induced by New World *Leishmania* species in C57BL/6 mice. *Parasitol Res* (2004) **94**:207–12. doi:10.1007/s00436-004-1193-6
67. Yang Z, Mosser DM, Zhang X. Activation of the MAPK, ERK, following *Leishmania amazonensis* infection of macrophages. *J Immunol* (2007) **178**:1077–85. doi:10.4049/jimmunol.178.2.1077
68. Hernández-Ruiz J, Salaiza-Suazo N, Carrada G, Escoto S, Ruiz-Remigio A, Rosenstein Y, et al. CD8 cells of patients with diffuse cutaneous leishmaniasis display functional exhaustion: the latter is reversed, in vitro, by TLR2 agonists. *PLoS Negl Trop Dis* (2010) **4**:e871. doi:10.1371/journal.pntd.0000871
69. Figueiredo AB, Serafim TD, Marques-da-Silva EA, Meyer-Fernandes JR, Afonso LCC. *Leishmania amazonensis* impairs DC function by inhibiting CD40 expression via A2B adenosine receptor activation. *Eur J Immunol* (2012) **42**:1203–15. doi:10.1002/eji.201141926
70. Soong L. Subversion and utilization of host innate defense by *Leishmania amazonensis*. *Front Immunol* (2012) **3**:58. doi:10.3389/fimmu.2012.00058
71. Carlsen ED, Hay C, Henard C, Popov V, Garg NJ, Soong L. *Leishmania amazonensis* amastigotes trigger neutrophil activation but resist neutrophil microbicidal mechanisms. *Infect Immun* (2013) **81**:3966–74. doi:10.1128/IAI.00770-13
72. Henard CA, Carlsen ED, Hay C, Kima PE, Soong L. *Leishmania amazonensis* amastigotes highly express a tryparedoxin peroxidase isoform that increases parasite resistance to macrophage antimicrobial defenses and fosters parasite virulence. *PLoS Negl Trop Dis* (2014) **8**:e3000. doi:10.1371/journal.pntd.0003000
73. Lacerda DI, Cysne-Finkelstein L, Nunes MP, De-Luca PM, Genestra MDS, Leon LLP, et al. Kinetoplastid membrane protein-11 exacerbates infection with *Leishmania amazonensis* in murine macrophages. *Mem Inst Oswaldo Cruz* (2012) **107**:238–45. doi:10.1590/S0074-02762012000200014
74. Kedzierski L, Zhu Y, Handman E. *Leishmania* vaccines: progress and problems. *Parasitology* (2006) **133**:S87. doi:10.1017/S0031182006001831
75. Morris RV, Shoemaker CB, David JR, Lanzaro GC, Titus RG. Sandfly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *J Immunol* (2001) **167**:5226–30. doi:10.4049/jimmunol.167.9.5226
76. Mbow ML, Bleyenberg JA, Hall LR, Titus RG. *Phlebotomus papatasi* sand fly salivary gland lysate down-regulates a Th1, but up-regulates a Th2, response in mice infected with *Leishmania major*. *J Immunol* (1998) **161**:5571–7.
77. de Moura TR, Oliveira F, Rodrigues GC, Carneiro MW, Fukutani KF, Novais FO, et al. Immunity to *Lutzomyia intermedia* saliva modulates the inflammatory environment induced by *Leishmania braziliensis*. *PLoS Negl Trop Dis* (2010) **6**:e712. doi:10.1371/journal.pntd.0000712
78. Norsworthy NB, Sun J, Elnaiem D, Lanzaro G, Soong L. Sand fly saliva enhances *Leishmania amazonensis* infection by modulating interleukin-10 production. *Infect Immun* (2004) **72**:1240–7. doi:10.1128/IAI.72.3.1240-1247.2004
79. Weinkopff T, de Oliveira CI, de Carvalho AM, Hauyon-La Torre Y, Muniz AC, Miranda JC, et al. Repeated exposure to *Lutzomyia intermedia* sand fly saliva induces local expression of interferon-inducible genes both at the site of injection in mice and in human blood. *PLoS Negl Trop Dis* (2014) **8**:e2627. doi:10.1371/journal.pntd.0002627
80. Kamhawi S, Belkaid Y, Modi G, Rowton E, Sacks D. Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. *Science* (2000) **290**:1351–4. doi:10.1126/science.290.5495.1351
81. de Moura TR, Oliveira F, Carneiro MW, Miranda JC, Clarêncio J, Barral-Netto M, et al. Functional transcriptomics of wild-caught *Lutzomyia intermedia* salivary glands: identification of a protective salivary protein against *Leishmania braziliensis* infection. *PLoS Negl Trop Dis* (2013) **7**:e2242. doi:10.1371/journal.pntd.0002242
82. Teixeira C, Gomes R, Oliveira F, Meneses C, Gilmore DC, Elnaiem DE, et al. Characterization of the early inflammatory infiltrate at the feeding site of infected sand flies in mice protected from vector-transmitted *Leishmania major* by exposure to uninfected bites. *PLoS Negl Trop Dis* (2014) **8**:e2781. doi:10.1371/journal.pntd.0002781
83. Oliveira F, Rowton E, Aslan H, Gomes R, Castrovinci PA, Alvarenga PH, et al. A sand fly salivary protein vaccine shows efficacy against vector-transmitted cutaneous leishmaniasis in nonhuman primates. *Sci Transl Med* (2015) **7**:290ra90. doi:10.1126/scitranslmed.aaa3043
84. Carregaro V, Costa DL, Brodskyn C, Barral AM, Barral-Netto M, Cunha FQ, et al. Dual effect of *Lutzomyia longipalpis* saliva on *Leishmania braziliensis* infection is mediated by distinct saliva-induced cellular recruitment into BALB/c mice ear. *BMC Microbiol* (2013) **13**:102. doi:10.1186/1471-2180-13-102

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The Contribution of Immune Evasive Mechanisms to Parasite Persistence in Visceral Leishmaniasis

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Leishmania is a genus of protozoan parasites that give rise to a range of diseases called Leishmaniasis that affects annually an estimated 1.3 million people from 88 countries. *Leishmania donovani* and *Leishmania (L.) infantum chagasi* are responsible to cause the visceral leishmaniasis. The parasite can use assorted strategies to interfere with the host homeostasis to establish persistent infections that without treatment can be lethal. In this review, we highlight the mechanisms involved in the parasite subversion of the host protective immune response and how alterations of host tissue physiology and vascular remodeling during VL could affect the organ-specific immunity against *Leishmania* parasites.

Keywords: leishmaniasis, treatment, *Leishmania donovani*, host protective responses, immune evasive mechanisms

INTRODUCTION

Leishmaniasis is a complex of mammalian neglected tropical diseases, caused by over 20 different parasitic protozoans of genera *Leishmania*. Transmission can occur as zoonotically or anthroponotically, usually by the bite of female by ~30 different species phlebotomine sandflies (1). Three main manifestations can occur that include the cutaneous (CL), mucocutaneous affecting the skin and mucous membranes, and visceral leishmaniasis (VL) (1).

These diseases are endemics in 98 countries, and around 350 million people are at risk. The estimate of annual new cases is around two million (2). VL is a disease that is fatal if untreated; around 500,000 new cases are estimated and 50,000 deaths reported annually (3). The disease is caused by *Leishmania donovani* complex in East Africa and the Indian subcontinent and *Leishmania infantum* in Europe, North Africa, and Latin America (4).

Two different types of VL can occur, which differ in the way of transmission: the zoonotic VL that is transmitted from animal to vector to human and the anthtoponotic VL where transmission from human to vector to human. So, humans are an occasional host and animals, especially dogs, play the role of reservoir of the parasite. In areas of *L. infantum,* the zoonotic VL is found, while in areas of *L. donovani* transmission, anthroponotic VL is found (5).

Visceral leishmaniasis is also known as kala-azar and is characterized by irregular fever, anemia, hepatosplenomegaly, pancytopenia, weight loss, and hypergammaglobulinemia. It is widely confined to East Africa, Indian subcontinent, Brazil, and regions bordering the Mediterranean. Dermal

leishmanoid (PKDL) is a macular, maculo-papular, or nodular rash representing a complication of VL that is usually noted after treatment in Sudan and less often in other East African countries and in the Indian Subcontinent. This often affects immunosuppressed individuals in *L. infantum* endemic areas (6). These lesions can appear anywhere on the body, but most commonly occur on the face (7). The interval between treated VL and PKDL is 0–6 months in Sudan and between 6 months to 3 years in India. As the nodular lesions contain many parasites (8), and such cases are the putative reservoir for anthroponotic VL between epidemic cycles, this form of disease is more infectious (6).

For treatment of leishmaniasis few drugs are available at moment. They include: pentavalent antimonium [Sodium atibogluconate (Pentostam®)] and meglumine antimoniate (Glucantime®), pentamidine, amphotericin B, liposomal amphotericin B, miltefosine, and paramomycin (9, 10). These face limitations for actual treatment, in that most of them require hospitalization that increases the cost, and they are highly toxic (9).

The mechanism of resistance to pentavalent antimonials is the focus of much research; they have been the standard drugs despite their high toxicity (7). Those drugs are not in use now in Bihar State, India, because of the high rate of drug resistance, where more than 65% of previously untreated patients fail to respond or readily relapse. Sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) are still in use elsewhere. Administration is intravenous or intramuscular, and they show the some efficacy when used in equipollent doses (7).

Fatigue, body aches, electrocardiographic abnormalities, raised aminotransferase levels, and chemical pancreatitis are frequently reported secondary effects. Fatal pancreatitis has been reported in patients with VL and HIV infection (11). AmBisome, a liposome formulation of amphotericin B, is the current standard treatment for VL, particularly against *L. donovani* in Bihar, and just one dose treatment was efficient in the treatment in rural public hospitals in Bangladesh (2). The effectiveness of treatment was less against *L. donovani* in East Africa and *L. infantum* in Latin America. The situation was not different when the treatment was with paromomycin that was efficient in the Indian subcontinent but did not work in East Africa (7).

The mechanisms of parasite evasion in VL are not only caused by down modulation of host protective immune response directly. Several reports showed that tissue physiological and vascular remodeling alterations caused by the disease also contribute to parasite replication and persistence. In this review, we discuss: (1) how the parasite subvert the host immune system by infecting specific keys cells and (2) how changes in the tissue structure and physiology could affect organ-specific immunity during VL.

Following the deposition of infective metacyclic promastigotes into the dermis, the skin innate immune system detects invading promastigotes, recruits inflammatory cells to sites of invasion within minutes, and promotes the induction of adaptive immunity (12). Initial sensing of the parasite involves pattern recognition receptors. The host skin immune system initially senses the parasite through pattern recognition receptors and complement receptors present on different cell types including neutrophils, macrophages, dendritic cells (DCs), and natural killer (NK) cells. Several Toll-like receptors (TLRs) such as TLR2, TLR3 (13), TLR4 (14), TLR7 (15), and TLR9 (14) have been shown to contribute to innate sensing and recognition of *Leishmania* by various innate immune cells. This recognition leads to activation of intracellular signaling pathways that are necessary for the initiation of inflammatory responses and control of parasite proliferation by the innate immune response (16).

Neutrophils are essential cells involved in inflammatory response and contribute to phagocytosis and killing of microbial pathogens. However, the precise role of these cells in VL remains to be addressed. McFarlane et al. (17) demonstrated that neutrophil depletion at the beginning of *L. donovani* infection leads to increase in parasite burden in the spleen and bone marrow but not in the liver, enhanced splenomegaly, a delay in the maturation of hepatic granulomas, a decrease in inducible nitric oxide synthase (iNOS) expression within granulomas, and increased levels of IL-4 and IL-10 with significant increase in the ratio of *L. donovani*-specific serum IgG1/IgG2a levels (17).

Although promastigotes are capable of directly invading DCs and macrophages following their deposition by infected sandflies, several TLRs have been shown to contribute to this process and play a vital role in the production of proinflammatory cytokines that are critical for immunity (18). Also activation of inflammasome and production of IL-1β are important for restriction *in vivo* infection with *L. infantum* in murine model (19). Polymorphisms within the human *IL1B* gene are associated with clinical severity of the disease (20).

Experimental studies in mice suggest that the control of VL may be associated with the development of parasite-specific, cell-mediated immune responses involving both $CD4^+$ and $CD8^+$ T cells (21). These cells produce IFN-γ, which activates infected macrophages, leading to the production of NO and other free radicals that kill the parasites. DCs activate $CD8^+$ T cells through mechanisms that involve antigen cross presentation (22). Also, IL-17 producing γδ T cells suppress early control of parasite growth in the liver, and inflammatory monocytes were an important target for the suppressive effects of IL-17 (23).

In VL, both $CD4^+$ and $CD8^+$ T cells have been implicated in the resistance and healing capacity against *L. donovani*. The production of IFN-γ by helper $CD4^+$ T cells and/or $CD8^+$ lymphocytes is associated with protection (24). The Th1 and Th17 profile are correlated with infection resolution (25–28) and Th2 response contribute to susceptibility and disease progression (29). High levels of IL-10 are another regulatory cytokine involved immune suppression inducing parasite persistence and chronicity of disease (29). In humans, IL-27 promoted the production of IL-10 and inhibited secretion of IL-17 by $CD4^+$ T cells (30). Recently, Ansari et al. (31) showed elevated circulating levels of IL-27 and elevated expression of IL-27p28 and EBI-3 transcripts in VL patients. Owens et al. (32) demonstrated that $CD11c^{hi}$ DCs promote expansion and maintenance of T cells inducing the production of IL-10 and IL-27 *in vivo*.

In infected individuals with active symptoms of VL was observed high levels of IFN-γ and IL-10, the main source of IFN-γ production found in both innate and cellular responses. On the other hand, IL-10 was restricted to $CD8^+$ T and B cells (33). In splenic aspirate cells from VL patients, anti-IL-10 antibodies promoted killing of parasite and increased the secretion of IFN-γ

and TNF-α in splenic cells *ex vivo* (34). Recently demonstrated in healed visceral human leishmaniasis patients, $CD8^+$ T cells were activated and the granzyme B levels were found increased when compared to naive group and active VL (35).

Suppression of T cell response is thought to be involved in the pathogenesis of VL. Regulatory T cell (Treg)-mediated immune suppression is reported in animal models of *Leishmania* infection. IL-10 receptor blockade mice were resistant to *L. donovani* infection (36). Also, low levels expression of CD40 in DC induced severity to infection by activation of Treg and the production of IL-10 (37). In immunocompromised *aly/aly* mice infected with *L. donovani* $CD4^+$ $Foxp3^+$ Treg cells were increased in the liver inducing progression of granuloma formation (38). Majumder et al. (14) showed that mice vaccinated with soluble leishmanial antigen (SLA)-pulsed CpG-ODN-stimulated dendritic cells (SLA-CpG-DCs) decreased the number of Treg cells; and consequently, there was low production of TGF-β. Interestingly, IL-$17^{-/-}$ mice infected with *L. infantum* failed to control parasitemia, increasing the proliferation of Treg cells and production of IL-10 (38). In humans, Treg cells produced high levels of IL-10 indicating immune suppression among VL patients (39). This mechanism will be useful to determine drug treatment and disease prognostic.

Studies investigating the immunoregulatory function, CTLA-4 (CD152 – cytotoxic T lymphocyte antigen-4) has a role regulatory in activation of T cells, including Treg cells (40, 41), and PD-1 (programmed cell death-1) is broadly expressed on activated T cells, regulatory T cells, and other hematopoietic cells (42). Administration of monoclonal antibodies against CTLA-4 reduced the burden of parasite in the liver in VL and increased the frequency of IFN-γ and IL-4 producing T cells in the liver (42). Blockade of the PD-1 during *L. infantum* in dogs, $CD8^+$ and $CD4^+$ T cells recovered functionality and increased reactive oxygen species production of phagocytes (43). Identification of the mechanism of blocking CTLA-4 or PD-1 reverts the downregulation of T cell response to infection. Ligand for the inhibitory receptor PD-1 (B7-H1) constitutively expressed in T cells showed interaction between B7-1: CTLA-4 and the PD-L1 (B7-H1): PD-1 pathways (44). The blockade of B7-H1, the ligand for the inhibitory receptor PD-1, was found to increase survival of $CD8^+$ T cells and induce protective immunity (45). Recently, HIV-1-coinfected patients with VL Treg cells expressed high levels of CTLA-4, showing impaired immunologic profile explaining persistence and/or relapse of the disease (46).

LIVER, BONE MARROW, AND SPLEEN: THREE ORGANS, DIFFERENT IMMUNE RESPONSES

One of the hallmarks of VL is hepatosplenomegaly (1, 21, 22, 47). There is a fine line between immune responses that effectively control parasite growth and induce long-term immunity and those that allow parasite persistence and associated disease (29). Thus, differences in splenic and hepatic tissue microenvironments dictate differences in the ability to generate effective immune responses and parasite control in these organs.

The liver is one of the primary target organs in VL. In experimental models of VL, infection in the liver is self-resolving within 2–3 months (22). This resolution of disease is associated with the development of granuloma formation mediated by a Th1 immune response both in humans and dogs as well (48–50). The development of inflammatory granulomas around infected liver macrophages leading to immunity is a T-cell-driven event. This Th1-dominated response is mediated by TLR7, TLR8, TLR9, IL-1, and IL-18 *via* the MyD88 signaling pathway (15). An efficient granuloma formation involves the expression of inducible iNOS by macrophages (22, 51), which is regulated by several pro-inflammatory (Th1) cytokines, such as IL-12, IFN-γ, TNF-α, lymphotoxin, granulocyte/macrophage colony-stimulating factor, IL-2 (52, 53) as well as intact and functional NK and NKT cells (54–56).

Leishmania parasites have developed strategies to evade the host immune defenses: invasion of cell types to modulate cell host function to replicate and to downregulate the host immunity for its persistence (24). In a murine model of *L. donovani* infection, liver-resident macrophages (Kupffer cells) infected have a different trancriptomic network profile compared to uninfected Kupffer cells isolated from the same mouse (57). Retinoid X receptor alpha (RXRα) was identified as a key hub within this network, and its pharmacological pertubation with agonists of RXRα enhanced the innate resistance of Kupffer cells to *Leishmania* infection *in vivo* (57). Also Hepatic stellate cells infected *in vitro* and *in vivo* with *L. donovani* produces immunoregulatory cytokines that induces $CD4^+$ T cells to become Treg that leads to parasite persitence (58).

Although initially unaffected (due to efficient local immune response), the liver is slowly damaged as the disease progresses (59). Consequently, VL leads to hepatic dysfunction, such as coagulation defects, increased serum concentrations of several liver-specific enzymes, and changes in the cholesterol biosynthesis (60, 61). The liver is the main source of cholesterol biosynthesis in mammals (62) and the decreased serum cholesterol was associated with VL severity and parasite persitence (63, 64). Ghosh et al. (65) identify that the *L. donovani* infection downregulates miR-122 in hepatic tissue, lowering serum cholesterol and increasing parasite burden. The pathology is reversed when hepatic levels of miR-122 are restored with increased serum cholesterol and reduction of liver parasite burden.

In VL, the spleen also becomes chronically infected by mechanisms that are less well understood. In EVL, the spleen becomes enlarged and splenomegaly can account for up to 15% of the body weight of infected mice in as little as 6–8 weeks postinfection (22). The persistence of parasites in the spleen is associated with changes in the splenic lymphoid microenvironment, and concomitant increases in the rate of T-cell apoptosis, decreased responsiveness to leishmanial antigens, and drug resistance (21, 22, 66–68).

The spleen is composed by red (RP) and white pulp (WP), separated by an interface called the marginal zone (MZ). The splenic RP contains macrophages that recycle iron blood from aging red blood cells. The WP is organized similarly to a lymph node, containing T-cell and B-cell follicles. It is in the WP where antigen-specific immune responses are generated (69).

During VL, there is an intense vascular remodeling in the RP and WP (68, 70–72). This vascular change causes disruption to both the gp38$^+$ fibroblastic reticular cell network, which guides T cell and DC migration to the T cell zone, and the follicular DC network in the B cell follicles (73, 74). As a consequence, DCs fails to migrate to T cell zone, resulting in an diminishes priming of T cells (73). Dalton et al. (75) showed that by using a receptor tyrosine kinase inhibitor, sunitinib maleate (Sm), vascular remodeling and splenomegaly associated with VL can be blocked, and the pathology can be reversed. The use of Sm alone did not cause a reduction in parasite burden in the spleen; but when combined with conventional antimonial drugs, enhanced leishmanicidal activity with enhanced immune response mediated by CD4$^+$ T cells producing IFN-γ and TNF (75).

Bone marrow is also affected during the chronic phase of VL in both patients and experimental models (76, 77). In patients, bone marrow shows moderate to severe megaloblastosis, megakaryocitic hyperplasia, and increased number of plasma cells. All parameters were correlated to parasite load (78). Calvo et al. (79) identified that splenic sequestration and ineffective hematopoiesis appear to be the main etiopathogenetic factors in the bone marrow changes and peripheral cytopenias. This is also observed in experimental models. Lafuse et al. (80) identified increased BFU-E and CFU-E progenitor populations in the spleen and bone marrow and differentially altered erythroid gene expression in these organs. In murine model, there is a correlation in the hematopoietic activity with parasite load in the bone marrow (81). Stromal macrophages are the main target for *L. donovani* infection *in vivo* and *in vitro*; and as a consequence of the selective induction of GM-CSF and TNF-α production, infected stromal macrophages preferentially support increased levels of myelopoiesis (82). Also, Singal and Singh (83) demonstrated that *L. donovani* amastigotes antigen could also induce both *in vitro* and *in vivo* myelopoiesis. If this preferential increase of myelopoeisis may merely serve to increase the number of phagocytes, which are the host cells targets for parasite replication, as well as for increasing the phagocytic uptake of the parasite, further studies are needed to elucidate this question.

CONCLUSION AND PERSPECTIVES

Despite the global public health importance of leishmaniasis, progress in developing vaccines against the disease has lagged because of some key technical hurdles, including the fact that the disease occurs mostly in the world's poorest countries, and the absence of financial incentives to pharmaceutical companies. Chemotherapy for VL has changed little in 50 years; in areas where drug resistance has yet occurred. The conventional drug treatment still involves parenteral administration of antimonial compounds (Pentostam and Glucantime). Amphotericin B, particularly in liposomal formulation (84), has become the drug of choice in developed countries and where antimony resistance is problematic; but issues of cost and toxicity remain. Also, there are already clinical cases of treatment failure related to liposomal amphotericin B (67, 85). The onset of immunosuppression is a critical event during the progression of VL in a susceptible population. A more comprehensive study would be very helpful for a better understanding about how morpho-physiological tissue alterations and pathogen factors would affect organ-specific immunity during VL. Recently, the use of Systems Biology has been increased (86). Different *in silico* approaches are available for identification of interactions between pathogens and hosts and factors for parasite dissemination and disease progression, as well as to the selection of promising antigens as vaccine candidates, since experimental methods are difficult and time consuming (87, 88). A new approach to develop treatment strategy against VL in resistance cases has to take into account not only by the development of new leishmanicidal drugs but also by the drugs that could reverse the anergic immune response and pathophysiological changes during VL, such as hypocholesterolemia and splenic neovascularization. The use of an anti-vascular therapy (with Sm, for instance) could be an alternative choice to splenectomy in cases of failure treatment for lipossomal Amphotericin B (67, 85).

AUTHOR CONTRIBUTIONS

EOF, FML and DFF wrote the paper. DFF and AM made substantial contributions to the conception of the work. CGF-L revised the manuscript.

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REFERENCES

1. Pearson RD, Sousa AQ. Clinical spectrum of Leishmaniasis. *Clin Infect Dis* (1996) **22**(1):1–13. doi:10.1093/clinids/22.1.1
2. WHO. *Leishmaniasis*. (2015). Available from: http://www.who.int/leishmaniasis/en/
3. Desjeux P. The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg* (2001) **95**(3):239–43. doi:10.1016/S0035-9203(01)90223-8
4. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Natl Acad Sci U S A* (2007) **104**(22):9375–80. doi:10.1073/pnas.0703678104
5. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) **7**(5):e35671. doi:10.1371/journal.pone.0035671
6. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* (2007) **5**(11):873–82. doi:10.1038/nrmicro1748
7. Barrett MP, Croft SL. Management of trypanosomiasis and leishmaniasis. *Br Med Bull* (2012) **104**:175–96. doi:10.1093/bmb/lds031
8. Addy M, Nandy A. Ten years of kala-azar in west Bengal, Part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? *Bull World Health Organ* (1992) **70**(3):341–6.
9. Maltezou HC. Drug resistance in visceral leishmaniasis. *J Biomed Biotechnol* (2010) **2010**:617521. doi:10.1155/2010/617521

10. Sundar S, Chakravarty J. Leishmaniasis: an update of current pharmacotherapy. *Expert Opin Pharmacother* (2013) **14**(1):53–63. doi:10.1517/14656566.2013.755515
11. Santos J, Rivero A, Marquez M. [Acute pancreatitis with a fatal evolution due to antimonials in patients with visceral leishmaniasis and HIV infection]. *An Med Interna* (2000) **17**(10):562–3.
12. Liu D, Uzonna JE. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* (2012) **2**:83. doi:10.3389/fcimb.2012.00083
13. Flandin JF, Chano F, Descoteaux A. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. *Eur J Immunol* (2006) **36**(2):411–20. doi:10.1002/eji.200535079
14. Majumder S, Bhattacharjee A, Paul Chowdhury B, Bhattacharyya Majumdar S, Majumdar S. Antigen-pulsed CpG-ODN-activated dendritic cells induce host-protective immune response by regulating the T regulatory cell functioning in *Leishmania donovani*-infected mice: critical role of CXCL10. *Front Immunol* (2014) **5**:261. doi:10.3389/fimmu.2014.00261
15. Paun A, Bankoti R, Joshi T, Pitha PM, Stager S. Critical role of IRF-5 in the development of T helper 1 responses to *Leishmania donovani* infection. *PLoS Pathog* (2011) **7**(1):e1001246. doi:10.1371/journal.ppat.1001246
16. Faria MS, Reis FC, Lima AP. Toll-like receptors in *Leishmania* infections: guardians or promoters? *J Parasitol Res* (2012) **2012**:930257. doi:10.1155/2012/930257
17. McFarlane E, Perez C, Charmoy M, Allenbach C, Carter KC, Alexander J, et al. Neutrophils contribute to development of a protective immune response during onset of infection with *Leishmania donovani*. *Infect Immun* (2008) **76**(2):532–41. doi:10.1128/IAI.01388-07
18. Srivastava A, Singh N, Mishra M, Kumar V, Gour JK, Bajpai S, et al. Identification of TLR inducing Th1-responsive *Leishmania donovani* amastigote-specific antigens. *Mol Cell Biochem* (2012) **359**(1–2):359–68. doi:10.1007/s11010-011-1029-5
19. Lima-Junior DS, Costa DL, Carregaro V, Cunha LD, Silva AL, Mineo TW, et al. Inflammasome-derived IL-1beta production induces nitric oxide-mediated resistance to *Leishmania*. *Nat Med* (2013) **19**(7):909–15. doi:10.1038/nm.3221
20. Moravej A, Rasouli M, Kalani M, Asaei S, Kiany S, Najafipour S, et al. IL-1beta (-511T/C) gene polymorphism not IL-1beta (+3953T/C) and LT-alpha (+252A/G) gene variants confers susceptibility to visceral leishmaniasis. *Mol Biol Rep* (2012) **39**(6):6907–14. doi:10.1007/s11033-012-1517-z
21. Goto H, Prianti M. Immunoactivation and immunopathogeny during active visceral leishmaniasis. *Rev Inst Med Trop Sao Paulo* (2009) **51**(5):241–6. doi:10.1590/S0036-46652009000500002
22. Kaye PM, Svensson M, Ato M, Maroof A, Polley R, Stager S, et al. The immunopathology of experimental visceral leishmaniasis. *Immunol Rev* (2004) **201**:239–53. doi:10.1111/j.0105-2896.2004.00188.x
23. Sheel M, Beattie L, Frame TC, de Labastida Rivera F, Faleiro RJ, Bunn PT, et al. IL-17A-producing gammadelta T cells suppress early control of parasite growth by monocytes in the liver. *J Immunol* (2015) **195**(12):5707–17. doi:10.4049/jimmunol.1501046
24. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* (2011) **9**(8):604–15. doi:10.1038/nrmicro2608
25. Reiner SL, Locksley RM. Cytokines in the differentiation of Th1/Th2 CD4+ subsets in leishmaniasis. *J Cell Biochem* (1993) **53**(4):323–8. doi:10.1002/jcb.240530409
26. Pitta MG, Romano A, Cabantous S, Henri S, Hammad A, Kouriba B, et al. IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*. *J Clin Invest* (2009) **119**(8):2379–87. doi:10.1172/JCI38813
27. Sacramento LA, Cunha FQ, de Almeida RP, da Silva JS, Carregaro V. Protective role of 5-lipoxigenase during *Leishmania infantum* infection is associated with Th17 subset. *Biomed Res Int* (2014) **2014**:264270. doi:10.1155/2014/264270
28. Ghosh K, Sharma G, Saha A, Kar S, Das PK, Ukil A. Successful therapy of visceral leishmaniasis with curdlan involves T-helper 17 cytokines. *J Infect Dis* (2013) **207**(6):1016–25. doi:10.1093/infdis/jis771
29. Stanley AC, Engwerda CR. Balancing immunity and pathology in visceral leishmaniasis. *Immunol Cell Biol* (2007) **85**(2):138–47. doi:10.1038/sj.icb7100011
30. Murugaiyan G, Mittal A, Lopez-Diego R, Maier LM, Anderson DE, Weiner HL. IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *J Immunol* (2009) **183**(4):2435–43. doi:10.4049/jimmunol.0900568
31. Ansari NA, Kumar R, Gautam S, Nylen S, Singh OP, Sundar S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. *J Immunol* (2011) **186**(7):3977–85. doi:10.4049/jimmunol.1003588
32. Owens BM, Beattie L, Moore JW, Brown N, Mann JL, Dalton JE, et al. IL-10-producing Th1 cells and disease progression are regulated by distinct CD11c(+) cell populations during visceral leishmaniasis. *PLoS Pathog* (2012) **8**(7):e1002827. doi:10.1371/journal.ppat.1002827
33. Peruhype-Magalhaes V, Martins-Filho OA, Prata A, Silva Lde A, Rabello A, Teixeira-Carvalho A, et al. Mixed inflammatory/regulatory cytokine profile marked by simultaneous raise of interferon-gamma and interleukin-10 and low frequency of tumour necrosis factor-alpha(+) monocytes are hallmarks of active human visceral Leishmaniasis due to *Leishmania chagasi* infection. *Clin Exp Immunol* (2006) **146**(1):124–32. doi:10.1111/j.1365-2249.2006.03171.x
34. Gautam S, Kumar R, Maurya R, Nylen S, Ansari N, Rai M, et al. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. *J Infect Dis* (2011) **204**(7):1134–7. doi:10.1093/infdis/jir461
35. Kaushal H, Bras-Goncalves R, Negi NS, Lemesre JL, Papierok G, Salotra P. Role of CD8(+) T cells in protection against *Leishmania donovani* infection in healed Visceral Leishmaniasis individuals. *BMC Infect Dis* (2014) **14**:653. doi:10.1186/s12879-014-0653-6
36. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, et al. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. *Infect Immun* (2002) **70**(11):6284–93. doi:10.1128/IAI.70.11.6284-6293.2002
37. Martin S, Agarwal R, Murugaiyan G, Saha B. CD40 expression levels modulate regulatory T cells in *Leishmania donovani* infection. *J Immunol* (2010) **185**(1):551–9. doi:10.4049/jimmunol.0902206
38. Tiwananthagorn S, Iwabuchi K, Ato M, Sakurai T, Kato H, Katakura K. Involvement of CD4(+) Foxp3(+) regulatory T cells in persistence of *Leishmania donovani* in the liver of alymphoplastic aly/aly mice. *PLoS Negl Trop Dis* (2012) **6**(8):e1798. doi:10.1371/journal.pntd.0001798
39. Rai AK, Thakur CP, Singh A, Seth T, Srivastava SK, Singh P, et al. Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis. *PLoS One* (2012) **7**(2):e31551. doi:10.1371/journal.pone.0031551
40. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol* (2004) **34**(11):2996–3005. doi:10.1002/eji.200425143
41. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* (2005) **6**(2):152–62. doi:10.1038/ni1160
42. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* (2008) **26**:677–704. doi:10.1146/annurev.immunol.26.021607.090331
43. Esch KJ, Juelsgaard R, Martinez PA, Jones DE, Petersen CA. Programmed death 1-mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. *J Immunol* (2013) **191**(11):5542–50. doi:10.4049/jimmunol.1301810
44. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* (2007) **27**(1):111–22. doi:10.1016/j.immuni.2007.05.016
45. Joshi T, Rodriguez S, Perovic V, Cockburn IA, Stager S. B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against *Leishmania donovani* infections. *PLoS Pathog* (2009) **5**(5):e1000431. doi:10.1371/journal.ppat.1000431
46. Vallejo A, Abad-Fernandez M, Moreno S, Moreno A, Perez-Elias MJ, Dronda F, et al. High levels of CD4(+) CTLA-4(+) Treg cells and CCR5 density in

HIV-1-infected patients with visceral leishmaniasis. *Eur J Clin Microbiol Infect Dis* (2015) **34**(2):267–75. doi:10.1007/s10096-014-2229-1
47. Melby PC, Tabares A, Restrepo BI, Cardona AE, McGuff HS, Teale JM. *Leishmania donovani*: evolution and architecture of the splenic cellular immune response related to control of infection. *Exp Parasitol* (2001) **99**(1):17–25. doi:10.1006/expr.2001.4640
48. Murray HW. Tissue granuloma structure-function in experimental visceral leishmaniasis. *Int J Exp Pathol* (2001) **82**(5):249–67. doi:10.1046/j.1365-2613.2001.00199.x
49. Melo FA, Moura EP, Ribeiro RR, Alves CF, Caliari MV, Tafuri WL, et al. Hepatic extracellular matrix alterations in dogs naturally infected with *Leishmania (Leishmania) chagasi*. *Int J Exp Pathol* (2009) **90**(5):538–48. doi:10.1111/j.1365-2613.2009.00681.x
50. Sanchez MA, Diaz NL, Zerpa O, Negron E, Convit J, Tapia FJ. Organ-specific immunity in canine visceral leishmaniasis: analysis of symptomatic and asymptomatic dogs naturally infected with *Leishmania chagasi*. *Am J Trop Med Hyg* (2004) **70**(6):618–24.
51. Beattie L, Peltan A, Maroof A, Kirby A, Brown N, Coles M, et al. Dynamic imaging of experimental *Leishmania donovani*-induced hepatic granulomas detects Kupffer cell-restricted antigen presentation to antigen-specific CD8 T cells. *PLoS Pathog* (2010) **6**(3):e1000805. doi:10.1371/journal.ppat.1000805
52. Costa AS, Costa GC, Aquino DM, Mendonca VR, Barral A, Barral-Netto M, et al. Cytokines and visceral leishmaniasis: a comparison of plasma cytokine profiles between the clinical forms of visceral leishmaniasis. *Mem Inst Oswaldo Cruz* (2012) **107**(6):735–9. doi:10.1590/S0074-02762012000600005
53. Beattie L, Phillips R, Brown N, Owens BM, Chauhan N, Dalton JE, et al. Interferon regulatory factor 7 contributes to the control of *Leishmania donovani* in the mouse liver. *Infect Immun* (2011) **79**(3):1057–66. doi:10.1128/IAI.00633-10
54. Stanley AC, Zhou Y, Amante FH, Randall LM, Haque A, Pellicci DG, et al. Activation of invariant NKT cells exacerbates experimental visceral leishmaniasis. *PLoS Pathog* (2008) **4**(2):e1000028. doi:10.1371/journal.ppat.1000028
55. Beattie L, Svensson M, Bune A, Brown N, Maroof A, Zubairi S, et al. *Leishmania donovani*-induced expression of signal regulatory protein alpha on Kupffer cells enhances hepatic invariant NKT-cell activation. *Eur J Immunol* (2010) **40**(1):117–23. doi:10.1002/eji.200939863
56. Maroof A, Beattie L, Zubairi S, Svensson M, Stager S, Kaye PM. Posttranscriptional regulation of Il10 gene expression allows natural killer cells to express immunoregulatory function. *Immunity* (2008) **29**(2):295–305. doi:10.1016/j.immuni.2008.06.012
57. Beattie L, d'El-Rei Hermida M, Moore JW, Maroof A, Brown N, Lagos D, et al. A transcriptomic network identified in uninfected macrophages responding to inflammation controls intracellular pathogen survival. *Cell Host Microbe* (2013) **14**(3):357–68. doi:10.1016/j.chom.2013.08.004
58. Khadem F, Gao X, Mou Z, Jia P, Movassagh H, Onyilagha C, et al. Hepatic stellate cells regulate liver immunity to visceral leishmaniasis through P110delta-dependent induction and expansion of regulatory T cells in mice. *Hepatology* (2015) **63:** 620–32. doi:10.1002/hep.28130
59. Al-Saffar NR, Al-Mudhaffer SA. Lactate dehydrogenase and other enzymes in kala-azar patients in Iraq. *Indian J Med Res* (1979) **70**:598–608.
60. Tonin AA, Calado AM, Bottari NB, Dalenogare D, Thome GR, Duarte T, et al. Novel markers of inflammatory response and hepatic dysfunction in canine leishmaniasis. *Comp Immunol Microbiol Infect Dis* (2015) **44**: 61–4. doi:10.1016/j.cimid.2015.09.004
61. el Hag IA, Hashim FA, el Toum IA, Homeida M, el Kalifa M, el Hassan AM. Liver morphology and function in visceral leishmaniasis (Kala-azar). *J Clin Pathol* (1994) **47**(6):547–51. doi:10.1136/jcp.47.6.547
62. Tennent DM, Zanetti ME, Atkinson DI, Kuron GW, Opdyke DF. The endogenous origin of blood cholesterol. *J Biol Chem* (1957) **228**(1):241–5.
63. Ghosh J, Lal CS, Pandey K, Das VN, Das P, Roychoudhury K, et al. Human visceral leishmaniasis: decrease in serum cholesterol as a function of splenic parasite load. *Ann Trop Med Parasitol* (2011) **105**(3):267–71. doi:10.1179/136485911X12899838683566
64. Carvalho MD, Alonso DP, Vendrame CM, Costa DL, Costa CH, Werneck GL, et al. Lipoprotein lipase and PPAR alpha gene polymorphisms, increased very-low-density lipoprotein levels, and decreased high-density lipoprotein levels as risk markers for the development of visceral leishmaniasis by *Leishmania infantum*. *Mediators Inflamm* (2014) **2014**:230129. doi:10.1155/2014/230129
65. Ghosh J, Bose M, Roy S, Bhattacharyya SN. *Leishmania donovani* targets Dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver infection. *Cell Host Microbe* (2013) **13**(3):277–88. doi:10.1016/j.chom.2013.02.005
66. Cavalcanti AS, Ribeiro-Alves M, Pereira Lde O, Mestre GL, Ferreira AB, Morgado FN, et al. Parasite load induces progressive spleen architecture breakage and impairs cytokine mRNA expression in *Leishmania infantum*-naturally infected dogs. *PLoS One* (2015) **10**(4):e0123009. doi:10.1371/journal.pone.0123009
67. Dos-Santos WL, Pagliari C, Santos LG, Almeida VA, e Silva TL, Coutinho Jde J Jr, et al. A case of conventional treatment failure in visceral leishmaniasis: leukocyte distribution and cytokine expression in splenic compartments. *BMC Infect Dis* (2014) **14**:491. doi:10.1186/1471-2334-14-491
68. Santana CC, Vassallo J, de Freitas LA, Oliveira GG, Pontes-de-Carvalho LC, dos-Santos WL. Inflammation and structural changes of splenic lymphoid tissue in visceral leishmaniasis: a study on naturally infected dogs. *Parasite Immunol* (2008) **30**(10):515–24. doi:10.1111/j.1365-3024.2008.01051.x
69. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. *Immunity* (2013) **39**(5):806–18. doi:10.1016/j.immuni.2013.10.010
70. Yurdakul P, Dalton J, Beattie L, Brown N, Erguven S, Maroof A, et al. Compartment-specific remodeling of splenic micro-architecture during experimental visceral leishmaniasis. *Am J Pathol* (2011) **179**(1):23–9. doi:10.1016/j.ajpath.2011.03.009
71. Dalton JE, Glover AC, Hoodless L, Lim EK, Beattie L, Kirby A, et al. The neurotrophic receptor Ntrk2 directs lymphoid tissue neovascularization during *Leishmania donovani* infection. *PLoS Pathog* (2015) **11**(2):e1004681. doi:10.1371/journal.ppat.1004681
72. Dalton JE, Kaye PM. Immunomodulators: use in combined therapy against leishmaniasis. *Expert Rev Anti Infect Ther* (2010) **8**(7):739–42. doi:10.1586/eri.10.64
73. Ato M, Stager S, Engwerda CR, Kaye PM. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat Immunol* (2002) **3**(12):1185–91. doi:10.1038/ni861
74. Smelt SC, Engwerda CR, McCrossen M, Kaye PM. Destruction of follicular dendritic cells during chronic visceral leishmaniasis. *J Immunol* (1997) **158**(8):3813–21.
75. Dalton JE, Maroof A, Owens BM, Narang P, Johnson K, Brown N, et al. Inhibition of receptor tyrosine kinases restores immunocompetence and improves immune-dependent chemotherapy against experimental leishmaniasis in mice. *J Clin Invest* (2010) **120**(4):1204–16. doi:10.1172/JCI41281
76. Varma N, Naseem S. Hematologic changes in visceral leishmaniasis/kala azar. *Indian J Hematol Blood Transfus* (2010) **26**(3):78–82. doi:10.1007/s12288-010-0027-1
77. Faleiro RJ, Kumar R, Hafner LM, Engwerda CR. Immune regulation during chronic visceral leishmaniasis. *PLoS Negl Trop Dis* (2014) **8**(7):e2914. doi:10.1371/journal.pntd.0002914
78. Marwaha N, Sarode R, Gupta RK, Garewal G, Dash S. Clinico-hematological characteristics in patients with kala azar. A study from north-west India. *Trop Geogr Med* (1991) **43**(4):357–62.
79. Calvo JM, Hernandez JM, Palencia J, Sierra E. [Visceral leishmaniasis presenting with peripheral leukocytosis and lymphocytosis]. *Sangre (Barc)* (1994) **39**(1):57–8.
80. Lafuse WP, Story R, Mahylis J, Gupta G, Varikuti S, Steinkamp H, et al. *Leishmania donovani* infection induces anemia in hamsters by differentially altering erythropoiesis in bone marrow and spleen. *PLoS One* (2013) **8**(3):e59509. doi:10.1371/journal.pone.0059509
81. Cotterell SE, Engwerda CR, Kaye PM. Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with *Leishmania donovani*. *Infect Immun* (2000) **68**(4):1840–8. doi:10.1128/IAI.68.4.1840-1848.2000
82. Cotterell SE, Engwerda CR, Kaye PM. *Leishmania donovani* infection of bone marrow stromal macrophages selectively enhances myelopoiesis, by a mechanism involving GM-CSF and TNF-alpha. *Blood* (2000) **95**(5):1642–51.

83. Singal P, Singh PP. *Leishmania donovani* amastigote components-induced colony-stimulating factors production. *Parasitol Int* (2005) **54**(1):9–20. doi:10.1016/j.parint.2004.08.001
84. Sibley LD. Invasion and intracellular survival by protozoan parasites. *Immunol Rev* (2011) **240**(1):72–91. doi:10.1111/j.1600-065X.2010.00990.x
85. Dutra RA, Dutra LF, Reis Mde O, Lambert RC. Splenectomy in a patient with treatment-resistant visceral leishmaniasis: a case report. *Rev Soc Bras Med Trop* (2012) **45**(1):130–1. doi:10.1590/S0037-86822012000100027
86. Flower DR, Macdonald IK, Ramakrishnan K, Davies MN, Doytchinova IA. Computer aided selection of candidate vaccine antigens. *Immunome Res* (2010) **6**(Suppl 2):S1. doi:10.1186/1745-7580-6-S2-S1
87. Albergante L, Timmis J, Beattie L, Kaye PM. A Petri net model of granulomatous inflammation: implications for IL-10 mediated control of *Leishmania donovani* infection. *PLoS Comput Biol* (2013) **9**(11):e1003334. doi:10.1371/journal.pcbi.1003334
88. Blohmke CJ, O'Connor D, Pollard AJ. The use of systems biology and immunological big data to guide vaccine development. *Genome Med* (2015) **7**(1):114. doi:10.1186/s13073-015-0236-1

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Host Lipid Bodies as Platforms for Intracellular Survival of Protozoan Parasites

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Pathogens induce several changes in the host cell signaling and trafficking mechanisms in order to evade and manipulate the immune response. One prominent pathogen-mediated change is the formation of lipid-rich organelles, termed lipid bodies (LBs) or lipid droplets, in the host cell cytoplasm. Protozoan parasites, which contribute expressively to the burden of infectious diseases worldwide, are able to induce LB genesis in non-immune and immune cells, mainly macrophages, key players in the initial resistance to the infection. Under host–parasite interaction, LBs not only accumulate in the host cytoplasm but also relocate around and move into parasitophorous vacuoles. There is increasing evidence that protozoan parasites may target host-derived LBs either for gaining nutrients or for escaping the host immune response. Newly formed, parasite-induced LBs may serve as lipid sources for parasite growth and also produce inflammatory mediators that potentially act in the host immune response deactivation. In this mini review, we summarize current knowledge on the formation and role of host LBs as sites exploited by intracellular protozoan parasites as a strategy to maintain their own survival.

Keywords: infectious diseases, lipid droplets, inflammation, phagocytosis, lipid mediators, parasite survival, parasitophorous vacuole

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INTRODUCTION

Protozoan parasitic infections comprise devastating infectious diseases, such as malaria, visceral leishmaniasis, toxoplasmic encephalitis, and trypanosomiasis, which still account for a large proportion of death and disability worldwide (1). Many protozoan parasites have an obligate intracellular existence. The infection is initiated when the parasite enter the host target cell and is internalized within a plasma membrane-derived vacuole, the parasitophorous vacuole (PV) (2). Within the host, protozoan parasites that are mainly intracellular will only cause infectious disease if they are able to survive and multiply within the PV (2, 3).

The events of the PV formation and progression generally occur in parallel with accentuated genesis of lipid-rich organelles, termed lipid bodies (LBs) or lipid droplets, in the host cell cytoplasm [reviewed in Ref. (4, 5)]. It is now well documented that experimental and clinical infections with a range of protozoan parasites trigger LB accumulation (**Table 1**) and an intriguing interaction with the PV [reviewed in Ref. (5)]. Other pathogens, such as bacteria and viruses, also induce LB formation within different cell types, indicating that LB accumulation in response to infectious diseases is a broad event and may have implications for microbial pathogenesis (5–8).

TABLE 1 | Protozoan parasite-induced lipid body formation in host cells.

Parasite	Cell type	Organism/ model	Reference
Leishmania amazonensis	Peritoneal macrophages	Mouse	(9)
	Dendritic leukocytes	Mouse	(10)
Leishmania major	Blood-marrow-derived macrophages	Mouse	(11, 12)
Plasmodium berghei	Hepatocytes	Mouse	(13)
	Renal tubular cells	Mouse	(14)
Plasmodium chabaudi	Hepatocytes	Mouse	(15)
Toxoplasma gondii	Fibroblasts	Human	(16)
	Skeletal muscle cells	Mouse	(17)
Trypanosoma cruzi	Heart macrophages	Rat	(18, 19)
	Peritoneal macrophages	Rat	(18, 19)
	Uterine macrophages	Rat	(19)
	Peritoneal macrophages	Mouse	(20)
	Placental cells	Human	(21)

While the successful replication within the PV is under influence of several factors, there is increasing evidence that LB organelles are important for the rapid intracellular reproduction of protozoan parasites (5). Protozoan parasites require large amounts of lipids necessary for membrane biogenesis of new progenies [reviewed in Ref. (22, 23)] and may take advantage of these organelles as high-energy substrate sources. LBs within infected cells are also able to produce inflammatory mediators that potentially can inhibit the host Th1 response, thus, favoring parasite growth [reviewed in Ref. (4)]. In this mini review, we will discuss the role of host LBs as organelles modulated by intracellular protozoan parasites for survival.

LB STRUCTURE AND VISUALIZATION

Lipid bodies are common organelles distributed in the cytoplasm of prokaryotic and eukaryotic cells (5). As a general feature, LBs contain a core rich in neutral lipids, surrounded by a monolayer of phospholipids with structural proteins – the perilipin (PLIN) family proteins (5). Proteins are not restricted to the LB surface. It is documented that many types of proteins are present in the LB internum depending on the cell type (24–26).

In spite of variations in the LB composition, these organelles are seen in the cell cytoplasm as compartmentalized round sites by light microscopy or transmission electron microscopy (TEM). While LB imaging under light microscopy usually requires the use of specific lipid probes, ultrastructural observation does not need any additional labeling because LBs lack a true delimiting unit membrane structure, which enables unambiguous identification by TEM (**Figure 1**) (4, 8, 26).

PROTOZOAN PARASITES INDUCE LB ACCUMULATION IN HOST CELLS

Several protozoan parasites induce LB formation in a variety of host immune and non-immune cells (**Table 1**). Under host–parasite interaction, LBs not only accumulate but also increase in size and undergo ultrastructural changes in the host cell cytoplasm. These structural modifications of LBs have been well demonstrated during *in vitro* and *in vivo* studies with *Trypanosoma cruzi*, the causal agent of Chagas' disease (18, 19). This pathology elicits a strong inflammatory response characterized by elevated infiltration of macrophages in the target organs, mainly the heart (28). Histopathological studies conducted during the acute phase of this disease showed that increased myocardial parasitism is paralleled by increased size of LBs within inflammatory macrophages in which LBs can reach up to 4 μm in diameter (19).

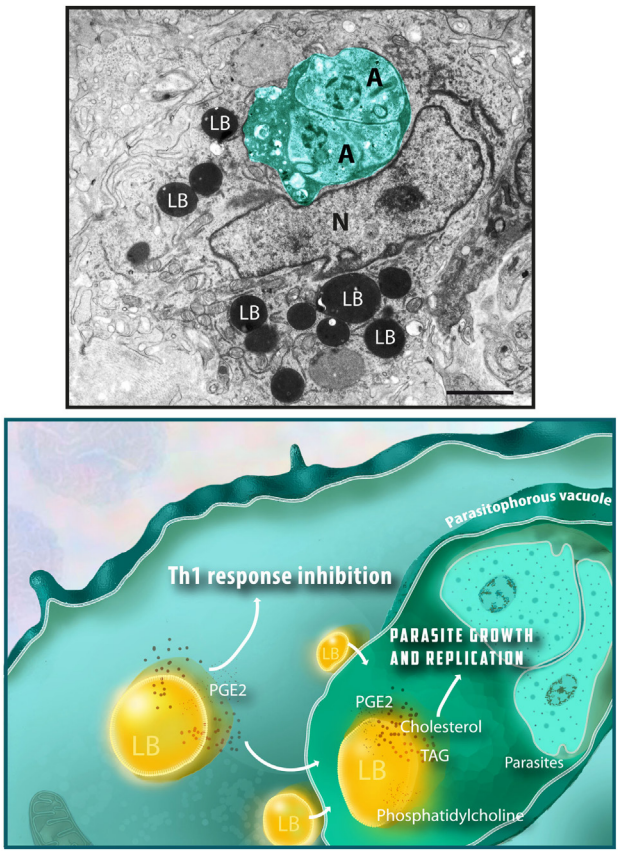

FIGURE 1 | Lipid bodies (LBs) accumulate in the host cell cytoplasm in response to interaction with protozoan parasites and favor parasite survival. Top panel shows an electron micrograph of a heart inflammatory macrophage infected with *Trypanosoma cruzi*. Note the presence of dividing amastigotes (A) within the parasitophorous vacuole (outlined in green) and the high number of LBs in the cytoplasm. LBs typically appear as round electron dense organelles in the cell cytoplasm. A model to explain how protozoan parasite-induced LB formation favors parasite growth and replication is shown in the bottom panel. Interaction of LBs with the parasitophorous vacuole, an event also triggered by the parasite infection, leads to discharge of LB contents, such as cholesterol, triacylglycerol (TAG), and phosphatidylcholine, which serve as lipid sources for parasite growth. Newly formed LBs are also sites for prostaglandin E2 synthesis (PGE2), a potent inflammation mediator that potentially inhibit the host Th1 immune response, thus decreasing the microbicidal capacity of the parasitophorous vacuole. Nu, nucleus. Scale bar, 600 nm. Top panel was reprinted from Ref. (27) with permission.

Moreover, LBs show varied electron-density in response to the *T. cruzi* infection, a morphological change likely associated with mobilization and/or *in situ* synthesis of lipid mediators [reviewed in Ref. (8)]. The capability of host LBs to generate these compounds will be discussed later.

Increase of LB size in parallel with augmented number of these organelles was also found in dendritic leukocytes hosting live *Leishmania amazonensis* amastigotes (10). Overall, as noted in diverse cell types (**Table 1**), it is clear that protozoan parasites are able to trigger LB formation in host cells. But how is the mechanism leading to LB genesis? Similar to other studies with mycobacteria (29, 30), it has been demonstrated that parasite uptake potentiates LB formation within host cells but is not essential for triggering this event. After 24 h of infection with *T. cruzi*, peritoneal macrophages with internalized parasites as well as non-parasitized cells show increased number of LBs compared to control, non-infected cells, suggesting a bystander amplification of the cell response (20). Accordingly, inhibition of parasite phagocytosis did not abolish LB genesis during infection with *Leishmania major* (11). These authors have recently showed that while phagocytosis of latex beads by macrophages did not trigger LB formation, this phenomenon is equally induced by both live and heat-killed parasites (11). Altogether, these results imply that soluble factors in parasitized cells may act in a paracrine manner to produce LB in non-parasitized bystander cells (11). While LB formation occurs through a toll-like receptor-2 (TLR2)-dependent mechanism as documented during *T. cruzi* infection in macrophages (20), the identification of downstream signaling pathways involved in this event during parasitic infections awaits further investigation.

NEWLY FORMED LBs ARE RECRUITED TO THE PROXIMITY OF PARASITES

Interestingly, protozoan parasites trigger a redistribution of the newly formed LBs around parasite-containing phagosomes. As documented by studies with *T. cruzi* (18, 19), *L. major* (11, 12), *L. amazonensis* (10), and *Toxoplasma gondii* (17, 31), LBs accumulate in close proximity to PVs or even move into these vacuoles, suggesting that these pathogens take advantage of these organelles.

Indeed, TEM analyses have enabled the identification of intimate contact between LBs and the PV membrane (10, 17–20, 31, 32). In 2003, our group was the first to observe that during an *in vivo* experimental infection with a pathogen (*T. cruzi*), LBs were internalized into parasite-containing phagosomes (18). Following studies found the same event for infections with different species of bacteria [reviewed in Ref. (5)] and with the protozoan parasites *T. gondii* (17) and *L. major* (11).

How LBs translocate across the phagosome membrane? This mechanism has been mainly addressed in studies with bacteria. LB-associated proteins, secreted by bacteria seem to be involved in capturing LB into bacteria-containing vacuoles, while LB translocation seems to involve displacement of the LB structural protein PLIN2/adipose differentiation-related protein (ADRP), which is constitutively associated with the surface of LBs (33). Although studies of membrane sites between pathogen-containing compartments and intracellular host organelles has been gaining more attention in the literature [reviewed in Ref. (34)], the mechanistic details underlying the intriguing LB–phagosome interaction remains to be fully defined.

HOST LIPIDS, LBs, AND INTRACELLULAR PARASITE GROWTH

Lipid metabolic pathways have been demonstrated as major networks modulated by protozoan parasites in host cells. Numerous studies with *T. gondii*, malaria parasites (*Plasmodium berghei, Plasmodium falciparum*, *and Plasmodium yoelii)*, *L. amazonensis*, *Cryptosporidium parvum*, and *Eimeria bovis* have documented that these parasites are able to (i) induce accumulation of lipids, such as neutral lipids [triacylglycerol (TAG), diacylglycerol, and cholesterol esters], cholesterol, and/or phospholipids, especially phosphatidylcholine in host cells (10, 12, 35–37); (ii) acquire lipid resources, such as cholesterol and phospholipids from their host environment into the PV (16, 36, 38–40), and/or (iii) use host lipids to synthesize complex own lipids or even own LBs (16, 38, 41).

Thus, although the nutritional requirements of these parasites are intricate, overall it is believed that host lipids are central to support successful parasite replication within the PV (16, 32, 37, 38, 42, 43). Host lipid acquisition is also considered crucial to PV maturation. This vacuole undergoes a pronounced membranous remodeling associated with formation of an internal network of tubules and vesicles as observed during the infection with *T. gondii* (42, 44). By using different approaches, including fluorescence recovery after photobleaching (FRAP) microscopy, Caffaro and Boothroyd demonstrated that host cells are major lipid contributors to the PV remodeling and that lipids are transferred in a continuous way from the host into the PV (42).

How is the contribution of host LBs as lipid sources for protozoan parasites? Consistent with parasite-induced host LB formation (**Table 1**), LB genesis in parallel to host lipid reprograming/accumulation (10, 12) and host LB–PV interaction (10–12, 17–19, 31), it is believed that these organelles act as important lipid sources for parasite growth.

Several classes of lipids, including neutral lipids, cholesterol, and phospholipids, make up LBs (45). Because protozoan parasites are not competent or have limited ability to synthesize lipids [reviewed in Ref. (22, 46)], LBs could be an essential source of both TAG and cholesterol for these parasites (10). These molecules are important as precursors for membrane neogenesis for newly formed parasites [reviewed in Ref. (22, 46)]. Moreover, the phospholipid monolayer of LBs consists of numerous phospholipid species of which phosphatidylcholine is the most abundant (47). Thus, it is likely that LB-derived lipids are transferred from host to the PV and taken up by replicating parasites. Indeed, by using a fluorescent probe (BODIPY-phosphatidylcholine) and live imaging microscopy, Charron and Sibley showed that this

probe moved from host plasma membrane and/or host LBs, seen as dispersed puncta in the host cytoplasm, to LBs formed within the parasite (16). However, the potential relocation of other lipids from host LBs to the parasite still needs to be better explored in future studies.

Does depletion of host lipids and/or LBs impact protozoan parasites development within host cells? In *T. gondii* infection, inhibition of cholesterol esterification in the host cell blocks parasite growth (38). Exposure to cholesterol esterification inhibitors led to reduction of cholesteryl ester synthesis, morphological changes in parasite LBs, and deformations of the parasite plasma membrane with discharge of parasite content into the PV (38). Accordingly, the use of inhibitors targeting the host cellular cholesterol *de novo* synthesis and processing repressed both *E. bovis* proliferation and LB formation within host endothelial cells (43). On the other hand, depletion of cholesterol content did not impact malaria parasites (*P. berghei and P. yoelii*) development within hepatocytes (39). In this case, the authors consider that the parasite may exploit alternative sources in these cells to sustain infectivity (39). In fact, protozoan parasites can divert lipids from other sources than the intracellular environment. *C. parvum*, the causal agent of cryptosporidiosis, a life-threatening diarrheal disease in immunocompromised individuals (48), scavenges cholesterol from plasma low-density protein (LDL) and micelles, and to a lesser extent from the cholesterol pathway within enterocytes (40). Removal of cholesterol from the media, and to lesser extent from host intracellular pools, obstructs parasite reproduction (40).

During the experimental infection with *T. cruzi*, our group demonstrated that the use of C75, an inhibitor of fatty acid synthase, led to both inhibition of LB formation and parasite division within macrophages (20). While C75 had no direct cytotoxic effect on the parasite, intracellular parasite replication was likely affected by an accentuated reduction of the LB numbers (around 66%) after cell treatment with C75 (20).

HOST LBs PRODUCE PGE2 IN RESPONSE TO INFECTION WITH PROTOZOAN PARASITES

One interesting aspect of LBs is that they are able to change their composition in response to inflammatory events as documented in cells from the immune system [reviewed in Ref. (49)]. LBs contain stores of arachidonic acid (AA), indicating that these organelles are potentially able to initiate cascades that culminate in the formation of inflammatory mediators (eicosanoids) (50, 51). Eicosanoid-generating enzymes (52–55), and *in situ* synthesis of eicosanoids (prostaglandins and leukotrienes) were indeed documented in these organelles within activated leukocytes and other cells from the immune system during inflammatory conditions [for example, see Ref. (29, 56–58)].

During the infection with *T. cruzi* (18), *T. gondii* (17, 31), and *L. amazonensis* (9), significant correlations between LB formation and enhanced generation of eicosanoids, specifically prostaglandin E2 (PGE2), by host cells have been observed. Moreover, *T. gondii* infection also elicited a time-dependent increase of cyclooxygenase-2 (COX-2) mRNA levels, indicating that the PGE2 may be a product of an active COX pathway within host cells (17).

By investigating the intracellular specific localization of both COX-2 and PGE2 within *T. cruzi*-infected macrophages, we found that both molecules were immunolocalized in LBs, indicating that LBs act as sources of PGE2 (20). Interestingly, cell treatment with non-steroidal anti-inflammatory drugs (NSAIDs) inhibited both LB formation and LB-derived PGE2 synthesis in a mechanism independent of COX inhibition (20), as previously documented (59). Therefore, accumulation of LBs in infected host cells may modulate the production of an innate immune response with production of PGE2, which in turn may contribute to a permissive environment for pathogen proliferation. For example, during the infection with *T. gondii*, the increased numbers of LBs within macrophages correlated with high PGE2 levels, decreased nitric oxide (NO) production and parasite survival (31). In fact, high concentrations of PGE2 potentially inhibit the Th1 response, tumor necrosis factor alpha (TNF-α) and/or NO production (60–62). This scenario is also detected during infections with mycobacteria (29, 30), demonstrating that pathogen-induced LB formation associated with PGE2 synthesis is a broader event that can potentially support intracellular pathogen survival. Taken together, these data suggest a model by which LBs are acting as potential stations for the survival of protozoan parasites within host cells, as depicted in **Figure 1**.

FINAL REMARKS AND FUTURE DIRECTIONS

Several key issues remain to be addressed to better understand the link between LBs and parasite survival within host cells during infections with intracellular parasitic protozoans. It is now clear that host LBs interact with PVs and that the parasite has a remarkable ability to sequester host lipids. What are the molecular mechanisms involved in LB translocation into the PV and how the LB content is extracted by the parasite? In patients with chronic mycobacterial infection (*Mycobacterium leprae*), round classical LBs are observed in contact with pathogen-containing phagosomes and intact bacteria are seen completely enmeshed in accumulated lipid content within the vacuole (63). Are LBs important for pathogen survival in chronic parasitic infections? Considering that LBs are sources for parasite development, could these organelles be target by therapeutic treatment? How significant is the contribution of host LBs and/or parasite LBs to parasite development? Moreover, the mechanisms by which new molecules, including natural products, can affect pathways of the parasite lipid metabolism and both host and parasite LB formation have yet to be fully appreciated.

AUTHOR CONTRIBUTIONS

RM prepared the manuscript. All authors contributed in part to writing and editing the manuscript and approved the final version.

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REFERENCES

1. Bar AK, Phukan N, Pinheiro J, Simoes-Barbosa A. The interplay of host microbiota and parasitic protozoans at mucosal interfaces: implications for the outcomes of infections and diseases. *PLoS Negl Trop Dis* (2015) **9**(12):e0004176. doi:10.1371/journal.pntd.0004176
2. Sibley LD. Invasion and intracellular survival by protozoan parasites. *Immunol Rev* (2011) **240**(1):72–91. doi:10.1111/j.1600-065X.2010.00990.x
3. Liévin-Le Moal V, Loiseau PM. *Leishmania* hijacking of the macrophage intracellular compartments. *FEBS J* (2015) **282**(4):598–607. doi:10.1111/febs.13601
4. Melo RCN, D'Avila H, Wan HC, Bozza PT, Dvorak AM, Weller PF. Lipid bodies in inflammatory cells: structure, function, and current imaging techniques. *J Histochem Cytochem* (2011) **59**(5):540–56. doi:10.1369/0022155411404073
5. Melo RCN, Dvorak AM. Lipid body-phagosome interaction in macrophages during infectious diseases: host defense or pathogen survival strategy? *PLoS Pathog* (2012) **8**(7):e1002729. doi:10.1371/journal.ppat.1002729
6. Saka HA, Valdivia R. Emerging roles for lipid droplets in immunity and host-pathogen interactions. *Annu Rev Cell Dev Biol* (2012) **28**:411–37. doi:10.1146/annurev-cellbio-092910-153958
7. Nicolaou G, Goodall AH, Erridge C. Diverse bacteria promote macrophage foam cell formation via toll-like receptor-dependent lipid body biosynthesis. *J Atheroscler Thromb* (2012) **19**(2):137–48. doi:10.5551/jat.10249
8. Dias FF, Zarantonello VC, Parreira GG, Chiarini-Garcia H, Melo RCN. The intriguing ultrastructure of lipid body organelles within activated macrophages. *Microsc Microanal* (2014) **20**(3):869–78. doi:10.1017/S143192761400066X
9. Pinheiro RO, Nunes MP, Pinheiro CS, D'Avila H, Bozza PT, Takiya CM, et al. Induction of autophagy correlates with increased parasite load of *Leishmania amazonensis* in BALB/c but not C57BL/6 macrophages. *Microbes Infect* (2009) **11**(2):181–90. doi:10.1016/j.micinf.2008.11.006
10. Lecoeur H, Giraud E, Prevost MC, Milon G, Lang T. Reprogramming neutral lipid metabolism in mouse dendritic leucocytes hosting live *Leishmania amazonensis* amastigotes. *PLoS Negl Trop Dis* (2013) **7**(6):e2276. doi:10.1371/journal.pntd.0002276
11. Rabhi S, Rabhi I, Trentin B, Piquemal D, Regnault B, Goyard S, et al. Lipid droplet formation, their localization and dynamics during *Leishmania* major macrophage infection. *PLoS One* (2016) **11**(2):e0148640. doi:10.1371/journal.pone.0148640
12. Rabhi I, Rabhi S, Ben-Othman R, Rasche A, Daskalaki A, Trentin B, et al. Transcriptomic signature of *Leishmania* infected mice macrophages: a metabolic point of view. *PLoS Negl Trop Dis* (2012) **6**(8):e1763. doi:10.1371/journal.pntd.0001763
13. Rodríguez-Acosta A, Finol HJ, Pulido-Méndez M, Márquez A, Andrade G, González N, et al. Liver ultrastructural pathology in mice infected with *Plasmodium berghei*. *J Submicrosc Cytol Pathol* (1998) **30**(2):299–307.
14. Pulido-Méndez M, Finol HJ, Girón ME, Aguilar I. Ultrastructural pathological changes in mice kidney caused by *Plasmodium berghei* infection. *J Submicrosc Cytol Pathol* (2006) **38**(2–3):143–8.
15. Dumont ME, Dei-Cas E, Maurois P, Slomianny C, Prensier G, Houcke-Lecomte M, et al. [Histopathology of the liver and kidney during malaria: relation to malaria-induced dyslipoproteinemia]. *Ann Parasitol Hum Comp* (1988) **63**(3):171–83.
16. Charron AJ, Sibley LD. Host cells: mobilizable lipid resources for the intracellular parasite *Toxoplasma gondii*. *J Cell Sci* (2002) **115**(Pt 15):3049–59.
17. Gomes AF, Magalhaes KG, Rodrigues RM, de Carvalho L, Molinaro R, Bozza PT, et al. *Toxoplasma gondii*-skeletal muscle cells interaction increases lipid droplet biogenesis and positively modulates the production of IL-12, IFN-g and PGE2. *Parasit Vectors* (2014) **7**:47. doi:10.1186/1756-3305-7-47
18. Melo RCN, D'Avila H, Fabrino DL, Almeida PE, Bozza PT. Macrophage lipid body induction by Chagas disease in vivo: putative intracellular domains for eicosanoid formation during infection. *Tissue Cell* (2003) **35**(1):59–67. doi:10.1016/S0040-8166(02)00105-2
19. Melo RCN, Fabrino DL, Dias FF, Parreira GG. Lipid bodies: structural markers of inflammatory macrophages in innate immunity. *Inflamm Res* (2006) **55**(8):342–8. doi:10.1007/s00011-006-5205-0
20. D'Avila H, Freire-de-Lima CG, Roque NR, Teixeira L, Barja-Fidalgo C, Silva AR, et al. Host cell lipid bodies triggered by *Trypanosoma cruzi* infection and enhanced by the uptake of apoptotic cells are associated with prostaglandin E generation and increased parasite growth. *J Infect Dis* (2011) **204**(6):951–61. doi:10.1093/infdis/jir432
21. Fabro AE, Calzolari G. [Increase of lipids in human chagasic placentas: cytochemical and biochemical study]. *Rev Fac Cien Med Univ Nac Cordoba* (1990) **48**(1–2):25–32.
22. Helms JB, Kaloyanova DV, Strating JR, van Hellemond JJ, van der Schaar HM, Tielens AG, et al. Targeting of the hydrophobic metabolome by pathogens. *Traffic* (2015) **16**(5):439–60. doi:10.1111/tra.12280
23. Coppens I. Contribution of host lipids to *Toxoplasma* pathogenesis. *Cell Microbiol* (2006) **8**(1):1–9. doi:10.1111/j.1462-5822.2005.00647.x
24. Welte MA. Proteins under new management: lipid droplets deliver. *Trends Cell Biol* (2007) **17**(8):363–9. doi:10.1016/j.tcb.2007.06.004
25. Wan HC, Melo RCN, Jin Z, Dvorak AM, Weller PF. Roles and origins of leukocyte lipid bodies: proteomic and ultrastructural studies. *FASEB J* (2007) **21**(1):167–78. doi:10.1096/fj.06-6711com
26. Melo RCN, Weller PF. Unraveling the complexity of lipid body organelles in human eosinophils. *J Leukoc Biol* (2014) **96**(5):703–12. doi:10.1189/jlb.3RU0214-110R
27. Melo RCN, Machado CRS. *Trypanosoma cruzi*: peripheral blood monocytes and heart macrophages in the resistance to acute experimental infection in rats. *Exp Parasitol* (2001) **97**(1):15–23. doi:10.1006/expr.2000.4576
28. Melo RCN. Acute heart inflammation: ultrastructural and functional aspects of macrophages elicited by *Trypanosoma cruzi* infection. *J Cell Mol Med* (2009) **13**(2):279–94. doi:10.1111/j.1582-4934.2008.00388.x
29. D'Avila H, Melo RCN, Parreira GG, Werneck-Barroso E, Castro-Faria-Neto HC, Bozza PT. *Mycobacterium bovis* bacillus Calmette-Guerin induces TLR2-mediated formation of lipid bodies: intracellular domains for eicosanoid synthesis in vivo. *J Immunol* (2006) **176**(5):3087–97. doi:10.4049/jimmunol.176.5.3087
30. Mattos KA, D'Avila H, Rodrigues LS, Oliveira VG, Sarno EN, Atella GC, et al. Lipid droplet formation in leprosy: toll-like receptor-regulated organelles involved in eicosanoid formation and *Mycobacterium leprae* pathogenesis. *J Leukoc Biol* (2010) **87**(3):371–84. doi:10.1189/jlb.0609433

31. Mota LA, Roberto Neto J, Monteiro VG, Lobato CS, Oliveira MA, Cunha M, et al. Culture of mouse peritoneal macrophages with mouse serum induces lipid bodies that associate with the parasitophorous vacuole and decrease their microbicidal capacity against *Toxoplasma gondii*. *Mem Inst Oswaldo Cruz* (2014) **109**(6):767–74.
32. Combs TP, Nagajyothi, Mukherjee S, de Almeida CJ, Jelicks LA, Schubert W, et al. The adipocyte as an important target cell for *Trypanosoma cruzi* infection. *J Biol Chem* (2005) **280**(25):24085–94. doi:10.1074/jbc.M412802200
33. Cocchiaro JL, Kumar Y, Fischer ER, Hackstadt T, Valdivia RH. Cytoplasmic lipid droplets are translocated into the lumen of the *Chlamydia trachomatis* parasitophorous vacuole. *Proc Natl Acad Sci U S A* (2008) **105**(27):9379–84. doi:10.1073/pnas.0712241105
34. Dumoux M, Hayward RD. Membrane contact sites between pathogen-containing compartments and host organelles. *Biochim Biophys Acta* (2016). doi:10.1016/j.bbalip.2016.01.018
35. Simoes AP, Roelofsen B, Op den Kamp JA. Incorporation of free fatty acids can explain alterations in the molecular species composition of phosphatidylcholine and phosphatidylethanolamine in human erythrocytes as induced by *Plasmodium falciparum*. *Cell Biol Int Rep* (1992) **16**(6):533–45. doi:10.1016/S0309-1651(05)80052-9
36. Itoe MA, Sampaio JL, Cabal GG, Real E, Zuzarte-Luis V, March S, et al. Host cell phosphatidylcholine is a key mediator of malaria parasite survival during liver stage infection. *Cell Host Microbe* (2014) **16**(6):778–86. doi:10.1016/j.chom.2014.11.006
37. Hamid PH, Hirzmann J, Kerner K, Gimpl G, Lochnit G, Hermosilla CR, et al. Eimeria bovis infection modulates endothelial host cell cholesterol metabolism for successful replication. *Vet Res* (2015) **46**:100. doi:10.1186/s13567-015-0230-z
38. Nishikawa Y, Quittnat F, Stedman TT, Voelker DR, Choi JY, Zahn M, et al. Host cell lipids control cholesteryl ester synthesis and storage in intracellular *Toxoplasma*. *Cell Microbiol* (2005) **7**(6):849–67. doi:10.1111/j.1462-5822.2005.00518.x
39. Labaied M, Jayabalasingham B, Bano N, Cha SJ, Sandoval J, Guan G, et al. Plasmodium salvages cholesterol internalized by LDL and synthesized de novo in the liver. *Cell Microbiol* (2011) **13**(4):569–86. doi:10.1111/j.1462-5822.2010.01555.x
40. Ehrenman K, Wanyiri JW, Bhat N, Ward HD, Coppens I. *Cryptosporidium parvum* scavenges LDL-derived cholesterol and micellar cholesterol internalized into enterocytes. *Cell Microbiol* (2013) **15**(7):1182–97. doi:10.1111/cmi.12107
41. Jackson KE, Klonis N, Ferguson DJ, Adisa A, Dogovski C, Tilley L. Food vacuole-associated lipid bodies and heterogeneous lipid environments in the malaria parasite, *Plasmodium falciparum*. *Mol Microbiol* (2004) **54**(1):109–22. doi:10.1111/j.1365-2958.2004.04284
42. Caffaro CE, Boothroyd JC. Evidence for host cells as the major contributor of lipids in the intravacuolar network of *Toxoplasma*-infected cells. *Eukaryot Cell* (2011) **10**(8):1095–9. doi:10.1128/EC.00002-11
43. Hamid PH, Hirzmann J, Hermosilla C, Taubert A. Differential inhibition of host cell cholesterol de novo biosynthesis and processing abrogates *Eimeria bovis* intracellular development. *Parasitol Res* (2014) **113**(11):4165–76. doi:10.1007/s00436-014-4092-5
44. Sibley LD, Niesman IR, Parmley SF, Cesbron-Delauw MF. Regulated secretion of multi-lamellar vesicles leads to formation of a tubulo-vesicular network in host-cell vacuoles occupied by *Toxoplasma gondii*. *J Cell Sci* (1995) **108** (Pt 4):1669–77.
45. Farese RV Jr, Walther TC. Lipid droplets finally get a little R-E-S-P-E-C-T. *Cell* (2009) **139**(5):855–60. doi:10.1016/j.cell.2009.11.005
46. Wenk MR. Lipidomics of host-pathogen interactions. *FEBS Lett* (2006) **580**(23):5541–51. doi:10.1016/j.febslet.2006.07.007
47. Penno A, Hackenbroich G, Thiele C. Phospholipids and lipid droplets. *Biochim Biophys Acta* (2013) **1831**(3):589–94. doi:10.1016/j.bbalip.2012.12.001
48. Tzipori S, Ward H. Cryptosporidiosis: biology, pathogenesis and disease. *Microbes Infect* (2002) **4**(10):1047–58. doi:10.1016/S1286-4579(02)01629-5
49. Melo RCN, Weller PF. Lipid droplets in leukocytes: organelles linked to inflammatory responses. *Exp Cell Res* (2016) **340**(2):193–7. doi:10.1016/j.yexcr.2015.10.028
50. Weller PF, Dvorak AM. Arachidonic acid incorporation by cytoplasmic lipid bodies of human eosinophils. *Blood* (1985) **65**(5):1269–74.
51. Weller PF, Monahan-Earley RA, Dvorak HF, Dvorak AM. Cytoplasmic lipid bodies of human eosinophils. Subcellular isolation and analysis of arachidonate incorporation. *Am J Pathol* (1991) **138**(1):141–8.
52. Dvorak AM, Morgan E, Schleimer RP, Ryeom SW, Lichtenstein LM, Weller PF. Ultrastructural immunogold localization of prostaglandin endoperoxide synthase (cyclooxygenase) to non-membrane-bound cytoplasmic lipid bodies in human lung mast cells, alveolar macrophages, type II pneumocytes, and neutrophils. *J Histochem Cytochem* (1992) **40**(6):759–69. doi:10.1177/40.6.1316915
53. Dvorak AM, Morgan ES, Tzizik DM, Weller PF. Prostaglandin endoperoxide synthase (cyclooxygenase): ultrastructural localization to nonmembrane-bound cytoplasmic lipid bodies in human eosinophils and 3T3 fibroblasts. *Int Arch Allergy Immunol* (1994) **105**(3):245–50. doi:10.1159/000236764
54. Bozza PT, Yu W, Penrose JF, Morgan ES, Dvorak AM, Weller PF. Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. *J Exp Med* (1997) **186**(6):909–20. doi:10.1084/jem.186.6.909
55. Bozza PT, Yu W, Cassara J, Weller PF. Pathways for eosinophil lipid body induction: differing signal transduction in cells from normal and hypereosinophilic subjects. *J Leukoc Biol* (1998) **64**(4):563–9.
56. Bandeira-Melo C, Phoofolo M, Weller PF. Extranuclear lipid bodies, elicited by CCR3-mediated signaling pathways, are the sites of chemokine-enhanced leukotriene C4 production in eosinophils and basophils. *J Biol Chem* (2001) **276**(25):22779–87. doi:10.1074/jbc.M101436200
57. Vieira-de-Abreu A, Assis EF, Gomes GS, Castro-Faria-Neto HC, Weller PF, Bandeira-Melo C, et al. Allergic challenge-elicited lipid bodies compartmentalize in vivo leukotriene C4 synthesis within eosinophils. *Am J Respir Cell Mol Biol* (2005) **33**(3):254–61. doi:10.1165/rcmb.2005-0145OC
58. Pacheco P, Vieira-de-Abreu A, Gomes RN, Barbosa-Lima G, Wermelinger LB, Maya-Monteiro CM, et al. Monocyte chemoattractant protein-1/CC chemokine ligand 2 controls microtubule-driven biogenesis and leukotriene B4-synthesizing function of macrophage lipid bodies elicited by innate immune response. *J Immunol* (2007) **179**(12):8500–8. doi:10.4049/jimmunol.179.12.8500
59. Bozza PT, Payne JL, Morham SG, Langenbach R, Smithies O, Weller PF. Leukocyte lipid body formation and eicosanoid generation: cyclooxygenase-independent inhibition by aspirin. *Proc Natl Acad Sci U S A* (1996) **93**(20):11091–6. doi:10.1073/pnas.93.20.11091
60. Renz H, Gong J-H, Schmidt A, Nain M, Gemsa D. Release of tumor necrosis factor-alpha from macrophages. Enhancement and suppression are dose-dependently regulated by prostaglandin E2 and cyclic nucleotides. *J Immunol* (1988) **141**(7):2388–93.
61. Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol* (1991) **146**(1):108–13.
62. Kalinski P. Regulation of immune responses by prostaglandin E2. *J Immunol* (2012) **188**(1):21–8. doi:10.4049/jimmunol.1101029
63. Mattos KA, Oliveira VC, Berredo-Pinho M, Amaral JJ, Antunes LC, Melo RCN, et al. Mycobacterium leprae intracellular survival relies on cholesterol accumulation in infected macrophages: a potential target for new drugs for leprosy treatment. *Cell Microbiol* (2014) **16**(6):797–815. doi:10.1111/cmi.12279

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MINI REVIEW
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Modulation of Cell Sialoglycophenotype: A Stylish Mechanism Adopted by *Trypanosoma cruzi* to Ensure Its Persistence in the Infected Host

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Trypanosoma cruzi, the etiological agent of Chagas disease exhibits multiple mechanisms to guarantee its establishment and persistence in the infected host. It has been well demonstrated that *T. cruzi* is not able to synthesize sialic acids (Sia). To acquire the monosaccharide, the parasite makes use of a multifunctional enzyme called *trans*-sialidase (Tc-TS). Since this enzyme has no analogous in the vertebrate host, it has been used as a target in drug therapy development. Tc-TS preferentially catalyzes the transfer of Sia from the host glycoconjugates to the terminal β-galactopyranosyl residues of mucin-like molecules present on the parasite's cell surface. Alternatively, the enzyme can sialylate/re-sialylate glycoconjugates expressed on the surface of host cells. Since its discovery, several studies have shown that *T. cruzi* employs the Tc-TS activity to modulate the host cell sialoglycophenotype, thus favoring its perpetuation in the infected vertebrate. In this review, we summarize the dynamic of host/parasite sialoglycophenotype modulation, highlighting its role in the subversion of host immune response in order to promote the establishment of persistent chronic infection.

Keywords: ***Trypanosoma cruzi*, *trans*-sialidase, immune response, CD8$^+$ T cell, glycoconjugates, sialic acid**

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THE IMPORTANCE OF SIALO-CONTAINING GLYCANS IN CELL BIOLOGY

All living cells possess a complex and dense coating composed of glycoconjugates (Varki, 1993). Such structures exist as glycolipids, glycoproteins, and proteoglycans. Among other functions, cell surface glycans mediate cell–cell recognition and control interactions between cells and other components in their local environment. Throughout evolution, glycans were designated several roles based on their physical features and structural diversity (Varki, 2011). Advances in glycobiology researches have provided the means to correlate glycan structures to physiological and pathological conditions (Lowe and Marth, 2003; Baum et al., 2014; Schmaus et al., 2014; Kato and Ishiwa, 2015; Stowell et al., 2015). The result is that glycoscience is emerging as a major contributor

to the understanding of biology and medicine. The functional specificity of glycans is often dictated by the monosaccharides at the outermost ends of glycan chains (Berger et al., 1982; Varki, 1993; Hart, 2013). In mammals, the glycan chain of glycoproteins and glycolipids often end with sialic acids (Sia), a group of sugars with distinctive chemical properties that determine their functions (Traving and Schauer, 1998). Discovered about 70 years ago, Sia is a generic term referring to nine-carbon alpha (α)-keto sugars with a carboxyl group linked directly to the ring as well as its *N* or *O* substituted derivatives. There are over 50 Sia described, while the two most commonly found in mammals are *N*-acetyl and *N*-glycolyl neuraminic acid (Varki and Schauer, 2009). It is estimated that a cell exhibits tens of millions Sia molecules, with the concentration in the glycocalyx approaching 100 mM (Varki and Gagneux, 2012). Thanks to its bulky size and the electron density of the carboxyl group, Sia provides an important source of negative charge, which could alter the biophysical properties of cellular interactions. In eukaryotic cells, Sia residues are responsible for mediating many important interactions, including but not limited to regulation of transmembrane receptor function, altering membrane transport, control of glycoproteins (and even cell) half-life (Schauer, 2000). One of the most notable functions of Sia is to regulate immune response, playing an important role in self and non-self recognition (Traving and Schauer, 1998). It should not come as a surprise that several pathogenic microorganisms, such as viruses, bacteria, and protozoa have evolved strategies to exploit this feature (Neu et al., 2011; Nishikawa et al., 2013; Chang and Nizet, 2014). One of the more stylish mechanisms of cell surface sialylation is exploited by the protozoal *Trypanosoma cruzi,* the etiological agent of Chagas disease (Previato et al., 1985; Zingales et al., 1987; Schenkman et al., 1991, 1992; Mucci et al., 2006; Muia et al., 2010).

Trypanosoma cruzi trans-SIALIDASE AND HOST CELL INVASION

Incapable of synthetizing Sia, *T. cruzi* has developed an elegant mechanism to take advantage of the Sia residues displayed in its mammalian host sialoglycans in order to avoid detection and elimination by immune cells (Pereira-Chioccola and Schenkman, 1999; Dc-Rubin and Schenkman, 2012; Freire-de-Lima et al., 2015). Since it was shown that *T. cruzi* could not use the activated monosaccharide precursor CMP-Neu5Ac, as it was first considered (Schauer et al., 1983), it was proposed a novel metabolic mechanism of *trans*-glycosylation, by which *T. cruzi* would incorporate Sia onto its own cell surface (Previato et al., 1985). Incorporation of Sia on the surface molecules of *T. cruzi* parasite is catalyzed by the enzyme called *trans*-sialidase (Tc-TS) Previato et al., 1985; Schenkman et al., 1991). It was demonstrated *in vitro* and *in vivo* (Zingales et al., 1987; Previato et al., 1990) the activity of Tc-TS was characterized as specific for α-2,3 Sia, being responsible for the transfer of Sia residues from the mammal host surface sialylated molecules to the parasite mucin-like acceptors (Schenkman et al., 1991; Parodi et al., 1992; Ruiz Rde et al., 1993; Previato et al., 1994).

In *T. cruzi*, the relevance of the acquisition of Sia is not completely known. However, there are proposals that such an event might help trypomastigote forms adhere and penetrate into non-phagocytic cells (Ming et al., 1993; Schenkman et al., 1993). Sia also provides a strong negatively charged cover that protects parasites against human lytic antibodies specific for α-galactosyl residues present on the trypomastigote surface (Pereira-Chioccola et al., 2000).

The transfer of a Sia residue onto an acceptor hinders the access of a second residue when two potential acceptor sites are present on the same oligosaccharide (Previato et al., 1995). Tc-TS can also operate as a viral and bacterial sialidase, irreversibly transferring Sia to water in the absence of a carbohydrate acceptor (Vandekerckhove et al., 1992). Analysis of the crystal structure revealed that the molecular architecture of the Tc-TS active site preserves numerous conserved features of microbial sialidases (Buschiazzo et al., 2002). Furthermore, all sialidases studied so far, including Tc-TS, catalyze sialoside hydrolysis with configuration retention (Todeschini et al., 2000).

There has been significant progress in understanding the importance of Tc-TS on the biology of *T. cruzi*. However, obstacles such as the lack of knockout strains, as well as the absence of specific Tc-TS inhibitors pose difficulties for the progress in the area. In regard to the Tc-TS inhibitors, many of them are not strong enough to serve as a scaffold to the development of drug targeting Tc-TS. In addition, most are derivatives of the substrate Sia or a transition state analog known as 2,3-dehydro-3-deoxy-*N*-acetylneuraminic acid (DANA), with *in vitro* IC(50) values near the milimolar range and low *in vivo* efficacy (Uemura et al., 1992; Amaya et al., 2003; Arioka et al., 2010; Dc-Rubin and Schenkman, 2012). Recent studies suggest that high affinity inhibitory monoclonal antibodies (mAb) for Tc-TS might provide a rational framework for novel approaches in the design of chemotherapeutic drugs (Buschiazzo et al., 2012).

Over the last years, several papers described by Campetella's group demonstrated that active Tc-TS (aTS) induces systemic effects during the acute phase of the disease (Mucci et al., 2002, 2005; Tribulatti et al., 2005; Risso et al., 2007; Ruiz Díaz et al., 2015), until the elicitation of broad neutralizing antibodies (Pitcovsky et al., 2002). Buschiazzo et al. (2012) reported the identification and detailed characterization of the neutralizing mouse mAb 13G9, which was able to recognize and inhibit Tc-TS with high specificity. The crystal structure of the complex involving the antigen-binding fragment and the globular region of Tc-TS demonstrated that not obstructing the enzyme's catalytic site, the antibody inhibited the movement of an assisting tyrosine (Y_{119}), which plays an important role in the *trans*-glycosidase mechanism (Buschiazzo et al., 2002). The authors suggest that such results may bring into light new strategies for chemotherapy in Chagas disease and for disclosure of aTS function in *T. cruzi* pathogenesis and biology.

Besides encoding aTS, the genes of the TS family also encode its inactive analog (iTS). iTS has a His at position 342, while active members contain a Tyr at the same position (Cremona et al., 1995; Egima et al., 1996). It has been suggested that iTS may mediate the initial contact between *T. cruzi* and host

cells, functioning as an adhesin that contains two sugar-binding distinct sites, for Sia and β-galactose (Todeschini et al., 2004). Recently, Ruiz Díaz et al. (2015) demonstrated that both aTS and iTS besides to induce a Th2-like phenotype in naive T cells, also compromise the emergence of Th1 cells. Additionally, both isoforms were associated with the parasite's ability to reduce IL-2 production and IL-2Ra expression by T cells. The authors suggest that TS proteins are able to manipulate the $TCD4^+$ response throughout their maturation stages to favor parasite survival and infection.

The bivalent nature of iTS could promote a glycan cross-linking believed to be essential for cellular signal transduction. Tc-TS has essentially two different domains. The N-terminal, which contains the catalytic domain of the enzyme (Ribeirao et al., 1997), and the C-terminal, which is composed basically of 12 amino acids-long units repeated in tandem, termed SAPA (shed acute-phase antigen; Affranchino et al., 1989), which is not required for TS activity (Campetella et al., 1994). Tc-TS is linked to the parasite membrane via glycosylphosphatidylinositol (GPI) anchor and shed into the bloodstream during the infection (Schenkman et al., 1994). Nowadays, it is well recognized that Tc-TS is an important virulence factor involved in cell invasion and pathogenesis of Chagas disease (Mendonça-Previato et al., 2010; Dc-Rubin and Schenkman, 2012; Miller and Roitberg, 2013; Freire-de-Lima et al., 2015). However, the studies regarding the importance of Tc-TS for cell invasion and parasite survival were paramount to understanding how *T. cruzi* is capable of subverting the immune system by disturbing the host cells sialoglycophenotype.

ROLES OF SIALIC ACIDS ON T CELL BIOLOGY

It is well documented that in T cells, different lectin families recognize distinct sialylated ligands on glycoproteins or glycolipids to regulate their functions (Rabinovich et al., 2002a,b; Ley and Kansas, 2004; Crocker et al., 2007; Crocker and Redelinghuys, 2008). Examples include siglecs, a family of Sia-binding lectins that belong to the superfamily of I-type lectins (Crocker and Redelinghuys, 2008). Most siglecs bind α-2,3 and/or α-2,6-linked Sia on penultimate Gal residues on cell surface glycoproteins or glycolipids; however, some members bind to repeating Sia chains (Varki and Angata, 2006). In the immune system, siglecs mainly function as controllers of cell signaling, although roles for some siglecs in pathogen recognition and innate immunity have been proposed (von Gunten and Bochner, 2008). Erdmann et al. (2009) demonstrated that sialylated ligands on *T. cruzi* interact with Siglec-E (sialic acid-binding Ig-like lectin-E) on dendritic cells (DC), and such interaction suppressed the production of the proinflammatory cytokine IL-12 and subsequent T cell activation. Recently, it was proposed that sulfates from cruzipain, another important antigen expressed by *T. cruzi*, might play an essential role in the interaction with Siglec-E on inflammatory cells, favoring the persistence of the parasite in its mammalian hosts (Ferrero et al., 2016).

Sia plays crucial roles in the regulation of host immunity, as evidenced by the sialylation changes sustained by naïve T cells during thymic selection, a tightly regulated process, essential for establishing central tolerance (Daniels et al., 2001; Moody et al., 2001; Cao and Crocker, 2011; Paulson et al., 2012). The alterations suffered on cell surface *N*- and *O*-linked glycans, including glycoprotein sialylation, during T cell development and differentiation (Daniels et al., 2002) might regulate the T cell response through a direct effect on the intrinsic properties of specific proteins, or by modulating the binding of a disparate set of cell surface proteins to a specific carbohydrate moiety. It is important to bring out that many of the changes in the degree of sialylation of carbohydrate chains observed in T cells during their development can be monitored with plant lectins (Sharon, 1983; Daniels et al., 2002). It has been shown *in vitro* and *in vivo* that T cell activation is accompanied by loss of Sia from core 1 *O*-glycans (Siaα2,3Galβ1,3GalNAc-Ser/Thr; Galvan et al., 1998; Bi and Baum, 2009), which leads to exposure of asialo core 1 *O*-glycans. Such residues can be detected with the plant lectin peanut agglutinin (PNA), that recognizes Galβ1,3GalNAc sequences on several glycoproteins including CD8, CD43, and CD45 (Wu et al., 1996). It is a known fact that the loss of cell-surface Sia during T cell activation enhances TCR reactivity with antigens (Galvan et al., 1998; Harrington et al., 2000; Bi and Baum, 2009). Indeed, T cell cytolytic activity may be boosted by sialidase treatment, with a simultaneous reduction in cell surface negative charge (Sadighi Akha et al., 2006). In addition, de-sialylated $CD8^+$ T cells undergo more rounds of cell division following contact with antigen (Pappu and Shrikant, 2004). Recognition and disposal of pathogens rely upon the concerted actions of adaptive and innate immunological mechanisms, including recruitment of different cell types as well as production and release of cytokines, antibodies or other effector molecules (Janeway, 2001). In regards to adaptive response, while the importance of $CD8^+$ T cells is well described when it comes to the control of several viral and bacterial infections, it is seldom mentioned that eukaryotic pathogens, such as protozoans, are also subject to the action of cytotoxic lymphocytes. Chagas disease is probably the most well understood example of the role $CD8^+$ T cells play during protozoan infections. In its vertebrate hosts, *T. cruzi* is perpetually changing between its intracellular amastigote forms and extracellular trypomastigote ones. There is a great range of cells that are subject to invasion by the parasite, including but not limited to fibroblasts, macrophages, myocytes, and adipocytes. During the intracellular stages, *T. cruzi* antigens are presented by antigen presenting cells (APCs) through by MHC class I molecules, providing many opportunities for detection by $CD8^+$ T cells (Garg et al., 1997). Several reports confirmed the importance of $CD8^+$ T cell cytotoxicity regarding Chagas disease (Tarleton, 2007, 2015; Padilla et al., 2009; Dos Santos Virgilio et al., 2014). One of the most important was the identification of a number of epitopes expressed in proteins belonging to the TS family genes (Martin et al., 2006). Until that point, there was a lack

of immunodominant T cell epitopes, but now it is accepted that members of the TS gene family are a good target for $CD8^+$ T cells. That discovery has not only led to a better understanding of how the immune system reacts to *T. cruzi* infection, but in a more practical venue has also fueled the research on vaccine development (Machado et al., 2006; Araujo et al., 2014; Bontempi et al., 2015). Although it is universally accepted that $CD8^+$ T cell response is an important factor in mediating host survival during *T. cruzi* infection, there is a substantial delay in the appearance of antigen specific $CD8^+$ T cells following infection (Martin et al., 2006; Tzelepis et al., 2006). This delay differs from the quick response of $CD8^+$ T cells in other bacterial, viral, and, even protozoal infections (Kaech et al., 2002), suggesting that a mechanism of immune subversion is operative.

Trypanosoma cruzi trans-SIALIDASE MODULATES T CELL SIALOGLYCOPHENOTYPE

As mentioned above, Sia content of T cell glycoproteins and glycolipids is regulated during both T cell development in the thymus and activation in the periphery (Moody et al., 2001; Pappu and Shrikant, 2004). Each T cell subset expresses a specific set of glycan-modifying enzymes that regulate the pattern of sialylation on the cell surface (Bi and Baum, 2009). Besides being able to sialylate mucin-like molecules expressed on the parasite surface, Tc-TS is also capable of re-sialylating host cell asialoglycoconjugates (Mucci et al., 2006). Regarding T cells, such event is able to modulate both their function and half-life. Several papers published elsewhere have demonstrated that alterations in Sia residues on thymocytes and mature T cells are able to induce apoptosis (Leguizamon et al., 1999; Mucci et al., 2002, 2005, 2006). Given the way TS works, it comes as a logical conclusion that the parasite's ability to modulate the T cell sialophenotype is a powerful tool in evading detection and elimination by the host immune system. Pioneering studies on the impact of Tc-TS on T cells (Chuenkova and Pereira, 1995) demonstrated that intravenous injection of minute amounts of native Tc-TS was able to increase the parasitemia and mortality of *T. cruzi*-infected mice. The authors suggested that such effects were entirely dependent on Tc-TS activity, since the same events did not occur in mice primed with viral or bacterial sialidases. Further *in vitro* experiments demonstrated that recombinant Tc-TS binds host T-lymphocytes, activating $CD4^+$ T cells through CD43 engagement (Todeschini et al., 2002a,b). However, molecular evidences revealed that CD45 might be the main acceptor of Tc-TS during *T. cruzi* infection (Muia et al., 2010). *In vivo* studies have highlighted the impact of Tc-TS on B and T cells sialoglycophenotypes (Freire-de-Lima et al., 2010; Bermejo et al., 2013), reinforcing the idea that the Tc-TS may act as a virulence factor during *T. cruzi* infection. In B cells, the sialylation of cell surface molecules by Tc-TS elicits the production of IL-17. Interestingly, such event triggers a signaling pathway that differs from the one classically associated with IL-17, bypassing the activation of RORγt and RORα (Bermejo et al., 2013). As for T cells, Freire-de-Lima et al. (2010) demonstrated the relevance of Tc-TS on the sialoglycophenotype of splenic $CD8^+$ T cells during *T. cruzi* infection. *T. cruzi*-infected or *Plasmodium berghei*-infected mice were sacrificed on the eighth day post-infection (dpi), and the sialoglycophenotype of $CD8^+$ T cells was assessed. As expected, $CD8^+$ T cells from *P. berghei*-infected mice were highly positive for PNA (PNA^{high}). Interestingly, the glycophenotype PNA intermediate (PNA^{int}) exhibited by $CD8^+$ T cells derived from *T. cruzi*-infected mice, becomes PNA^{low} after intravenous administration recombinant aTS. Such event was able to inhibit the cytotoxic response mediated by antigen specific $CD8^+$ T cells, supporting the idea that re-sialylation of asialoglycans on the surface of activated T cells might be a sophisticated evasion mechanism adopted by *T. cruzi* to subvert the host immune response (Freire-de-Lima et al., 2010). In addition, we found that $CD8^+$ T cells from *T. cruzi*-infected mice primed with recombinant iTS exhibited a glycophenotype PNA^{high}, suggesting that iTS might have been able to compete with the native aTS by serum sialoglycoproteins, compromising the expected event of re-sialylation that takes place during the acute phase of Chagas disease (Freire-de-Lima et al., 2010). Although the evidence is quite compelling, new experiments with ST3 Gal-I KO mice must be conducted in order to understand the effects triggered by Tc-Ts on $CD8^+$ T cells during *T. cruzi* infection. Several immunobiological effects mediated by Tc-TS have been described elsewhere (Mucci et al., 2006; Ruiz Díaz et al., 2015), but the chemical evidence of the enzyme's essential role in the parasite is still lacking due to the absence of specific inhibitors of Tc-TS. As it stands Tc-TS is a promising target in drug design, an area in which further research is urgently needed.

CONCLUSION

Over the last years, several papers have demonstrated the importance of differential sialylation for $CD8^+$ T cells with respect to their maturation (Moody et al., 2001; Naito-Matsui et al., 2014), activation (Pappu and Shrikant, 2004), and cytotoxic responses (Sadighi Akha et al., 2006). However, few studies provided insights into the impact of surface sialylation of $CD8^+$ T cells on infectious diseases. Infection with *T. cruzi* is of particular interest in this context because the parasite releases into the host plasma large amounts of proteins belonging to the TS family (Frasch, 1994; Schenkman et al., 1994; Giorgi and de Lederkremer, 2011; Freire-de-Lima et al., 2012, 2015). Recent studies demonstrated that cell surface asialoglycans on B and T lymphocytes might be sialylated by the action of Tc-TS (Freire-de-Lima et al., 2010; Bermejo et al., 2013). Such events were able to disturb the host immune response and allow for the continuation of the parasite in the infected host. Since it is universally accepted that Tc-TS acts as a virulence factor during *T. cruzi* infection, further efforts are needed in this lively area to better understand the catalytic mechanism, as well as the functional properties of the Sia-dependent enzyme. Certainly, new impacting findings will contribute to further research on

the enzyme as a therapeutic target in the fight against Chagas' disease.

AUTHOR CONTRIBUTIONS

Wrote the paper: LFL, LMF, VAS, KMC, AM, CGFL, JOP and LM-P. All authors read and approved the final version of the manuscript.

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REFERENCES

Affranchino, J. L., Ibanez, C. F., Luquetti, A. O., Rassi, A., Reyes, M. B., Macina, R. A., et al. (1989). Identification of a *Trypanosoma cruzi* antigen that is shed during the acute phase of Chagas' disease. *Mol. Biochem. Parasitol.* 34, 221–228. doi: 10.1016/0166-6851(89)90050-9

Amaya, M. F., Buschiazzo, A., Nguyen, T., and Alzari, P. M. (2003). The high resolution structures of free and inhibitor-bound *Trypanosoma rangeli* sialidase and its comparison with *T. cruzi* trans-sialidase. *J. Mol. Biol.* 325, 773–784. doi: 10.1016/S0022-2836(02)01306-2

Araujo, A. F., de Oliveira, G., Vasconcelos, J. F., Ersching, J., Dominguez, M. R., Vasconcelos, J. R., et al. (2014). Genetic vaccination against experimental infection with myotropic parasite strains of *Trypanosoma cruzi*. *Mediators Inflamm.* 2014:605023. doi: 10.1155/2014/605023

Arioka, S., Sakagami, M., Uematsu, R., Yamaguchi, H., Togame, H., Takemoto, H., et al. (2010). Potent inhibitor scaffold against *Trypanosoma cruzi* trans-sialidase. *Bioorg. Med. Chem.* 18, 1633–1640. doi: 10.1016/j.bmc.2009.12.062

Baum, L. G., Garner, O. B., Schaefer, K., and Lee, B. (2014). Microbe-host interactions are positively and negatively regulated by galectin-glycan interactions. *Front. Immunol.* 5:284. doi: 10.3389/fimmu.2014.00284

Berger, E. G., Buddecke, E., Kamerling, J. P., Kobata, A., Paulson, J. C., and Vliegenthart, J. F. (1982). Structure, biosynthesis and functions of glycoprotein glycans. *Experientia* 38, 1129–1162. doi: 10.1007/BF01959725

Bermejo, D. A., Jackson, S. W., Gorosito-Serran, M., Acosta-Rodriguez, E. V., Amezcua-Vesely, M. C., Sather, B. D., et al. (2013). *Trypanosoma cruzi* trans-sialidase initiates a program independent of the transcription factors RORgammat and Ahr that leads to IL-17 production by activated B cells. *Nat. Immunol.* 14, 514–522. doi: 10.1038/ni.2569

Bi, S., and Baum, L. G. (2009). Sialic acids in T cell development and function. *Biochim. Biophys. Acta* 1790, 1599–1610. doi: 10.1016/j.bbagen.2009.07.027

Bontempi, I. A., Vicco, M. H., Cabrera, G., Villar, S. R., Gonzalez, F. B., Roggero, E. A., et al. (2015). Efficacy of a trans-sialidase-ISCOMATRIX subunit vaccine candidate to protect against experimental Chagas disease. *Vaccine* 33, 1274–1283. doi: 10.1016/j.vaccine.2015.01.044

Buschiazzo, A., Amaya, M. F., Cremona, M. L., Frasch, A. C., and Alzari, P. M. (2002). The crystal structure and mode of action of trans-sialidase, a key enzyme in *Trypanosoma cruzi* pathogenesis. *Mol. Cell.* 10, 757–768. doi: 10.1016/S1097-2765(02)00680-9

Buschiazzo, A., Muia, R., Larrieux, N., Pitcovsky, T., Mucci, J., and Campetella, O. (2012). *Trypanosoma cruzi* trans-sialidase in complex with a neutralizing antibody: structure/function studies towards the rational design of inhibitors. *PLoS Pathog.* 8:e1002474. doi: 10.1371/journal.ppat.1002474

Campetella, O. E., Uttaro, A. D., Parodi, A. J., and Frasch, A. C. (1994). A recombinant *Trypanosoma cruzi* trans-sialidase lacking the amino acid repeats retains the enzymatic activity. *Mol. Biochem. Parasitol.* 64, 337–340. doi: 10.1016/0166-6851(94)00036-0

Cao, H., and Crocker, P. R. (2011). Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation? *Immunology* 132, 18–26. doi: 10.1111/j.1365-2567.2010.03368.x

Chang, Y. C., and Nizet, V. (2014). The interplay between Siglecs and sialylated pathogens. *Glycobiology* 24, 818–825. doi: 10.1093/glycob/cwu067

Chuenkova, M., and Pereira, M. E. (1995). *Trypanosoma cruzi* trans-sialidase: enhancement of virulence in a murine model of Chagas' disease. *J. Exp. Med.* 181, 1693–1703. doi: 10.1084/jem.181.5.1693

Cremona, M. L., Sanchez, D. O., Frasch, A. C., and Campetella, O. (1995). A single tyrosine differentiates active and inactive *Trypanosoma cruzi* trans-sialidases. *Gene* 160, 123–128. doi: 10.1016/0378-1119(95)00175-6

Crocker, P. R., Paulson, J. C., and Varki, A. (2007). Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* 7, 255–266. doi: 10.1038/nri2056

Crocker, P. R., and Redelinghuys, P. (2008). Siglecs as positive and negative regulators of the immune system. *Biochem. Soc. Trans.* 36, 1467–1471. doi: 10.1042/BST0361467

Daniels, M. A., Devine, L., Miller, J. D., Moser, J. M., Lukacher, A. E., Altman, J. D., et al. (2001). CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation. *Immunity* 15, 1051–1061. doi: 10.1016/S1074-7613(01)00252-7

Daniels, M. A., Hogquist, K. A., and Jameson, S. C. (2002). Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat. Immunol.* 3, 903–910. doi: 10.1038/ni1002-903

Dc-Rubin, S. S., and Schenkman, S. (2012). T rypanosoma cruzi trans-sialidase as a multifunctional enzyme in Chagas' disease. *Cell Microbiol* 14, 1522–1530. doi: 10.1111/j.1462-5822.2012.01831.x

Dos Santos Virgilio, F., Pontes, C., Dominguez, M. R., Ersching, J., Rodrigues, M. M., and Vasconcelos, J. R. (2014). CD8(+) T cell-mediated immunity during *Trypanosoma cruzi* infection: a path for vaccine development? *Mediators Inflamm.* 2014:243786. doi: 10.1155/2014/243786

Egima, C. M., Briones, M. R., Freitas Junior, L. H., Schenkman, R. P., Uemura, H., and Schenkman, S. (1996). Organization of trans-sialidase genes in *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* 77, 115–125. doi: 10.1016/0166-6851(96)02582-0

Erdmann, H., Steeg, C., Koch-Nolte, F., Fleischer, B., and Jacobs, T. (2009). Sialylated ligands on pathogenic *Trypanosoma cruzi* interact with Siglec-E (sialic acid-binding Ig-like lectin-E). *Cell Microbiol.* 11, 1600–1611. doi: 10.1111/j.1462-5822.2009.01350.x

Ferrero, M. R., Heins, A. M., Soprano, L. L., Acosta, D. M., Esteva, M. I., Jacobs, T., et al. (2016). Involvement of sulfates from cruzipain, a major antigen of *Trypanosoma cruzi*, in the interaction with immunomodulatory molecule Siglec-E. *Med. Microbiol. Immunol.* 205, 21–35. doi: 10.1007/s00430-015-0421-2

Frasch, A. C. (1994). Trans-sialidase, SAPA amino acid repeats and the relationship between *Trypanosoma cruzi* and the mammalian host. *Parasitology* 108(Suppl.), S37–S44. doi: 10.1017/S0031182000075703

Freire-de-Lima, L., Alisson-Silva, F., Carvalho, S. T., Takiya, C. M., Rodrigues, M. M., DosReis, G. A., et al. (2010). *Trypanosoma cruzi* subverts host cell sialylation and may compromise antigen-specific CD8+ T cell responses. *J. Biol. Chem.* 285, 13388–13396. doi: 10.1074/jbc.M109.096305

Freire-de-Lima, L., Fonseca, L. M., Oeltmann, T., Mendonca-Previato, L., and Previato, J. O. (2015). The trans-sialidase, the major *Trypanosoma cruzi* virulence factor: three decades of studies. *Glycobiology* 25, 1142–1149. doi: 10.1093/glycob/cwv057

Freire-de-Lima, L., Oliveira, I. A., Neves, J. L., Penha, L. L., Alisson-Silva, F., Dias, W. B., et al. (2012). Sialic acid: a sweet swing between mammalian host and *Trypanosoma cruzi*. *Front. Immunol.* 3:356. doi: 10.3389/fimmu.2012.00356

Galvan, M., Murali-Krishna, K., Ming, L. L., Baum, L., and Ahmed, R. (1998). Alterations in cell surface carbohydrates on T cells from virally infected mice can distinguish effector/memory CD8+ T cells from naive cells. *J. Immunol.* 161, 641–648.

Garg, N., Nunes, M. P., and Tarleton, R. L. (1997). Delivery by *Trypanosoma cruzi* of proteins into the MHC class I antigen processing and presentation pathway. *J. Immunol.* 158, 3293–3302.

Giorgi, M. E., and de Lederkremer, R. M. (2011). Trans-sialidase and mucins of *Trypanosoma cruzi*: an important interplay for the parasite. *Carbohydr. Res.* 346, 1389–1393. doi: 10.1016/j.carres.2011.04.006

Harrington, L. E., Galvan, M., Baum, L. G., Altman, J. D., and Ahmed, R. (2000). Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. *J. Exp. Med.* 191, 1241–1246. doi: 10.1084/jem.191.7.1241

Hart, G. W. (2013). Thematic minireview series on glycobiology and extracellular matrices: glycan functions pervade biology at all levels. *J. Biol. Chem.* 288:6903. doi: 10.1074/jbc.R113.453977

Janeway, C. A. Jr. (2001). How the immune system protects the host from infection. *Microbes Infect.* 3, 1167–1171. doi: 10.1073/pnas.131202998

Kaech, S. M., Hemby, S., Kersh, E., and Ahmed, R. (2002). Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 111, 837–851. doi: 10.1016/S0092-8674(02)01139-X

Kato, K., and Ishiwa, A. (2015). The role of carbohydrates in infection strategies of enteric pathogens. *Trop. Med. Health* 43, 41–52. doi: 10.2149/tmh.2014-25

Leguizamon, M. S., Mocetti, E., Garcia Rivello, H., Argibay, P., and Campetella, O. (1999). Trans-sialidase from *Trypanosoma cruzi* induces apoptosis in cells from the immune system in vivo. *J. Infect. Dis.* 180, 1398–1402. doi: 10.1086/315001

Ley, K., and Kansas, G. S. (2004). Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat. Rev. Immunol.* 4, 325–335. doi: 10.1038/nri1351

Lowe, J. B., and Marth, J. D. (2003). A genetic approach to Mammalian glycan function. *Annu. Rev. Biochem.* 72, 643–691. doi: 10.1146/annurev.biochem.72.121801.161809

Machado, A. V., Cardoso, J. E., Claser, C., Rodrigues, M. M., Gazzinelli, R. T., and Bruna-Romero, O. (2006). Long-term protective immunity induced against *Trypanosoma cruzi* infection after vaccination with recombinant adenoviruses encoding amastigote surface protein-2 and trans-sialidase. *Hum. Gene Ther.* 17, 898–908. doi: 10.1089/hum.2006.17.898

Martin, D. L., Weatherly, D. B., Laucella, S. A., Cabinian, M. A., Crim, M. T., Sullivan, S., et al. (2006). CD8+ T-Cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. *PLoS Pathog.* 2:e77. doi: 10.1371/journal.ppat.0020077

Mendonça-Previato, L., Todeschini, A. R., de Lima, L. F., and Previato, J. O. (2010). The trans-sialidase from *Trypanosoma cruzi* a putative target for trypanocidal agents. *Open Parasitol. J.* 4, 111–115. doi: 10.2174/1874421401004010111

Miller, B. R. III, and Roitberg, A. E. (2013). *Trypanosoma cruzi* trans-sialidase as a drug target against Chagas disease (American trypanosomiasis). *Future Med. Chem.* 5, 1889–1900. doi: 10.4155/fmc.13.129

Ming, M., Chuenkova, M., Ortega-Barria, E., and Pereira, M. E. (1993). Mediation of *Trypanosoma cruzi* invasion by sialic acid on the host cell and trans-sialidase on the trypanosome. *Mol. Biochem. Parasitol.* 59, 243–252. doi: 10.1016/0166-6851(93)90222-J

Moody, A. M., Chui, D., Reche, P. A., Priatel, J. J., Marth, J. D., and Reinherz, E. L. (2001). Developmentally regulated glycosylation of the CD8alphabeta coreceptor stalk modulates ligand binding. *Cell* 107, 501–512. doi: 10.1016/S0092-8674(01)00577-3

Mucci, J., Hidalgo, A., Mocetti, E., Argibay, P. F., Leguizamon, M. S., and Campetella, O. (2002). Thymocyte depletion in *Trypanosoma cruzi* infection is mediated by trans-sialidase-induced apoptosis on nurse cells complex. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3896–3901. doi: 10.1073/pnas.052496399

Mucci, J., Mocetti, E., Leguizamon, M. S., and Campetella, O. (2005). A sexual dimorphism in intrathymic sialylation survey is revealed by the trans-sialidase from *Trypanosoma cruzi*. *J. Immunol.* 174, 4545–4550. doi: 10.4049/jimmunol.174.8.4545

Mucci, J., Risso, M. G., Leguizamon, M. S., Frasch, A. C., and Campetella, O. (2006). The trans-sialidase from *Trypanosoma cruzi* triggers apoptosis by target cell sialylation. *Cell Microbiol.* 8, 1086–1095. doi: 10.1111/j.1462-5822.2006.00689.x

Muia, R. P., Yu, H., Prescher, J. A., Hellman, U., Chen, X., Bertozzi, C. R., et al. (2010). Identification of glycoproteins targeted by *Trypanosoma cruzi* trans-sialidase, a virulence factor that disturbs lymphocyte glycosylation. *Glycobiology* 20, 833–842. doi: 10.1093/glycob/cwq037

Naito-Matsui, Y., Takada, S., Kano, Y., Iyoda, T., Sugai, M., Shimizu, A., et al. (2014). Functional evaluation of activation-dependent alterations in the sialoglycan composition of T cells. *J. Biol. Chem.* 289, 1564–1579. doi: 10.1074/jbc.M113.523753

Neu, U., Bauer, J., and Stehle, T. (2011). Viruses and sialic acids: rules of engagement. *Curr. Opin. Struct. Biol.* 21, 610–618. doi: 10.1016/j.sbi.2011.08.009

Nishikawa, Y., Ogiso, A., Kameyama, K., Nishimura, M., Xuan, X., and Ikehara, Y. (2013). alpha2-3 Sialic acid glycoconjugate loss and its effect on infection with Toxoplasma parasites. *Exp. Parasitol.* 135, 479–485. doi: 10.1016/j.exppara.2013.08.009

Padilla, A. M., Bustamante, J. M., and Tarleton, R. L. (2009). CD8+ T cells in *Trypanosoma cruzi* infection. *Curr. Opin. Immunol.* 21, 385–390. doi: 10.1016/j.coi.2009.07.006

Pappu, B. P., and Shrikant, P. A. (2004). Alteration of cell surface sialylation regulates antigen-induced naive CD8+ T cell responses. *J. Immunol.* 173, 275–284. doi: 10.4049/jimmunol.173.1.275

Parodi, A. J., Pollevick, G. D., Mautner, M., Buschiazzo, A., Sanchez, D. O., and Frasch, A. C. (1992). Identification of the gene(s) coding for the trans-sialidase of *Trypanosoma cruzi*. *EMBO J.* 11, 1705–1710.

Paulson, J. C., Macauley, M. S., and Kawasaki, N. (2012). Siglecs as sensors of self in innate and adaptive immune responses. *Ann. N. Y. Acad. Sci.* 1253, 37–48. doi: 10.1111/j.1749-6632.2011.06362.x

Pereira-Chioccola, V. L., Acosta-Serrano, A., Correia de Almeida, I., Ferguson, M. A., Souto-Padron, T., Rodrigues, M. M., et al. (2000). Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies. *J. Cell Sci.* 113(Pt 7), 1299–1307.

Pereira-Chioccola, V. L., and Schenkman, S. (1999). Biological role of *Trypanosoma cruzi* trans-sialidase. *Biochem. Soc. Trans.* 27, 516–518. doi: 10.1016/S0169-4758(00)01698-7

Pitcovsky, T. A., Buscaglia, C. A., Mucci, J., and Campetella, O. (2002). A functional network of intramolecular cross-reacting epitopes delays the elicitation of neutralizing antibodies to *Trypanosoma cruzi* trans-sialidase. *J. Infect. Dis.* 186, 397–404. doi: 10.1086/341463

Previato, J., Andrade, A., Vermelho, A., Firmino, J., and Mendonça-Previato, L. (1990). Evidence for N-glycolylneuraminic acid incorporation by *Trypanosoma cruzi* from infected animal. *Mem. Inst. Oswaldo Cruz* 85, 38–39. doi: 10.1084/jem.175.2.567

Previato, J. O., Andrade, A. F., Pessolani, M. C., and Mendonca-Previato, L. (1985). Incorporation of sialic acid into *Trypanosoma cruzi* macromolecules. A proposal for a new metabolic route. *Mol. Biochem. Parasitol.* 16, 85–96. doi: 10.1016/0166-6851(85)90051-9

Previato, J. O., Jones, C., Goncalves, L. P., Wait, R., Travassos, L. R., and Mendonca-Previato, L. (1994). O-glycosidically linked N-acetylglucosamine-bound oligosaccharides from glycoproteins of *Trypanosoma cruzi*. *Biochem. J.* 301(Pt 1), 151–159. doi: 10.1093/glycob/11.1.47

Previato, J. O., Jones, C., Xavier, M. T., Wait, R., Travassos, L. R., Parodi, A. J., et al. (1995). Structural characterization of the major glycosylphosphatidylinositol membrane-anchored glycoprotein from epimastigote forms of *Trypanosoma cruzi* Y-strain. *J. Biol. Chem.* 270, 7241–7250. doi: 10.1074/jbc.270.13.7241

Rabinovich, G. A., Baum, L. G., Tinari, N., Paganelli, R., Natoli, C., Liu, F. T., et al. (2002a). Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol.* 23, 313–320. doi: 10.1016/S1471-4906(02)02232-9

Rabinovich, G. A., Rubinstein, N., and Toscano, M. A. (2002b). Role of galectins in inflammatory and immunomodulatory processes. *Biochim. Biophys. Acta* 1572, 274–284. doi: 10.1016/S0304-4165(02)00314-8

Ribeirao, M., Pereira-Chioccola, V. L., Eichinger, D., Rodrigues, M. M., and Schenkman, S. (1997). Temperature differences for trans-glycosylation and hydrolysis reaction reveal an acceptor binding site in the catalytic mechanism of *Trypanosoma cruzi* trans-sialidase. *Glycobiology* 7, 1237–1246. doi: 10.1093/glycob/7.8.1237

Risso, M. G., Pitcovsky, T. A., Caccuri, R. L., Campetella, O., and Leguizamon, M. S. (2007). Immune system pathogenesis is prevented by the neutralization of the systemic trans-sialidase from *Trypanosoma cruzi* during severe infections. *Parasitology* 134, 503–510. doi: 10.1017/S0031182006001752

Ruiz Díaz, P., Mucci, J., Meira, M. A., Bogliotti, Y., Musikant, D., Leguizamon, M. S., et al. (2015). *Trypanosoma cruzi* trans-sialidase prevents elicitation of Th1 cell response via interleukin 10 and downregulates Th1 effector cells. *Infect. Immun.* 83, 2099–2108. doi: 10.1128/IAI.00031-15

Ruiz Rde, C., Rigoni, V. L., Gonzalez, J., and Yoshida, N. (1993). The 35/50 kDa surface antigen of *Trypanosoma cruzi* metacyclic trypomastigotes, an adhesion molecule involved in host cell invasion. *Parasite Immunol.* 15, 121–125. doi: 10.1111/j.1365-3024.1993.tb00591.x

Sadighi Akha, A. A., Berger, S. B., and Miller, R. A. (2006). Enhancement of CD8 T-cell function through modifying surface glycoproteins in young and old mice. *Immunology* 119, 187–194. doi: 10.1111/j.1365-2567.2006.02420.x

Schauer, R. (2000). Achievements and challenges of sialic acid research. *Glycoconj. J.* 17, 485–499. doi: 10.1023/A:1011062223612

Schauer, R., Reuter, G., Muhlpfordt, H., Andrade, A. F., and Pereira, M. E. (1983). The occurrence of N-acetyl- and N-glycoloylneuraminic acid in *Trypanosoma cruzi*. *Hoppe Seylers Z. Physiol. Chem.* 364, 1053–1057. doi: 10.1515/bchm2.1983.364.2.1053

Schenkman, R. P., Vandekerckhove, F., and Schenkman, S. (1993). Mammalian cell sialic acid enhances invasion by *Trypanosoma cruzi*. *Infect. Immun.* 61, 898–902.

Schenkman, S., Eichinger, D., Pereira, M. E., and Nussenzweig, V. (1994). Structural and functional properties of *Trypanosoma* trans-sialidase. *Annu. Rev. Microbiol.* 48, 499–523. doi: 10.1146/annurev.mi.48.100194.002435

Schenkman, S., Jiang, M. S., Hart, G. W., and Nussenzweig, V. (1991). A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. *Cell* 65, 1117–1125. doi: 10.1016/0092-8674(91)90008-M

Schenkman, S., Pontes de Carvalho, L., and Nussenzweig, V. (1992). *Trypanosoma cruzi* trans-sialidase and neuraminidase activities can be mediated by the same enzymes. *J. Exp. Med.* 175, 567–575. doi: 10.1084/jem.175.2.567

Schmaus, A., Bauer, J., and Sleeman, J. P. (2014). Sugars in the microenvironment: the sticky problem of HA turnover in tumors. *Cancer Metastasis Rev.* 33, 1059–1079. doi: 10.1007/s10555-014-9532-2

Sharon, N. (1983). Lectin receptors as lymphocyte surface markers. *Adv. Immunol.* 34, 213–298. doi: 10.1016/S0065-2776(08)60380-6

Stowell, S. R., Ju, T., and Cummings, R. D. (2015). Protein glycosylation in cancer. *Annu. Rev. Pathol.* 10, 473–510.

Tarleton, R. L. (2007). Immune system recognition of *Trypanosoma cruzi*. *Curr. Opin. Immunol.* 19, 430–434. doi: 10.1016/j.coi.2007.06.003

Tarleton, R. L. (2015). CD8+ T cells in *Trypanosoma cruzi* infection. *Semin. Immunopathol.* 37, 233–238. doi: 10.1007/s00281-015-0481-9

Todeschini, A. R., Dias, W. B., Girard, M. F., Wieruszeski, J. M., Mendonca-Previato, L., and Previato, J. O. (2004). Enzymatically inactive trans-sialidase from *Trypanosoma cruzi* binds sialyl and beta-galactopyranosyl residues in a sequential ordered mechanism. *J. Biol. Chem.* 279, 5323–5328. doi: 10.1074/jbc.M310663200

Todeschini, A. R., Girard, M. F., Wieruszeski, J. M., Nunes, M. P., DosReis, G. A., Mendonca-Previato, L., et al. (2002a). trans-Sialidase from *Trypanosoma cruzi* binds host T-lymphocytes in a lectin manner. *J. Biol. Chem.* 277, 45962–45968. doi: 10.1074/jbc.M203185200

Todeschini, A. R., Mendonca-Previato, L., Previato, J. O., Varki, A., and van Halbeek, H. (2000). Trans-sialidase from *Trypanosoma cruzi* catalyzes sialoside hydrolysis with retention of configuration. *Glycobiology* 10, 213–221. doi: 10.1093/glycob/10.2.213

Todeschini, A. R., Nunes, M. P., Pires, R. S., Lopes, M. F., Previato, J. O., Mendonca-Previato, L., et al. (2002b). Costimulation of host T lymphocytes by a trypanosomal trans-sialidase: involvement of CD43 signaling. *J. Immunol.* 168, 5192–5198. doi: 10.4049/jimmunol.168.10.5192

Traving, C., and Schauer, R. (1998). Structure, function and metabolism of sialic acids. *Cell. Mol. Life. Sci.* 54, 1330–1349. doi: 10.1007/s000180050258

Tribulatti, M. V., Mucci, J., Van Rooijen, N., Leguizamon, M. S., and Campetella, O. (2005). The trans-sialidase from *Trypanosoma cruzi* induces thrombocytopenia during acute Chagas' disease by reducing the platelet sialic acid contents. *Infect. Immun.* 73, 201–207. doi: 10.1128/IAI.73.1.201-207.2005

Tzelepis, F., de Alencar, B. C., Penido, M. L., Gazzinelli, R. T., Persechini, P. M., and Rodrigues, M. M. (2006). Distinct kinetics of effector CD8+ cytotoxic T cells after infection with *Trypanosoma cruzi* in naive or vaccinated mice. *Infect. Immun.* 74, 2477–2481. doi: 10.1128/IAI.74.4.2477-2481.2006

Uemura, H., Schenkman, S., Nussenzweig, V., and Eichinger, D. (1992). Only some members of a gene family in *Trypanosoma cruzi* encode proteins that express both trans-sialidase and neuraminidase activities. *EMBO J.* 11, 3837–3844.

Vandekerckhove, F., Schenkman, S., Pontes de Carvalho, L., Tomlinson, S., Kiso, M., Yoshida, M., et al. (1992). Substrate specificity of the *Trypanosoma cruzi* trans-sialidase. *Glycobiology* 2, 541–548. doi: 10.1093/glycob/2.6.541

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 3, 97–130. doi: 10.1093/glycob/3.2.97

Varki, A. (2011). Evolutionary forces shaping the Golgi glycosylation machinery: why cell surface glycans are universal to living cells. *Cold Spring Harb. Perspect. Biol.* 3, 1–14. doi: 10.1101/cshperspect.a005462

Varki, A., and Angata, T. (2006). Siglecs–the major subfamily of I-type lectins. *Glycobiology* 16, 1R–27R. doi: 10.1093/glycob/cwj008

Varki, A., and Gagneux, P. (2012). Multifarious roles of sialic acids in immunity. *Ann. N. Y. Acad. Sci.* 1253, 16–36. doi: 10.1111/j.1749-6632.2012.06517.x

Varki, A., and Schauer, R. (2009). "Sialic acids," in *Essentials of Glycobiology*, 2nd Edn, eds A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, et al. (New York, NY: Cold Spring Harbor).

von Gunten, S., and Bochner, B. S. (2008). Basic and clinical immunology of Siglecs. *Ann. N. Y. Acad. Sci.* 1143, 61–82. doi: 10.1196/annals.1443.011

Wu, W., Harley, P. H., Punt, J. A., Sharrow, S. O., and Kearse, K. P. (1996). Identification of CD8 as a peanut agglutinin (PNA) receptor molecule on immature thymocytes. *J. Exp. Med.* 184, 759–764. doi: 10.1084/jem.184.2.759

Zingales, B., Carniol, C., de Lederkremer, R. M., and Colli, W. (1987). Direct sialic acid transfer from a protein donor to glycolipids of trypomastigote forms of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* 26, 135–144. doi: 10.1016/0166-6851(87)90137-X

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REVIEW
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Immune Response of Amebiasis and Immune Evasion by *Entamoeba histolytica*

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Entamoeba histolytica is a protozoan parasite and the causative agent of amebiasis. It is estimated approximately 1% of humans are infected with *E. histolytica*, resulting in an estimate of 100,000 deaths annually. Clinical manifestations of amebic infection range widely from asymptomatic to severe symptoms, including dysentery and extra-intestinal abscesses. Like other infectious diseases, it is assumed that only ~20% of infected individuals develop symptoms, and genetic factors of both the parasite and humans as well as the environmental factors, e.g., microbiota, determine outcome of infection. There are multiple essential steps in amebic infection: degradation of and invasion into the mucosal layer, adherence to the intestinal epithelium, invasion into the tissues, and dissemination to other organs. While the mechanisms of invasion and destruction of the host tissues by the amebae during infection have been elucidated at the molecular levels, it remains largely uncharacterized how the parasite survive in the host by evading and attacking host immune system. Recently, the strategies for immune evasion by the parasite have been unraveled, including immunomodulation to suppress IFN-γ production, elimination of immune cells and soluble immune mediators, and metabolic alterations against reactive oxygen and nitrogen species to fend off the attack from immune system. In this review, we summarized the latest knowledge on immune reaction and immune evasion during amebiasis.

Keywords: ***Entamoeba histolytica*, cysteine protease, glycosidase, mucin, phagocytosis, oxidative stress, metabolism**

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INTRODUCTION

Entamoeba histolytica is an enteric protozoan parasite that infects humans, and is the etiological agent of amebiasis. Amebiasis remains a worldwide health problem accounting for up to 100,000 deaths annually (1, 2). Transmission occurs via ingestion of food and water contaminated with amebic cysts (1, 3, 4). In endemic areas, exposure can be extremely high: an annual incidence of 40% was estimated among children in an urban slum in Bangladesh (5). In some parts of Asia and Australia, amebiasis is endemic among men who have sex with men (MSM) and can be transmitted sexually (6–9). Majority of infections with *E. histolytica* remain asymptomatic, while ~20% of the cases develop clinical manifestations, such as dysentery, which is characterized by colonic mucosal invasion and tissue destruction (10). Invasive disease includes dysentery and extra-intestinal amebiasis, most commonly amebic liver abscesses (ALAs), which occur in approximately 1% of symptomatic cases in developing countries and around 17% in Japan (11, 12).

When amebic trophozoites invade the colonic epithelium, they activate immune response in the human host. In order to survive in the host, the repression of host immune systems and the control of the environment of parasitism are crucial. For instance, during extraintestinal dissemination, the amebae must transiently survive in the blood vessels and the spleen, in which a network of immune cells and humoral factors are present, and the amebae are exposed to high concentrations of oxygen (*E. histolytica* are anaerobic or microaerophilic). To persist in such environment, amebae must subvert detection by antibody and complement, and resist oxidative and nitrosative attack. In this review, we summarize our current knowledge on immune response during amebic infection (**Figure 1**) and the parasite's strategies to evade from host immune system (**Figure 2**).

IMMUNE RESPONSE DURING AMEBIC INFECTION

Course of Amebic Infection

Entamoeba histolytica infection is initiated by parasite adherence to the colonic mucin layer. Trophozoites express a galactose

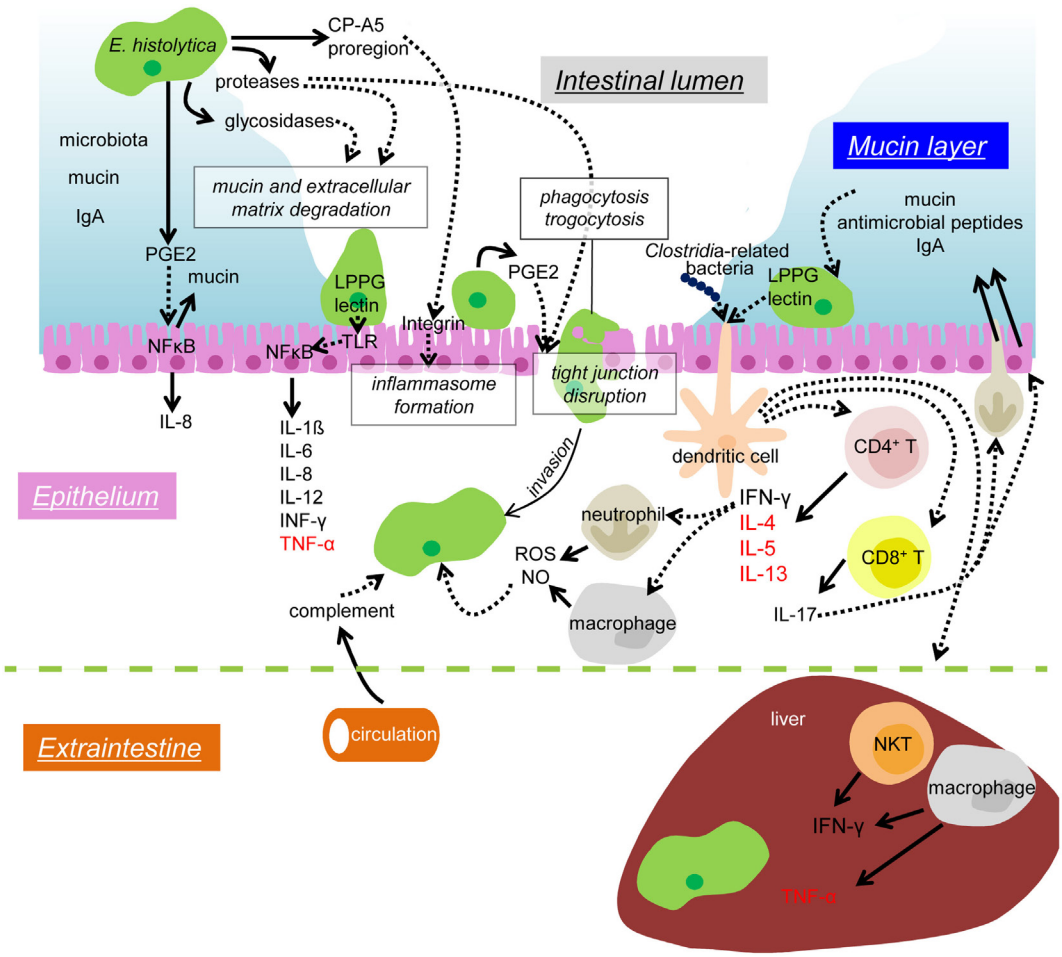

FIGURE 1 | Mechanisms of colonization and invasion by *E. histolytica* trophozoites and host immune responses to suppress and control amebic infection. In the lumen of the large intestine, the IEC layer is covered by the mucus layer (blue), which contains secreted mucin and IgA from the host and commensal microbiota. Proteases and glycosidases secreted from the amebae are involved in the degradation of mucin and extracellular matrix. The pro-domain of EhCP-A5 binds to and activate integrin and enhances the inflammasome formation of leading to pro-inflammatory responses. PGE2 also secreted from the amebae causes mucin hypersecretion and depletion of mucin from the IECs. PGE2 also elicits signaling in a cascade leading to NFκB activation in the IECs and induces IL-8 secretion. The Gal/GalNAc lectin (lectin) and LPPG on the ameba's surface binds to TLR2 and leads to NFκB activation and pro-inflammatory cytokine release for IEC. PGE2 also helps to disrupt tight junction function of the epithelium and enhances the amebic infiltration. Phagocytosis and trogocytosis are also involved in removal of host cells and invasion into the host tissue. Infiltrating trophozoites are attacked by complement from the circulation, ROS and NO from neutrophils and macrophages. The Gal/GalNAc lectin and LPPG activate CD4, CD8 T cells, and NKT cells, and, thus, enhances protective cellular immunity. CD4 T cells produce IFN-γ, IL-4, IL-5, and IL-13, and CD8 T cells produce IL-17. IL-17 induces neutrophil infiltration and enhances secretion of mucin, antimicrobial peptides, and IgA into the colonic lumen. When disseminated to the liver, the amebae are attached and removed by the dense mediated by IFN-γ secreted by NKT cells. TNF-α secreted from hepatic macrophages leads to abscess formation. Solid arrows depict secretion of soluble proteins and dotted arrows indicate interaction or signal transduction. Cytokines mainly beneficial for an elimination of the amebae are shown in black, while those involved in disease manifestations are shown in red.

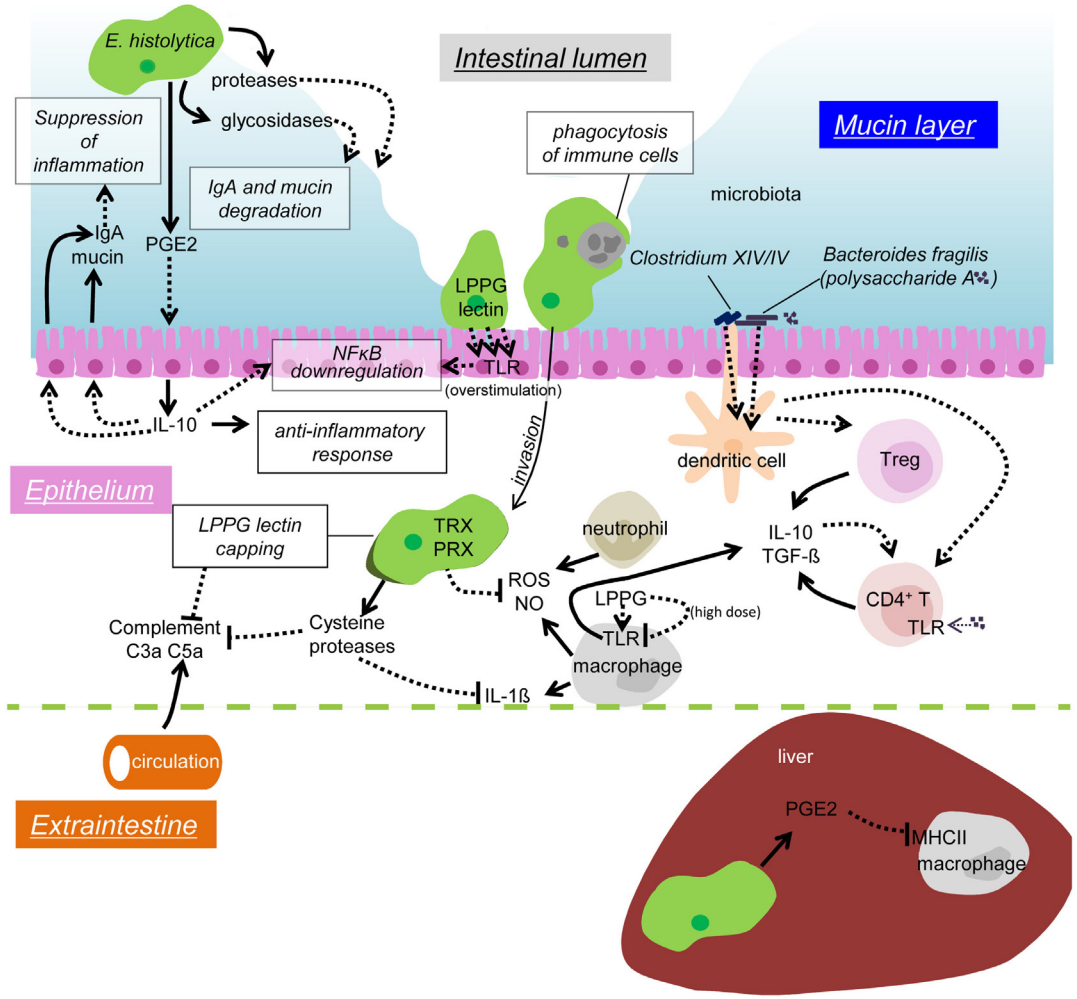

FIGURE 2 | Possible mechanisms of immune evasion during amebiasis. Secreted or surface proteases of the amebae degrade IgA in the mucosal layer. PGE2 from the amebae induces IL-10 secretion from the IECs, and in turn stimulates mucin and IgA secretion, which likely prevents unnecessary inflammation. Overstimulation of TLR causes downregulation of NFκB activation. Removal of infiltrating immune cells by phagocytosis/trogocytosis helps to reduce immune responses. Some commensal microbiota, namely *Clostridium* XIV and IV groups and *Bacteroides fragilis,* induce Treg cells to downregulate immune responses. Polysaccharide A from *B. fragilis* binds to TLR2 on CD4 T cells and induces IL-10 production. The amebae in the tissues and the blood stream evade from complement by surface receptor capping (LPPG, lectin) and degradation of C3a and C5a by cysteine proteases. Cysteine proteases also degrade IL-1β, antioxidative stress defense by the TRX and PRX systems fends off the attack from ROS and NO from activated neutrophils and macrophages. LPPG binds to TLR2 on monocytes and macrophages, which leads to secretion of cytokines, including IL-10 and TGF-β. High doses of LPPG downregulate TLR2 gene expression in monocyte and cause negative feedback of protective immune responses. PGE2 from the amebae and the host causes downregulation of MHC class II expression on macrophages in the liver, which results in anti-inflammation.

and *N*-acetyl-D-galactosamine specific lectin (Gal/GalNAc lectin) on the cell surface and attach to host mucin and colonic epithelial cells (13). The colonized parasites are capable of extensive tissue destruction. Beside the pore-forming proteins, amoebapores (14, 15), hydrolytic enzymes, particularly cysteine proteases (CP), are considered to be essential weapons of the parasite to penetrate the epithelium and destroy components of the host's extracellular matrix (ECM) (16–20). During and after penetration into the submucosal region, amebic trophozoites interact directly and indirectly with host immune and non-immune cells.

Humoral Immunity

While the mucosal layer in the gastrointestinal tract generally serves as a primary physical barrier against intestinal pathogens, the intestinal immune response is the secondary defense to *E. histolytica* infection. Mucosal immunoglobulins (Ig) are the major component of the human intestinal defense mechanism (21). Among them, secretory IgA is one of the most abundant Ig produced by plasma cells and functions by preventing pathogens from adhering and removing the mucosal barrier (21). Haque and colleagues showed that the presence of Gal/GalNAc lectin-specific IgA antibodies in the stool correlated with reduced

re-infection rates with *E. histolytica* in a study on susceptible children from Bangladesh (5, 22, 23). This implication was also confirmed with patients who had recovered from ALA. Increases in anti-Gal/GalNAc lectin IgA antibodies in post-ALA patients were associated with clearance of subsequent amebic infections, demonstrating that post-ALA patients developed a higher immune responsiveness and maintained immunological memory (24, 25). On the other hand, IgG levels have either protective or non-protective effects on the susceptibility to amebic infections depending upon major IgG subclasses induced by infection (i.e., IgG1 and IgG2 induced by Th2 and Th1, respectively) (26, 27).

Cell-Mediated Immunity

Cell-mediated immune responses are also important for host defense against *E. histolytica*. During the initial stage of infection, intestinal epithelial cells (IECs) bind to and recognize the carbohydrate recognition domain of the Gal/GalNAc lectin via toll-like receptor (TLR)-2/4, which activates NFκB and leads to the production of inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α (28–30). IECs are the second line of barriers against pathogens after the mucosal layer and the first line of host cells to encounter microbial/parasite antigens, they express an array of pathogen recognition receptors (PRRs), including TLRs (31). IFN-γ is involved in clearance of infection, whereas IL-4 and TNF-α are associated with disease (32–35). In fact, IFN-γ production by peripheral mononuclear cells was shown to be correlated with protection from future *E. histolytica* infection in children (36) and the serum level of IL-4 was high in patients with invasive amebiasis (27, 37). It has been also shown that IFN-γ-producing CD4+ T cells and IL-17-producing CD8+ T cells are involved protection in vaccinated mice (38, 39). IL-17 plays multiple roles in protection against amebic infection, including induction of secretion of mucin and antimicrobial peptides, increase in IgA transport across the intestinal epithelium, and promotion of neutrophil infiltration (40–43).

IFN-γ-activated neutrophils and macrophages have amebicidal activity *in vitro* (44, 45). *In vivo*, neutrophils predominated in amebic lesions where the macrophages were infrequent, suggesting importance of neutrophils for clearance of amebae (46). Production of reactive oxygen species (ROS) and nitric oxide (NO) via NAD(P)H oxidase complex and iNOS, respectively, play critical roles in killing trophozoites (45, 47). In experimental ALA, protection was mediated by IFN-γ from natural killer T cells (NKTs), while TNF-α-producing macrophages increased tissue damage (32, 33). Taken together, both humoral and cell-mediated immune responses play important roles against amebic infection.

MICROBIOTA-MEDIATED MODIFICATIONS OF PARASITE VIRULENCE AND HOST IMMUNE RESPONSE

Microbiota Affects Energy Metabolism and Growth of the Amebae

The adult human intestine has trillions of bacteria composed of more than hundreds of species. Recent studies have suggested that the intestinal bacterial microbiota may influence the outcome of protozoan infections (48, 49). The growth and survival of *E. histolytica* trophozoites depends on nutrients from the host and the microbiota. The bacterial microbiota produces glycosidases that degrade complex polysaccharides into forms available for the absorption by the amebae and the host (50). Microbial glycosidase activity determines the levels of free colonic carbohydrates (the glycobiome). Thus, microbiota potentially influences central energy metabolism of *E. histolytica* trophozoites. Since *E. histolytica* has numerous glycosidases, including amylases, β-hexosaminidases, and lysozymes encoded in its genome (51–55), and can degrade a panel of polysaccharides to yield monocarbohydrates, the activity and regulation of amebic glycosidases also influence available carbohydrate concentrations.

Microbiota Influences the Parasite's Virulence

The commensal bacteria are generally protective against enteric pathogens. However, *E. histolytica* infection requires the presence of enteric bacteria. Germ-free animals were resistant to *E. histolytica* infection, but the introduction of a single bacterial species restored amebic pathogenesis (56, 57).

It has been shown that axenization (i.e., removal of associating bacteria) of xenically cultivated trophozoites decreases virulence, and incubation of axenic trophozoites with live bacteria restored virulence in a contact-dependent manner (58, 59). Incubation of *E. histolytica* trophozoites with the enteropathogenic *Escherichia coli* (EHEC) or *Shigella dysenteriae* increased amebic adherence to and cytotoxicity against MDCK cell monolayer (60). These observations indicate the enteric microbiota influence *E. histolytica* virulence during human infection (also see Perturbation of the Enteric Microbiota by *E. histolytica*).

The microbiota-dependent glycobiome has an emerging role in regulating the virulence of enteric pathogenic bacteria, such as EHEC, *Clostridium difficile,* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) (61–63). EHEC has a fucose-responsive regulator of virulence genes, while *C. difficile* growth is promoted with high concentrations of free sialic acid reproduced by sialidase from associating bacteria (*Bacteroides thetaiotaomicron*). Similarly, the *in vivo* virulence of *S. typhimurium* was shown to be dependent on both fucose and sialic acid (63). It has been shown that glucose starvation enhances *E. histolytica* virulence, motility, and lectin expression via URE-3BP (64, 65). This finding suggests that the ameba has an ability to sense glucose (and possibly other monosugars) to modulate its virulence. The amebae and the bacterial microbiota influence each other by providing energy source and degrading available carbohydrates.

Microbiota Affects Host Immune Response

Gut microbiota plays a number of physiological roles involving digestion, metabolism, extraction of nutrients, synthesis of vitamins, prevention against colonization by pathogens, and immunomodulation (66–68). It has been demonstrated

that *Bacteroides fragilis* and cluster XIV and IV of *Clostridium* species induce the development of regulatory T (Treg) cells in the colon (69, 70). Treg cells have the ability to suppress inflammatory responses through the production of anti-inflammatory cytokines, including IL-10 and TGF-β, and are considered to be involved in self-tolerance (71, 72). The beneficial effect of *B. fragilis* depends on the expression of polysaccharide A, which is a unique surface polysaccharide that binds to TLR2 on CD4+ T cells (72). Microbiota-mediated immunomodulation is evident in several diseases, e. g., Rheumatoid arthritis, diabetes, obesity, and cancer (73–78). It seems that *E. histolytica* requires the intestinal microbiota for pathogenesis, and, conversely, the parasite also needs to disrupt the homeostasis of the microbiota during infection.

STRATEGIES FOR IMMUNE EVASION OF *E. histolytica*

Disruption of Host Physical Barriers and Soluble Immune Mediators by Hydrolases

Glycosidases

Hydrolases secreted by *E. histolytica* trophozoites are involved in the elimination of immune cells and degradation and/or activation of soluble immune mediators, as well as disruption of the host gut and liver epithelia (29, 55, 79–84). The mucosal layer between the lumen and the epithelia forms a physical barrier. Degradation of carbohydrates in the barrier is crucial for the initiation of colonization by the amebae. Human intestinal mucus is mainly composed of highly glycosylated mucins (85). Among >20 human mucins, MUC2 is the major gel-forming mucin secreted by goblet cells of the small and large intestines (86, 87). When the amebae colonize the colonic epithelia, they binds to secreted mucin oligosaccharides with the Gal/GalNAc lectin and penetrate through the mucosal layer. In this process, the amebae decompose the mucin barrier to finally reach and subsequently attach on IEC (13).

Secreted proteins by *E. histolytica* trophozoites displayed glycosidase activities, including β-*N*-acetyl-D-glucosaminidase, α-D-glucosidase, β-D-galactosidase, β-L-fucosidase, and α-*N*-acetyl-D-galactosaminidase (88). Among these glycosidases, β-*N*-acetyl-D-glucosaminidase showed the highest activity (88, 89). Thus, β-*N*-acetyl-D-glucosaminidase activity likely have a central role in degrading carbohydrates on mucin and exposing its protein backbone (88). It was previously demonstrated that the amount of intracellular and secreted β-*N*-acetyl-D-glucosaminidase activity increased by complement in the serum (90, 91). Huldt et al. also suggested that hexosaminidase activity plays a role in the amebic virulence (90).

Recently, it has been shown that knock down of a β-amylase gene by siRNA caused reduction in the degradation of the mucosal layer and the invasion into the human colon in an *ex vivo* experiment (55). Furthermore, the β-amylase gene was found upregulated after contact with colon tissues (55). Mucin degradation by amebic glycosidases may also affect the central metabolism of the amebae *per se* and also the microbiota equilibrium in the colon since highly glycosylated mucin is a carbon source for the amebae and the colonic microbiota (92, 93).

Cysteine Proteases

The *E. histolytica* genome has ~50 genes encoding CPs (20), which likely reflects robust biological importance of CPs. Of these, however, only four proteins, EhCP-A1, EhCP-A2, EhCP-A5, and EhCP-A7, are highly expressed under culture conditions and altogether account for more than 90% of the proteolytic activity in trophozoite extracts (94). After mucin was digested by amebic glycosidases, the protein backbone of mucin is degraded by robust CPs. Altogether, these mucin-digesting glycosidases and proteases are the ameba's first line strategy to overcome the innate defense of the mucus barrier.

As suggested by various studies, among the four major CPs, EhCP-A5 appears to play a pivotal role in virulence, including immunomodulation (80, 95–102). EhCP-A5 has a capacity to bind integrin via the RGD motif in the pro region, and elicits pro-inflammatory response in Caco-2 cells *in vitro* and the murine colon via NLRP3 inflammasome activation independent of the CP activity (100, 102, 103). CPs are also known to modulate cell-mediated immunity by activating pro-inflammatory cytokines and also modulate humoral immunity (see below).

Involvement of Glycosidases and Proteases for Extraintestinal Propagation

When *E. histolytica* trophozoites propagate extraintestinally, they take a route similar to that of cancer metastasis (104), which requires both glycosidases and proteases for the disintegration of the basement membrane and entry into circulation (105–107). In case of ALA, amebic glycosidases and proteases are also needed to survive in the blood vessels (see Degradation of Immunoglobulins and Complements), and to destroy Kupffer cells, the epithelial cells, ECM, and hepatocytes in the liver. Thibeaux and colleagues have recently demonstrated that EhCP-A5 secreted from the amebae activates host matrix metalloproteases (MMP), a well-known mediator of ECM degradation (84). Recombinant EhCP-A5 restored the invasiveness of the *EhCP-A5* gene-silenced trophozoites, suggesting that proteases from both the ameba and the host contribute to the tissue invasion process. In contrast to proteases, the roles of glycosidases in pathophysiology of amebiasis are not well demonstrated. It is evident in cancer metastasis that the level of serum β-hexosaminidase correlates with the likeliness of liver metastasis in variety of cancers, including colon, breast, stomach, pancreas, small bowel, kidney, testis, melanoma, lymphoma, and myeloma (108). Increased levels of tissue β-hexosaminidase were also reported for breast, kidney, pancreas, thyroid, colon, ovary, brain, salivary gland, stomach, and larynx cancers (109–112). Thus, it is conceivable by analogy that amebic glycosidases are involved in tissue invasion and extraintestinal dissemination.

Degradation of Immunoglobulins and Complements

As described above, the major component responsible for the intestinal immune response against amebic infection is secreted

Igs. It was demonstrated that anti-Gal/GalNAc lectin IgA reduces trophozoite colonization in the colon (5, 23, 25, 113–117). Intriguingly, *E. histolytica* surface-associated CP [most likely EhCP-A5, (118)] cleaves human IgA (16, 119). Amebic CPs are capable of cleaving both isotypes, i.e., IgA1 and IgA2 (119, 120). Furthermore, amebic CPs can also inactivate circulating IgG and, thus, believed to be involved in the survival during tissue invasion and extraintestinal propagation (18). Degradation of IgG in the blood could prevent activation of the classical pathway of the complement system and immune cells that harbor Fc receptors (19).

When the trophozoites are exposed to the intravascular immune system, complements are the major component that mediates trophozoite destruction. *E. histolytica* trophozoites evade from a complement attack by cleaving and inactivating anaphylatoxins C5a and C3a with CPs (79). C5a and C3a are potent activators of inflammation and enhance the release of histamine from mast cells, lysosomal enzymes from leukocytes, and pro-inflammatory cytokines, including IL-6 and TNF-α, from macrophages (121–123). C5a and C3a also increase vascular permeability and attract immune cells (122, 123). Reduction of these anaphylatoxins detracts from immune detection of the amebae in the blood and reduces inflammation in amebic lesions. It also partially explains the lack of severe inflammation in advanced colitis and ALA region.

Degradation of Cytokines

Cysteine proteases are also known to modulate cell-mediated immunity by activating pro-inflammatory cytokine IL-1β and inactivation of pro- and mature IL-18 (82, 124). It is not concluded, however, if these changes are protective against or deleterious for amebic infection.

Cell Surface Decorations to Evade Host Immunity

Glycosylphosphatidylinositol-Anchored Proteins

Entamoeba histolytica is also capable of evading from complement attach by decorating their surface with glycosylphosphatidylinositol (GPI)-anchored proteins. GPI is a glycolipid required for anchoring many proteins and glycoconjugates to the cell surface in most of eukaryotes (125–127). *E. histolytica* trophozoites expose on their cell surface a complex GPI-anchored glycoconjugate, designated lipopeptidophosphoglycan (LPPG) (128, 129). LPPG on the cell surface is a component of glycocalyx that is composed of oligosaccharides of glycoproteins and glycolipids and afford trophozoites protection by creating an impervious layer to complement (130, 131). It was demonstrated that complement-susceptible *Entamoeba dispar* trophozoites possess a much thinner structure of LPPG-containing glycocalyx, which is consistent with the premise that LPPG is important for the evasion from complement (130). It is also known that antibody against human CD59, a cell surface protein that prevents auto-lysis by inhibiting the formation of the membrane attack complex (MAC) antibody cross-reacts with Gal/GalNAc lectin and a 21 kDa surface protein (132, 133). Later, it was shown that the Gal/GalNAc lectin contains a CD59-like region on the cell surfaces that prevents MAC formation (132). These data suggest that the Gal/GalNAc lectin is a cross-reactive CD59 homolog of the ameba and have a similar function as CD59. In agreement with these results, global inhibition of GPI-anchor formation leaves *E. histolytica* trophozoites susceptible to complement-mediated lysis (131). However, functionality of 21 kDa protein as an inhibitor of MAC formation and its molecular identity has yet to be elucidated.

Surface Receptor Capping

Surface receptor capping is another strategy to hide from the immune system by disposing of the surface molecules that have been recognized by Igs or complements (134, 135). During cell movement, surface-bound immune complexes are translocated toward the uroid, where capped ligands accumulate (136). This polar re-distribution can be induced by concanavalin A (Con A) or anti-amebic polyclonal antibodies (137). It has been reported that serine protease, *E. histolytica* rhomboid protease (ROM1), is involved in the translocation of the complex to the base of the caps and subsequent release of the materials in the cap (135, 138). It is of note that ROM1 also cleaves the transmembrane domain of the heavy subunit of the Gal/GalNAc lectin (138). As the lectin heavy subunit is highly immunogenic, its release from the plasma membrane by ROM1 may interfere with host immune response directed to amebae.

Killing and Phago/Togocytosis of Immune Cells

Contact-Dependent Cell Killing

Immobilization and killing of immune cells also serves as an ameba's strategy for evasion from immune surveillance. Amebic trophozoites are able to kill a variety of cells, including neutrophils, T lymphocytes, macrophages, and a variety of tissue culture lines (116, 139–141). Adherence of the ameba triggers multiple intracellular events leading to cytotoxic effects to the mammalian cells. Such events include increased intracellular Ca^{2+}, production of ROS, loss of membrane integrity, DNA fragmentation, phosphatidylserine exposure on the cell surface, and caspase-3 activation (116, 117, 139–144). It was reported that after host cell killing, *E. histolytica* preferentially ingest the dead cells (117, 140, 143). This observation is consistent with the theory that clearance of dead cells and debris by phagocytosis helps to minimize pro-inflammatory responses (145, 146). A phagocytosis-defective line of *E. histolytica* apparently showed decreased virulence *in vitro* and *in vivo*, suggesting a potential causal link between phagocytosis and virulence (147, 148).

Huston and colleagues demonstrated that *E. histolytica* preferentially ingests apoptotic Jurkat cells via recognition of phosphatidylserine and collectins (140, 149). Amebic calreticulin was found to be the surface receptor for host C1q, and required for phagocytosis of apoptotic cells, but it did not directly mediate cell killing (150). A few recent studies have started to unveil the detailed molecular mechanisms involved in the ameba

phagocytosis (151, 152). However, the molecular events that take place in host immune cells in particular to suppress (or augment) immune response, together with a missing link between the surface receptor to the internalization machinery, remains totally unknown.

Trogocytosis

Ralston and colleagues have recently reported *E. histolytica* trophozoites ingested pieces of intact living cells via trogocytosis ("*trogo*" = nibbling) (153). When trophozoites were incubated with a combination of live and pre-killed host cells (Jurkat T cells), the live cells were ingested by trogocytosis, while the pre-killed host cells were ingested as a whole by canonical phagocytosis. Trogocytosis is an active process that resembles phagocytosis in some ways, i.e., it requires physiological temperature, actin rearrangements, Gal/GalNAc lectin, C2 domain-containing protein kinase, and phosphatidylinositol 3-phosphate kinase signaling, and it is accompanied with a rapid rise in intracellular Ca^{2+} concentrations. Trogocytosed host cells finally were killed. Trogocytosis of murine IEC was also evident in the *in vivo* animal model, suggesting that both trogocytosis of live host cells and phagocytosis of dead cells are important for pathogenesis and sustained parasitism of *E. histolytica*. Since amebic contact can potentially results in multiple outcomes: apoptosis and necrosis, followed by phagocytosis, or trogocytosis, it remains to be elucidated what factors and conditions differentiate these distinct manners of killing and ingestion of target host cells.

IFN-γ

Entamoeba histolytica regulates IFN-γ for survival in the host. In CBA mice, which are susceptible to *E. histolytica* cecal infection, the amebic infection led to upregulation of Th2 (IL-4, IL-5, and IL-13) and Th17 (IL-17) cytokine responses, while Th1 cytokines, IL-12p35 and IFN-γ, were suppressed (154). This indicates that suppression of INF-γ causes susceptibility of amebiasis. From cohort studies in Bangladesh, susceptible children with malnutrition showed lower IFN-γ levels (36, 155). Analysis of asymptomatic carriers of *E. histolytica* showed that carriers had higher levels of IFN-γ, while patients with invasive amebiasis displayed higher levels of IL-4 (35). The significance of IFN-γ in susceptibility is also implicated for ALA. It is known that more than 80% of all ALA cases occur in adult males (156–158), and the male predominance is attributable to testosterone (159). Lotter and colleagues showed that testosterone inhibits IFN-γ secretion from invariant natural killer T (iNKT) cells stimulated by LPPG, a physiological ligand for CD1d (159). iNKT cells are a subset of NKT cells that recognize lipid antigens in the context of CD1d and produce IFN-γ and IL-4. *E. histolytica* LPPG is presented on CD1d to invariant TCR and activates iNKT cells in combination with TLR signaling. αGalCer, a CD1d agonist, stimulates production of both IFN-γ and IL-4, whereas LPPG induces IFN-γ but not IL-4 production (33). These data suggest that iNKT cells provide a link between innate and adaptive immunity due to their capacity to produce large amounts of IFN-γ and IL-4 that can bias the immune response into either a Th1 or Th2 direction. Production of IFN-γ helps clearance of *E. histolytica* infection and controls abscess formation, whereas an adequate level of IFN-γ reduces the trophozoite number and pro-inflammatory response at a low level, and may balance for trophozoites to survive.

IL-10

It is known that anti-inflammatory cytokine, IL-10, plays a critical role to maintain the mucosal barrier. IL-10-deficient mice have compromised and highly permeable mucosal barriers and develop spontaneous intestinal inflammation in response to normal microflora (160). A murine amebic colitis model demonstrated that IL-10 from hematopoietic cells (CD4+ T cells) acting upon the non-hematopoietic compartment (IEC) is required for innate resistance to parasite invasion (161). Furthermore, it has been shown that IL-10 enhances MUC2 production, suppresses activation of antigen-presenting cells, induces B cell class-switching to IgA, has anti-apoptotic effects on IECs, reduces pro-inflammatory NFκB signaling in IECs, and promotes induction of CD4+ Treg cells (162–165). Interestingly, in asymptomatic carriers, no elevation of IL-10 level was observed. On the other hand, the IL-10 level was increased in dysenteric and ALA patients (27, 37). These studies indicate that invasion of the colon and liver by *E. histolytica* elicits an anti-inflammatory immune response and may successfully suppress immune reaction to the amebae. Altogether, the ameba needs to balance IL-10 and inflammatory cytokine levels to establish infection. It was shown that peritoneal monocytes and macrophages exposed to LPPG secreted TNF-α, IL-6, IL-8, IL-12, and IL-10 via TLR2 (166). It has been also shown that high doses of LPPG down-regulated TLR2 gene expression (166, 167). Thus, LPPG-driven signaling may activate a negative feedback loop that attenuates inflammatory responses. The mechanisms of the suppression of IL-10 production by the ameba remain to be elucidated (see below).

Suppression of NFκB in IECs

Entamoeba histolytica trophozoites secrete materials that induce a protective response in human IECs (168, 169), the first line of host cells to encounter microbial antigens, via PRRs, including TLRs. Upon binding to their ligand, PRRs trigger activation of a transcription factor NFκB. Gut homeostasis requires continuous activation of NFκB by TLR signaling in response to intestinal bacteria (170), commensal microbes can also disrupt NFκB signaling to attenuate pro-inflammatory IEC responses (171). It has been shown that secreted components from *E. histolytica* trophozoites induce a protective response in human IECs that primed by macrophage secretions through suppression of NFκB via heat shock protein response and increase resistance of IECs to apoptosis (168). Thus, it appears that *E. histolytica* elicits a stress response to IECs and promotes a hyporesponsive state toward trophozoites. The amebic factors that induce NFκB suppression have not yet determined. The factors that activate TLR2, i.e., LPPG and Gal/GalNAc lectin, are candidates involved in this pathway (172).

Prostaglandin PGE2

Entamoeba histolytica trophozoites produce and secrete prostaglandin 2 (PGE2), which have contact-independent effects on tight junction integrity and ion absorption. Secreted amebic PGE2 binds to prostaglandin E receptor 4 (EP4) on IECs, disrupts tight junctions, and increases luminal Cl^- secretion (173, 174). PGE2 secreted from the amebae elicits inflammatory response in IECs by increasing IL-8 production by IECs (173). PGE2 is a potent mucin secretagogue (175) that can overcome luminal barrier function by causing hypersecretion and, thus, depletion of the protective mucus barrier (176). On the contrary, it has been also reported that during invasive amebiasis, local PGE2 has anti-inflammatory effect. In animal model of chronic ALA, hepatic granuloma macrophages do not respond to IFN-γ and LPS and do not produce inflammatory cytokines, show decrease in MHC class II expression, and are unable to kill trophozoites (47, 177, 178). This suppression is local during chronic ALA and is directly caused by the parasite (47, 177). A culture supernatant and an unknown soluble protein component of *E. histolytica* trophozoites decrease class II major histocompatibility complex (MHC II) immune-associated (Ia) antigen expression through a PGE2-dependent manner (178). Inhibition of macrophage PGE2 synthesis can partially recover MHC II Ia expression and TNF-α expression (177, 178). However, inhibition of PGE2 synthesis does not recover iNOS expression or amebicidal activity in the deactivated macrophage (177). A continuous supply of parasite-derived PGE2 likely prevents iNOS expression and full recovery of MHC II and TNF-α, possibly through a concentration-dependent effect of PGE2. In short, ameba-secreted PGE2 represses inflammation in ALA, which is beneficial for survival, whereas it likely enhances destruction of the colon.

PERTURBATION OF THE ENTERIC MICROBIOTA BY *E. histolytica*

It has been reported that *E. histolytica* infection alters the microbiota composition. *E. histolytica*-induced dysbiosis was characterized by fewer *Bacteroides*, *Clostridia*, *Lactobacillus*, *Campylobacter*, and *Eubacterium* species, and increased *Bifidobacterium* species (179). *In vitro* experiments have shown that *E. histolytica* preferentially ingest some bacterial species (59, 180). It is known that amoebapores, a family of the major pore-forming peptides, have differential activity against bacteria and eukaryotes (15). Furthermore, *E. histolytica* infection induces production of colonic antimicrobial peptides, while the trophozoites degrade them (181). A recent study has shown that dendritic cells from the mouse intestine where *Clostridia*-related bacteria colonized provide IL-17A-dependent protection against amebic colitis (182). Detailed molecular events remain to be elucidated, however, by examining how alternations of the microbiota modulate host immune responses against amebic intestinal infection. Altogether, microbiota can be modulated by amebic infection, and in turn concentrations of carbohydrates (and other compounds) that affect growth and virulence of the amebae can strongly influence outcome of infection. It remains to be elucidated whether and how the amebae modulate the intestinal microbiota for their survival and parasitism.

STRATEGY FOR OXIDATIVE STRESS MANAGEMENT AND METABOLIC CONTROL

Lack of Respiration and Antioxidative Stress Management in *E. histolytica*

Entamoeba histolytica trophozoites are microaerophilic and consume oxygen. They tolerate low levels of oxygen tension. *E. histolytica* lacks a conventional respiratory electron transport chain that terminates in the reduction of O_2 to H_2O. However, it does respire and tolerates up to 5% oxygen in the gas phase (183–185). The parasite lacks most of the components of antioxidant defense mechanisms that are widely present in other prokaryotic and eukaryotic organisms, such as catalase, peroxidase, glutathione, and the glutathione-recycling enzymes glutathione peroxidase and glutathione reductase (184, 185). However, during tissue invasion, trophozoites must fend off reactive oxygen and nitrogen species produced by activated immune cells through the respiratory burst. Thus, trophozoites must use antioxidative stress defense to survive immune surveillance.

Anti-Oxidative Stress Response Contributes to Immune Evasion in *E. histolytica*

Entamoeba histolytica trophozoites contain high levels of cysteine, instead of glutathione, as the major thiol in the cell. They possess several enzymes to defend from oxidative stress, such as peroxiredoxin (Prx), superoxide dismutase, flavoprotein A, ferredoxin, thioredoxin (Trx), and Trx reductase (186, 187). The Trx/Trx reductase system is crucial for buffering sensitive proteins under oxidative stress (188). The amebicidal drugs, metronidazole and auranofin, are known to disrupt Trx (189, 190). Interestingly, the oxidative stress increases *E. histolytica* virulence. It has been shown that oxidative stress causes upregulation of a stress-induced adhesion factor and a phospholipid transporting P-type ATPase/flippase (187). Both genes are involved in adhesion and phagocytosis. Oxidative stress also alters metabolic flux, including glycerol and chitin biosynthesis, potentially triggering encystation (191). Furthermore, it has been shown that *E. histolytica* (HM-1:IMSS) responds more strongly to oxidative stress than *E. dispar* and *E. histolytica* non-virulent Rahman strain, and surface localization of Prx in HM-1:IMSS is associated with virulence (186). Altogether, antioxidative defense mechanisms in *E. histolytica* are associated with pathogenesis. For more details on the anti-oxidative management in *E. histolytica,* a recent review should be consulted (192).

CONCLUSION

Our understanding of molecular mechanisms of the parasite's pathogenesis, such as adherence to host cells, induction of apoptosis, degradation of mucin and ECM, tissue invasion, and phago/trogocytosis of host cells, has greatly advanced in recent years. So have mechanisms of immune evasion, such as induction of IL-10 and suppression of INF-γ, degradation of Igs, complement, and pro-inflammatory cytokines. In addition, defense against ROS and NO and evasion from antibody and complement-dependent

killing also plays important roles in survival in the host. Furthermore, mutual signaling among the three domains in the complex network of the parasite, the human, and the microbiota with polymorphic genetic backgrounds affect outcome of amebic infection. Further research is needed to elucidate the molecular basis of the complex interaction in the intestinal ecosystem.

AUTHOR CONTRIBUTIONS

KN-T and TN have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Ackers J, Clark CG, Diamond LS, Duchêne M, Cantellano ME, Jackson TF, et al. WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Mexico City, Mexico 28-29 January, 1997. *Epidemiol Bull* (1997) **18**:13–4.
2. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. *Lancet* (2012) **380**:2095–128. doi:10.1016/S0140-6736(12)61728-0
3. Ali IK, Clark CG, Petri WA Jr. Molecular epidemiology of amebiasis. *Infect Genet Evol* (2008) **8**:698–707. doi:10.1016/j.meegid.2008.05.004
4. Ximénez C, Morán P, Rojas L, Valadez A, Gómez A. Reassessment of the epidemiology of amebiasis: state of the art. *Infect Genet Evol* (2009) **9**:1023–32. doi:10.1016/j.meegid.2009.06.008
5. Haque R, Ali IM, Sack RB, Farr BM, Ramakrishnan G, Petri WA Jr. Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. *J Infect Dis* (2001) **183**:1787–93. doi:10.1086/320740
6. Hung CC, Chang SY, Ji DD. *Entamoeba histolytica* infection in men who have sex with men. *Lancet Infect Dis* (2012) **12**:729–36. doi:10.1016/S1473-3099(12)70147-0
7. Watanabe K, Gatanaga H, Escueta-de Cadiz A, Tanuma J, Nozaki T, Oka S. Amebiasis in HIV-1-infected Japanese men: clinical features and response to therapy. *PLoS Negl Trop Dis* (2011) **5**:e1318. doi:10.1371/journal.pntd.0001318
8. Haghighi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T. Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. *J Clin Microbiol* (2002) **40**:4081–90. doi:10.1128/JCM.40.11.4081-4090.2002
9. Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T. Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. *J Clin Microbiol* (2003) **41**:3748–56. doi:10.1128/JCM.41.8.3748-3756.2003
10. Haque R, Mondal D, Karim A, Molla IH, Rahim A, Faruque AS, et al. Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh. *Clin Infect Dis* (2009) **48**:1191–7. doi:10.1086/597580
11. Haque R, Huston CD, Hughes M, Houpt E, Petri WA Jr. Amebiasis. *N Engl J Med* (2003) **348**:1565–73. doi:10.1056/NEJMra022710
12. Infectious Disease Surveillance Center, National Institute of Infectious Diseases of Japan. *HIV/AIDS in Japan in the Year of 2013. IASR*. Vol. **35**. (2014). p. 223–4. Available from: http://www.nih.go.jp/niid/ja/id/969-disease-based/a/entamoeba-histolytica/idsc/iasr-in/4978-kj4151.html
13. Petri WA Jr, Haque R, Mann BJ. The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*. *Annu Rev Microbiol* (2002) **56**:39–64. doi:10.1146/annurev.micro.56.012302.160959
14. Leippe M. Amoebapores. *Parasitol Today* (1997) **13**:178–83. doi:10.1016/S0169-4758(97)01038-7
15. Bruhn H, Riekens B, Berninghausen O, Leippe M. Amoebapores and NK-lysin, members of a class of structurally distinct antimicrobial and cytolytic peptides from protozoa and mammals: a comparative functional analysis. *Biochem J* (2003) **375**:737–44. doi:10.1042/BJ20030250
16. Kelsall BL, Ravdin JI. Degradation of human IgA by *Entamoeba histolytica*. *J Infect Dis* (1993) **168**:1319–22. doi:10.1093/infdis/168.5.1319
17. Schulte W, Scholze H. Action of the major protease from *Entamoeba histolytica* on proteins of the extracellular matrix. *J Protozool* (1989) **36**:538–43. doi:10.1111/j.1550-7408.1989.tb01092.x
18. Tran VQ, Herdman S, Torian BE, Reed SL. The neutral cysteine proteinase of *Entamoeba histolytica* degrades IgG and prevents its binding. *J Infect Dis* (1998) **177**:508–11. doi:10.1086/517388
19. Que X, Reed SL. Cysteine proteinases and the pathogenesis of amebiasis. *Clin Microbiol Rev* (2000) **13**:196–206. doi:10.1128/CMR.13.2.196-206.2000
20. Tillack M, Biller L, Irmer H, Freitas M, Gomes MA, Tannich E, et al. The *Entamoeba histolytica* genome: primary structure and expression of proteolytic enzymes. *BMC Genomics* (2007) **8**:170. doi:10.1186/1471-2164-8-170
21. Lamm ME. Current concepts in mucosal immunity. IV. How epithelial transport of IgA antibodies relates to host defense. *Am J Physiol* (1998) **274**:G614–7.
22. Haque R, Duggal P, Ali IM, Hossain MB, Mondal D, Sack RB, et al. Innate and acquired resistance to amebiasis in bangladeshi children. *J Infect Dis* (2002) **186**:547–52. doi:10.1086/341566
23. Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM, et al. *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. *Infect Immun* (2006) **74**:904–9. doi:10.1128/IAI.74.2.904-909.2006
24. Abd-Alla MD, Jackson TF, Rogers T, Reddy S, Ravdin JI. Mucosal immunity to asymptomatic *Entamoeba histolytica* and *Entamoeba dispar* infection is associated with a peak intestinal antilectin immunoglobulin A antibody response. *Infect Immun* (2006) **74**:3897–903. doi:10.1128/IAI.02018-05
25. Ravdin JI, Abd-Alla MD, Welles SL, Reddy S, Jackson TF. Intestinal antilectin immunoglobulin A antibody response and immunity to *Entamoeba dispar* infection following cure of amebic liver abscess. *Infect Immun* (2003) **71**:6899–905. doi:10.1128/IAI.71.12.6899-6905.2003
26. Kaur U, Sharma AK, Sharma M, Vohra H. Distribution of *Entamoeba histolytica* Gal/GalNAc lectin-specific antibody response in an endemic area. *Scand J Immunol* (2004) **60**:524–8. doi:10.1111/j.0300-9475.2004.01512.x
27. Bernin H, Marggraff C, Jacobs T, Brattig N, Le VA, Blessmann J, et al. Immune markers characteristic for asymptomatically infected and diseased *Entamoeba histolytica* individuals and their relation to sex. *BMC Infect Dis* (2014) **14**:621. doi:10.1186/s12879-014-0621-1
28. Gay NJ, Gangloff M. Structure and function of toll receptors and their ligands. *Annu Rev Biochem* (2007) **76**:141–65. doi:10.1146/annurev.biochem.76.060305.151318
29. Bansal D, Ave P, Kerneis S, Frileux P, Boché O, Baglin AC, et al. An ex-vivo human intestinal model to study *Entamoeba histolytica* pathogenesis. *PLoS Negl Trop Dis* (2009) **3**:e551. doi:10.1371/journal.pntd.0000551
30. Galván-Moroyoqui JM, Del CarmenDomínguez-Robles M, Meza I. Pathogenic bacteria prime the induction of toll-like receptor signalling in human colonic cells by the Gal/GalNAc lectin carbohydrate recognition domain of *Entamoeba histolytica*. *Int J Parasitol* (2011) **41**:1101–12. doi:10.1016/j.ijpara.2011.06.003
31. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol* (2011) **30**:16–34. doi:10.3109/08830185.2010.529976
32. Helk E, Bernin H, Ernst T, Ittrich H, Jacobs T, Heeren J, et al. TNFα-mediated liver destruction by Kupffer cells and Ly6Chi monocytes during *Entamoeba*

histolytica infection. *PLoS Pathog* (2013) **9**:e1003096. doi:10.1371/journal.ppat.1003096
33. Lotter H, González-Roldán N, Lindner B, Winau F, Isibasi A, Moreno-Lafont M, et al. Natural killer T cells activated by a lipopeptidophosphoglycan from E*ntamoeba histolytica* are critically important to control amebic liver abscess. *PLoS Pathog* (2009) **5**(5):e1000434. doi:10.1371/journal.ppat.1000434
34. Peterson KM, Shu J, Duggal P, Haque R, Mondal D, Petri WA Jr. Association between TNF-α and *Entamoeba histolytica* diarrhea. *Am J Trop Med Hyg* (2010) **82**:620–5. doi:10.4269/ajtmh.2010.09-0493
35. Sánchez-Guillén Mdel C, Pérez-Fuentes R, Salgado-Rosas H, Ruiz-Argüelles A, Ackers J, Shire A, et al. Differentiation of *Entamoeba histolytica*/*Entamoeba dispar* by PCR and their correlation with humoral and cellular immunity in individuals with clinical variants of amoebiasis. *Am J Trop Med Hyg* (2002) **66**:731–7.
36. Haque R, Mondal D, Shu J, Roy S, Kabir M, Davis AN, et al. Correlation of interferon-γ production by peripheral blood mononuclear cells with childhood malnutrition and susceptibility to amebiasis. *Am J Trop Med Hyg* (2007) **76**:340–4.
37. Bansal D, Sehgal R, Chawla Y, Malla N, Mahajan RC. Cytokine mRNA expressions in symptomatic vs. asymptomatic amoebiasis patients. *Parasite Immunol* (2005) **27**:37–43. doi:10.1111/j.1365-3024.2005.00739.x
38. Guo X, Barrosom L, Becker SM, Lyerly DM, Vedvick TS, Reed SG, et al. Protection against intestinal amebiasis by a recombinant vaccine is transferable by T cells and mediated by gamma interferon. *Infect Immun* (2009) **77**:3909–18. doi:10.1128/IAI.00487-09
39. Guo X, Barroso L, Lyerly DM, Petri WA Jr, Houpt ER. CD4+ and CD8+ T cell- and IL-17-mediated protection against *Entamoeba histolytica* induced by a recombinant vaccine. *Vaccine* (2011) **29**:772–7. doi:10.1016/j.vaccine.2010.11.013
40. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* (2006) **203**(10):2271–9. doi:10.1084/jem.20061308
41. Nishida A, Lau CW, Zhang M, Andoh A, Shi HN, Mizoguchi E, et al. The membrane-bound mucin Muc1 regulates T helper 17-cell responses and colitis in mice. *Gastroenterology* (2012) **142**:865–74.e2. doi:10.1053/j.gastro.2011.12.036
42. Cao AT, Yao S, Gong B, Elson CO, Cong Y. Th17 cells upregulate polymeric Ig receptor and intestinal IgA and contribute to intestinal homeostasis. *J Immunol* (2012) **189**:4666–73. doi:10.4049/jimmunol.1200955
43. Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. *J Clin Immunol* (2010) **30**:196–203. doi:10.1007/s10875-010-9368-7
44. Ghadirian E, Denis M. In vivo activation of macrophages by IFN-γ to kill *Entamoeba histolytica* trophozoites in vitro. *Parasite Immunol* (1992) **14**:397–404. doi:10.1111/j.1365-3024.1992.tb00014.x
45. Lin JY, Chadee K. Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. *J Immunol* (1992) **148**:3999–4005.
46. Espinosa-Cantellano M, Martínez-Palomo A. Pathogenesis of intestinal amebiasis: from molecules to disease. *Clin Microbiol Rev* (2000) **13**:318–31. doi:10.1128/CMR.13.2.318-331.2000
47. Denis M, Chadee K. Cytokine activation of murine macrophages for in vitro killing of *Entamoeba histolytica* trophozoites. *Infect Immun* (1989) **57**:1750–6.
48. Berrilli F, Di Cave D, Cavallero S, D'Amelio S. Interactions between parasites and microbial communities in the human gut. *Front Cell Infect Microbiol* (2012) **2**:141. doi:10.3389/fcimb.2012.00141
49. Gilchrist CA, Petri SE, Schneider BN, Reichman DJ, Jiang N, Begum S, et al. Role of the gut microbiota of children in diarrhea due to the protozoan parasite *Entamoeba histolytica*. *J Infect Dis* (2015). doi:10.1093/infdis/jiv772
50. Ouwerkerk JP, de Vos WM, Belzer C. Glycobiome: bacteria and mucus at the epithelial interface. *Best Pract Res Clin Gastroenterol* (2013) **27**:25–38. doi:10.1016/j.bpg.2013.03.001
51. Nakada-Tsukui K, Tsuboi K, Furukawa A, Yamada Y, Nozaki T. A novel class of cysteine protease receptors that mediate lysosomal transport. *Cell Microbiol* (2012) **14**:1299–317. doi:10.1111/j.1462-5822.2012.01800.x
52. Furukawa A, Nakada-Tsukui K, Nozaki T. Novel transmembrane receptor involved in phagosome transport of lysozymes and β-hexosaminidase in the enteric protozoan *Entamoeba histolytica*. *PLoS Pathog* (2012) **8**:e1002539. doi:10.1371/journal.ppat.1002539
53. Furukawa A, Nakada-Tsukui K, Nozaki T. Cysteine protease-binding protein family 6 mediates the trafficking of amylases to phagosomes in the enteric protozoan *Entamoeba histolytica*. *Infect Immun* (2013) **81**:1820–9. doi:10.1128/IAI.00915-12
54. Marumo K, Nakada-Tsukui K, Tomii K, Nozaki T. Ligand heterogeneity of the cysteine protease binding protein family in the parasitic protist *Entamoeba histolytica*. *Int J Parasitol* (2014) **44**:625–35. doi:10.1016/j.ijpara.2014.04.008
55. Thibeaux R, Weber C, Hon CC, Dillies MA, Avé P, Coppée JY, et al. Identification of the virulence landscape essential for *Entamoeba histolytica* invasion of the human colon. *PLoS Pathog* (2013) **9**:e1003824. doi:10.1371/journal.ppat.1003824
56. Phillips BP, Wolfe PA, Rees CW, Gordon HA, Wright WH, Reyniers JA. Studies on the amebabacteria relationship in amebiasis: comparative results of the intracecal inoculation of germfree, monocontaminated, and conventional guinea pigswith *Entamoeba histolytica*. *Am J Trop Med Hyg* (1955) **4**:675–92.
57. Phillips BP, Gorstein F. Effects of different species of bacteria on the pathology of enteric amebiasis in monocontaminated guinea pigs. *Am J Trop Med Hyg* (1966) **15**:863–8.
58. Wittner M, Rosenbaum RM. Role of bacteria in modifying virulence of *Entamoeba histolytica*: studies of amebae from axenic cultures. *Am J Trop Med Hyg* (1970) **19**:755–61.
59. Mirelman D, Feingold C, Wexler A, Bracha R. Interactions between *Entamoeba histolytica*, bacteria and intestinal cells. *Ciba Found Symp* (1983) **99**:2–30.
60. Galván-Moroyoqui JM, Del Carmen Domínguez-Robles M, Franco E, Meza I. The interplay between *Entamoeba* and enteropathogenic bacteria modulates epithelial cell damage. *PLoS Negl Trop Dis* (2008) **2**:e266. doi:10.1371/journal.pntd.0000266
61. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* (2012) **3**:289–306. doi:10.4161/gmic.19897
62. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, et al. Fucose sensing regulates bacterial intestinal colonization. *Nature* (2012) **492**:113–7. doi:10.1038/nature11623
63. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature* (2013) **502**:96–9. doi:10.1038/nature12503
64. Tovy A, Hertz R, Siman-Tov R, Syan S, Faust DM, Guillen N, et al. Glucose starvation boosts *Entamoeba histolytica* virulence. *PLoS Negl Trop Dis* (2011) **5**:e1247. doi:10.1371/journal.pntd.0001247
65. Gilchrist CA, Baba DJ, Zhang Y, Crasta O, Evans C, Caler E, et al. Targets of the *Entamoeba histolytica* transcription factor URE3-BP. *PLoS Negl Trop Dis* (2008) **2**:e282. doi:10.1371/journal.pntd.0000282
66. Jumpertz R, Le DS, Turnbaugh PJ, Trinidad C, Bogardus C, Gordon JI, et al. Energy-balance studies reveal associations between gut microbes, caloric-load, and nutrient absorption in humans. *Am J Clin Nutr* (2011) **94**:58–65. doi:10.3945/ajcn.110.010132
67. Purchiaroni F, Tortora A, Gabrielli M, Bertucci F, Gigante G, Ianiro G, et al. The role of intestinal microbiota and the immune system. *Eur Rev Med Pharmacol Sci* (2013) **17**:323–33.
68. Donia MS, Fischbach MA. HUMAN MICROBIOTA. Small molecules from the human microbiota. *Science* (2015) **349**:1254766. doi:10.1126/science.1254766
69. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* (2008) **453**:620–5. doi:10.1038/nature07008
70. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* (2011) **331**:337–41. doi:10.1126/science.1198469
71. Sakaguchi S. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J Clin Invest* (2003) **112**:1310–2. doi:10.1172/JCI200320274
72. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* (2011) **332**:974–7. doi:10.1126/science.1206095

73. Hot A, Miossec P. Effects of interleukin (IL)-17A and IL-17F in human rheumatoid arthritis synoviocytes. *Ann Rheum Dis* (2011) **70**:727–32. doi:10.1136/ard.2010.143768
74. Scher JU, Abramson SB. The microbiome and rheumatoid arthritis. *Nat Rev Rheumatol* (2011) **7**:569–78. doi:10.1038/nrrheum.2011.121
75. Romano-Keeler J, Weitkamp JH, Moore DJ. Regulatory properties of the intestinal microbiome effecting the development and treatment of diabetes. *Curr Opin Endocrinol Diabetes Obes* (2012) **19**:73–80. doi:10.1097/MED.0b013e3283514d43
76. Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes* (2012) **3**:4–14. doi:10.4161/gmic.19320
77. Jin C, Henao-Mejia J, Flavell RA. Innate immune receptors: key regulators of metabolic disease progression. *Cell Metab* (2013) **17**:873–82. doi:10.1016/j.cmet.2013.05.011
78. Erdman SE, Poutahidis T. Gut bacteria and cancer. *Biochim Biophys Acta* (2015) **1856**:86–90. doi:10.1016/j.bbcan.2015.05.007
79. Reed SL, Ember JA, Herdman DS, DiScipio RG, Hugli TE, Gigli I. The extracellular neutral cysteine proteinase of *Entamoeba histolytica* degrades anaphylatoxins C3a and C5a. *J Immunol* (1995) **155**:266–74.
80. Ankri S, Stolarsky T, Bracha R, Padilla-Vaca F, Mirelman D. Antisense inhibition of expression of cysteine proteinases affects *Entamoeba histolytica*-induced formation of liver abscess in hamsters. *Infect Immun* (1999) **67**:421–2.
81. Stanley SL Jr. Amoebiasis. *Lancet* (2003) **361**:1025–34. doi:10.1016/S0140-6736(03)12830-9
82. Que X, Kim SH, Sajid M, Eckmann L, Dinarello CA, McKerrow JH, et al. A surface amebic cysteine proteinase inactivates interleukin-18. *Infect Immun* (2003) **71**:1274–80. doi:10.1128/IAI.71.3.1274-1280.2003
83. Kissoon-Singh V, Mortimer L, Chadee K. *Entamoeba histolytica* cathepsin-like enzymes: interactions with the host gut. *Adv Exp Med Biol* (2011) **712**:62–83. doi:10.1007/978-1-4419-8414-2_5
84. Thibeaux R, Avé P, Bernier M, Morcelet M, Frileux P, Guillén N, et al. The parasite *Entamoeba histolytica* exploits the activities of human matrix metalloproteinases to invade colonic tissue. *Nat Commun* (2014) **5**:5142. doi:10.1038/ncomms6142
85. Corfield AP. Mucins: a biologically relevant glycan barrier in mucosal protection. *Biochim Biophys Acta* (2015) **1850**:236–52. doi:10.1016/j.bbagen.2014.05.003
86. Carlstedt I, Herrmann A, Karlsson H, Sheehan J, Fransson L, Hansson G. Characterization of two different glycosylated domains from the insoluble mucin complex of rat small intestine. *J Biol Chem* (1993) **268**:18771–81.
87. Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, et al. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. *J Biol Chem* (1999) **274**:15828–36. doi:10.1074/jbc.274.22.15828
88. Moncada D, Keller K, Chadee K. *Entamoeba histolytica*-secreted products degrade colonic mucin oligosaccharides. *Infect Immun* (2005) **73**:3790–3. doi:10.1128/IAI.73.6.3790-3793.2005
89. Connaris S, Greenwell P. Glycosidases in mucin-dwelling protozoans. *Glycoconj J* (1997) **14**:879–82. doi:10.1023/A:1018554408558
90. Huldt G, Davies P, Allison AC, Schorlemmer HU. Interactions between *Entamoeba histolytica* and complement. *Nature* (1979) **18**:214–6. doi:10.1038/277214a0
91. Arias-Negrete S, Sabanero-López M, Villagómez-Castro JC. Biochemical analysis of *Entamoeba histolytica* HM1 strain resistant to complement lysis. *Arch Med Res* (1992) **23**:135–7.
92. Sonnenburg JL, Xu J, Leip DD, Chen CH, Westover BP, Weatherford J, et al. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science* (2005) **307**:1955–9. doi:10.1126/science.1109051
93. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* (2010) **464**:59–65. doi:10.1038/nature08821
94. Irmer H, Tillack M, Biller L, Handal G, Leippe M, Roeder T, et al. Major cysteine peptidases of *Entamoeba histolytica* are required for aggregation and digestion of erythrocytes but are dispensable for phagocytosisand cytopathogenicity. *Mol Microbiol* (2009) **72**:658–67. doi:10.1111/j.1365-2958.2009.06672.x
95. Bruchhaus I, Jacobs T, Leippe M, Tannich E. *Entamoeba histolytica* and *Entamoeba dispar*: differences in numbers and expression of cysteine proteinase genes. *Mol Microbiol* (1996) **22**:255–63. doi:10.1046/j.1365-2958.1996.00111.x
96. Willhoeft U, Hamann L, Tannich E. A DNA sequence corresponding to the gene encoding cysteine proteinase 5 in *Entamoeba histolytica* is present and positionally conserved but highly degenerated in *Entamoeba dispar*. *Infect Immun* (1999) **67**:5925–9.
97. Hellberg A, Nickel R, Lotter H, Tannich E, Bruchhaus I. Overexpression of cysteine proteinase 2 in *Entamoeba histolytica* or *Entamoeba dispar* increases amoeba-induced monolayer destruction in vitro but does not augment amoebic liver abscess formation in gerbils. *Cell Microbiol* (2001) **3**:13–20. doi:10.1046/j.1462-5822.2001.00086.x
98. Tillack M, Nowak N, Lotter H, Bracha R, Mirelman D, Tannich E, et al. Increased expression of the major cysteine proteinases by stable episomal transfection underlines the important role of EhCP5 for the pathogenicity of *Entamoeba histolytica*. *Mol Biochem Parasitol* (2006) **149**:58–64. doi:10.1016/j.molbiopara.2006.04.009
99. Freitas MA, Fernandes HC, Calixto VC, Martins AS, Silva EF, Pesquero JL, et al. *Entamoeba histolytica*: cysteine proteinase activity and virulence. Focus on cysteine proteinase 5 expression levels. *Exp Parasitol* (2009) **122**:306–9. doi:10.1016/j.exppara.2009.04.005
100. Hou Y, Mortimer L, Chadee K. *Entamoeba histolytica* cysteine proteinase 5 binds integrin on colonic cells and stimulates NFkappaB-mediated pro-inflammatory responses. *J Biol Chem* (2010) **285**:35497–504. doi:10.1074/jbc.M109.066035
101. Thibeaux R, Dufour A, Roux P, Bernier M, Baglin AC, Frileux P, et al. Newly visualized fibrillar collagen scaffolds dictate *Entamoeba histolytica* invasion route in the human colon. *Cell Microbiol* (2012) **14**:609–21. doi:10.1111/j.1462-5822.2012.01752.x
102. Mortimer L, Moreaum F, Cornick S, Chadee K. The NLRP3 Inflammasome is a pathogen sensor for invasive *Entamoeba histolytica* via activation of α5β1 integrin at the macrophage-amebae intercellular junction. *PLoS Pathog* (2015) **11**:e1004887. doi:10.1371/journal.ppat.1004887
103. Mortimer L, Moreau F, Cornick S, Chadee K. Gal-lectindependent contact activates the inflammasome by invasive *Entamoeba histolytica*. *Mucosal Immunol* (2014) **7**:829–41. doi:10.1038/mi.2013.100
104. Leroy A, Mareel M, De Bruyne G, Bailey G, Nelis H. Metastasis of *Entamoeba histolytica* compared to colon cancer: one more step in invasion. *Invasion Metastasis* (1995) **14**:177–91.
105. Bernacki RJ, Niedbala MJ, Korytnyk W. Glycosidases in cancer and invasion. *Cancer Metastasis Rev* (1985) **4**:81–101. doi:10.1007/BF00047738
106. Liotta LA. Tumor invasion and metastases: role of the basement membrane. *Am J Pathol* (1984) **117**:339–48.
107. Coghlin C, Murray GI. Current and emerging concepts in tumour metastasis. *J Pathol* (2010) **222**:1–15. doi:10.1002/path.2727
108. Plucinsky MC, Prorok JJ, Alhadeff JA. Variant serum beta-hexosaminidase as a biochemical marker of malignancy. *Cancer* (1986) **58**:1484–7. doi:10.1002/1097-0142(19861001)58:7<1484:AID-CNCR2820580718>3.0.CO;2-R
109. Bosmann HB, Hall TC. Enzyme activity in invasive tumors of human breast and colon. *Proc Natl Acad Sci USA* (1974) **71**:1833–7. doi:10.1073/pnas.71.5.1833
110. Gil-Martín E, Rodríguez-Berrocal J, Páez de la Cadena M, Fernández-Briera A. Alterations of glycosidases in human colonic adenocarcinoma. *Clin Biochem* (1997) **30**:17–25. doi:10.1016/S0009-9120(96)00123-3
111. Ramessur KT, Greenwell P, Nash R, Dwek MV. Breast cancer invasion is mediated by beta-N-acetylglucosaminidase (beta-NAG) and associated with a dysregulation in the secretory pathway of cancer cells. *Br J Biomed Sci* (2010) **67**:189–96.
112. Choromańska B, Luto M, Szajda SD, Waszkiewicz N, Kępka A, Janica J, et al. Activity of N-acetyl-β-hexosaminidase and its isoenzymes A and B in cancer. *Postepy Hig Med Dosw (Online)* (2011) **65**:752–8. doi:10.5604/17322693.966833
113. Chadee K, Petri WA Jr, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. *J Clin Invest* (1987) **80**:1245–54. doi:10.1172/JCI113199
114. Saffer LD, Petri WA Jr. Role of the galactose lectin of *Entamoeba histolytica* in adherence-dependent killing of mammalian cells. *Infect Immun* (1991) **59**:4681–3.

115. Guerrant RL, Brush J, Ravdin JI, Sullivan JA, Mandell GL. Interaction between *Entamoeba histolytica* and human polymorphonuclear neutrophils. *J Infect Dis* (1981) **143**:83–93. doi:10.1093/infdis/143.1.83
116. Ravdin JI, Moreau F, Sullivan JA, Petri WA Jr, Mandell GL. Relationship of free intracellular calcium to the cytolytic activity of *Entamoeba histolytica*. *Infect Immun* (1988) **56**:505–12.
117. Huston CD, Boettner DR, Miller-Sims V, Petri WA Jr. Apoptotic killing and phagocytosis of host cells by the parasite *Entamoeba histolytica*. *Infect Immun* (2003) **71**:964–72. doi:10.1128/IAI.71.2.964-972.2003
118. Jacobs T, Bruchhaus I, Dandekar T, Tannich E, Leippe M. Isolation and molecular characterization of a surface-bound proteinase of *Entamoeba histolytica*. *Mol Microbiol* (1998) **27**:269–76. doi:10.1046/j.1365-2958.1998.00662.x
119. Garcia-Nieto RM, Rico-Mata R, Arias-Negrete S, Avila EE. Degradation of human secretory IgA1 and IgA2 by *Entamoeba histolytica* surface-associated proteolytic activity. *Parasitol Int* (2008) **57**:417–23. doi:10.1016/j.parint.2008.04.013
120. Macpherson AJ, McCoy KD, Johansen F, Brandtzaeg P. The immune geography of IgA induction and function. *Mucosal Immunol* (2008) **1**:11–22. doi:10.1038/mi.2007.6
121. Walport MJ. Complement: second of two parts. *N Engl J Med* (2001) **344**:1140–4. doi:10.1056/NEJM200104123441506
122. Köhl J. Anaphylatoxins and infectious and non-infectious inflammatory diseases. *Mol Immunol* (2001) **38**:175–87. doi:10.1016/S0161-5890(01)00041-4
123. Gasque P. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* (2004) **41**:1089–98. doi:10.1016/j.molimm.2004.06.011
124. Zhang Z, Wang L, Seydel KB, Li E, Ankri S, Mirelman D, et al. *Entamoeba histolytica* cysteine proteinases with interleukin-1 beta converting enzyme (ICE) activity cause intestinal inflammation and tissue damage in amoebiasis. *Mol Microbiol* (2000) **37**:542–8. doi:10.1046/j.1365-2958.2000.02037.x
125. Mayor S, Riezman H. Sorting GPI-anchored proteins. *Nat Rev Mol Cell Biol* (2004) **5**:110–20. doi:10.1038/nrm1309
126. Fujita M, Kinoshita T. GPI-anchor remodeling: potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. *Biochim Biophys Acta* (2012) **1821**:1050–8. doi:10.1016/j.bbalip.2012.01.004
127. Saha S, Anilkumar AA, Mayor S. GPI-anchored protein organization and dynamics at the cell surface. *J Lipid Res* (2015). doi:10.1194/jlr.R062885
128. Isibasi A, Santa Cruz M, Soto Montano X, Ramirez A, Kumate J. Localization of a lipopeptidophosphoglycan extracted by phenol-water from trophozoites of the HK-9 strain of *Entamoeba histolytica*. *Arch Invest Med (Mex)* (1982) **13**:57–62.
129. Guha-Niyogi A, Sullivan DR, Turco SJ. Glycoconjugate structures of parasitic protozoa. *Glycobiology* (2001) **11**:45R–59R. doi:10.1093/glycob/11.4.45R
130. Bhattacharya A, Arya R, Clark CG, Ackers JP. Absence of lipophosphoglycan-like glycoconjugates in *Entamoeba dispar*. *Parasitology* (2000) **120**:31–5. doi:10.1017/S0031182099005259
131. Weber C, Blazquez S, Marion S, Ausseur C, Vats D, Krzeminski M, et al. Bioinformatics and functional analysis of an *Entamoeba histolytica* mannosyltransferase necessary for parasite complement resistance and hepatical infection. *PLoS Negl Trop Dis* (2008) **2**:e165. doi:10.1371/journal.pntd.0000165
132. Braga L, Ninomiya H, McCoy JJ, Eacker S, Wiedmer T, Pham C, et al. Inhibition of the complement membrane attack complex by the galactose-specific adhesion of *Entamoeba histolytica*. *J Clin Invest* (1992) **90**:1131–7. doi:10.1172/JCI115931
133. Ventura-Juárez J, Campos-Rodríguez R, Jarillo-Luna RA, Muñoz-Fernández L, Escario-G-Trevijano JA, Pérez-Serrano J, et al. Trophozoites of Entamoeba histolytica express a CD59-like molecule in human colon. *Parasitol Res* (2009) **104**:821–6. doi:10.1007/s00436-008-1262-3
134. Calderón J, de Lourdes Muñoz M, Acosta HM. Surface redistribution and release of antibody-induced caps in entamoebae. *J Exp Med* (1980) **151**:184–93. doi:10.1084/jem.151.1.184
135. Baxt LA, Baker RP, Singh U, Urban S. An *Entamoeba histolytica* rhomboid protease with atypical specificity cleaves a surface lectin involved in phagocytosis and immune evasion. *Genes Dev* (2008) **22**:1636–46. doi:10.1101/gad.1667708
136. Bailey GB, Day DB, McCoomer NE. Entamoeba motility: dynamics of cytoplasmic streaming, locomotion and translocation of surface bound particles, and organization of the actin cytoskeleton in *Entamoeba invadens*. *J Protozool* (1992) **39**:267–72. doi:10.1111/j.1550-7408.1992.tb01313.x
137. Espinosa-Cantellano M, Martínez-Palomo A. *Entamoeba histolytica*: mechanism of surface receptor capping. *Exp Parasitol* (1994) **79**:424–35. doi:10.1006/expr.1994.1104
138. Baker RP, Wijetilaka R, Urban S. Two *Plasmodium* rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. *PLoS Pathog* (2006) **2**:e113. doi:10.1371/journal.ppat.0020113
139. Ragland BD, Ashley LS, Vaux DL, Petri WA Jr. *Entamoeba histolytica*: target cells killed by trophozoites undergo DNA fragmentation which is not blocked by Bcl-2. *Exp Parasitol* (1994) **79**:460–7. doi:10.1006/expr.1994.1107
140. Huston CD, Houpt ER, Mann BJ, Hahn CS, Petri WA Jr. Caspase 3-dependent killing of host cells by the parasite *Entamoeba histolytica*. *Cell Microbiol* (2000) **2**:617–25. doi:10.1046/j.1462-5822.2000.00085.x
141. Sim S, Yong TS, Park SJ, Im KI, Kong Y, Ryu JS, et al. NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by *Entamoeba histolytica*. *J Immunol* (2005) **174**:4279–88. doi:10.4049/jimmunol.174.7.4279
142. Berninghausen O, Leippe M. Necrosis versus apoptosis as the mechanism of target cell death induced by *Entamoeba histolytica*. *Infect Immun* (1997) **65**:3615–21.
143. Boettner DR, Huston CD, Linford AS, Buss SN, Houpt E, Sherman NE, et al. *Entamoeba histolytica* phagocytosis of human erythrocytes involves PATMK, a member of the transmembrane kinase family. *PLoS Pathog* (2008) **4**:e8. doi:10.1371/journal.ppat.0040008
144. Ralston KS, Petri WA Jr. Tissue destruction and invasion by *Entamoeba histolytica*. *Trends Parasitol* (2011) **27**:254–63. doi:10.1016/j.pt.2011.02.006
145. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* (1998) **101**:890–8. doi:10.1172/JCI1112
146. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol* (2014) **14**:166–80. doi:10.1038/nri3607
147. Orozco E, Guarneros G, Martinez-Palomo A, Sánchez T. *Entamoeba histolytica*: phagocytosis as a virulence factor. *J Exp Med* (1983) **158**:1511–21. doi:10.1084/jem.158.5.1511
148. Rodríguez MA, Orozco E. Isolation and characterization of phagocytosis- and virulence-deficient mutants of *Entamoeba histolytica*. *J Infect Dis* (1986) **154**:27–32. doi:10.1093/infdis/154.1.27
149. Teixeira JE, Heron BT, Huston CD. C1q- and collectin-dependent phagocytosis of apoptotic host cells by the intestinal protozoan *Entamoeba histolytica*. *J Infect Dis* (2008) **198**:1062–70. doi:10.1086/591628
150. Vaithilingam A, Teixeira JE, Miller PJ, Heron BT, Huston CD. *Entamoeba histolytica* cell surface calreticulin binds human C1q and functions in amebic phagocytosis of host cells. *Infect Immun* (2012) **80**:2008–18. doi:10.1128/IAI.06287-11
151. Somlata, Bhattacharya S, Bhattacharya A. A C2 domain protein kinase initiates phagocytosis in the protozoan parasite *Entamoeba histolytica*. *Nat Commun* (2011) **2**:230. doi:10.1038/ncomms1199
152. Mansuri MS, Bhattacharya S, Bhattacharya A. A novel alpha kinase EhAK1 phosphorylates actin and regulates phagocytosis in *Entamoeba histolytica*. *PLoS Pathog* (2014) **10**:e1004411. doi:10.1371/journal.ppat.1004411
153. Ralston KS, Solga MD, Mackey-Lawrence NM, Somlata, Bhattacharya A, Petri WA Jr. Trogocytosis by *Entamoeba histolytica* contributes to cell killing and tissue invasion. *Nature* (2014) **508**:526–30. doi:10.1038/nature13242
154. Guo X, Stroup SE, Houpt E. Persistence of *Entamoeba histolytica* infection in CBA mice owes to intestinal IL-4 production and inhibition of protective IFN-γ. *Mucosal Immunol* (2008) **1**:139–46. doi:10.1038/mi.2007.18
155. Petri WA Jr, Mondal D, Peterson KM, Duggal P, Haque R. Association of malnutrition with amebiasis. *Nutr Rev* (2009) **67**:S207–15. doi:10.1111/j.1753-4887.2009.00242.x
156. Acuna-Soto R, Maguire JH, Wirth DF. Gender distribution in asymptomatic and invasive amebiasis. *Am J Gastroenterol* (2000) **95**:1277–83. doi:10.1111/j.1572-0241.2000.01525.x

157. Blessmann J, Van Linh P, Nu PA, Thi HD, Muller-Myhsok B, Buss H, et al. Epidemiology of amebiasis in a region of high incidence of amebic liver abscess in central Vietnam. *Am J Trop Med Hyg* (2002) **66**:578–83.
158. Blessmann J, Ali IK, Nu PA, Dinh BT, Viet TQ, Van AL, et al. Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. *J Clin Microbiol* (2003) **41**:4745–50. doi:10.1128/JCM.41.10.4745-4750.2003
159. Lotter H, Helk E, Bernin H, Jacobs T, Prehn C, Adamski J, et al. Testosterone increases susceptibility to amebic liver abscess in mice and mediates inhibition of IFNγ secretion in natural killer T cells. *PLoS One* (2013) **8**:e55694. doi:10.1371/journal.pone.0055694
160. Madsen KL, Malfair D, Gray D, Doyle JS, Jewell LD, Fedorak RN. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm Bowel Dis* (1999) **5**:262–70. doi:10.1097/00054725-199911000-00004
161. Hamano S, Asgharpour A, Stroup SE, Wynn TA, Leiter EH, Houpt E. Resistance of C57BL/6 mice to amoebiasis is mediated by nonhemopoietic cells but requires hemopoietic IL-10 production. *J Immunol* (2006) **177**:1208–13. doi:10.4049/jimmunol.177.2.1208
162. Ruiz PA, Shkoda A, Kim SC, Sartor RB, Haller D. IL-10 gene-deficient mice lack TGF-beta/Smad signaling and fail to inhibit proinflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic *Enterococcus faecalis*. *J Immunol* (2005) **174**:2990–9. doi:10.4049/jimmunol.174.5.2990
163. Schwerbrock NM, Makkink MK, van der Sluis M, Buller HA, Einerhand AW, Sartor RB, et al. Interleukin 10-deficient mice exhibit defective colonic Muc2 synthesis before and after induction of colitis by commensal bacteria. *Inflamm Bowel Dis* (2004) **10**:811–23. doi:10.1097/00054725-200411000-00016
164. Grazia Roncarolo M, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. *Immunol Rev* (2001) **182**:68–79. doi:10.1034/j.1600-065X.2001.1820105.x
165. Zhou P, Streutker C, Borojevic R, Wang Y, Croitoru K. IL-10 modulates intestinal damage and epithelial cell apoptosis in T cell-mediated enteropathy. *Am J Physiol* (2004) **287**:G599–604. doi:10.1152/ajpgi.00063.2004
166. Maldonaldo-Bernal C, Kirschning CJ, Rosenstein Y, Rocha LM, Rios-Sarabia N, Espinosa-Cantellano M, et al. The innate immune response to *Entamoeba histolytica* lipopeptidophosphoglycan is mediated by toll-like receptors 2 and 4. *Parasite Immunol* (2005) **2**:127–37. doi:10.1111/j.1365-3024.2005.00754.x
167. Maldonado C, Trejo W, Ramirez A, Carrera M, Sánchez J, López-Macías C, et al. Lipophosphopeptidoglycan of *Entamoeba histolytica* induces an anti-inflammatory innate immune response and downregulation of toll-like receptor 2 (TLR-2) gene expression in human monocytes. *Arch Med Res* (2000) **31**:S71–3. doi:10.1016/S0188-4409(00)00199-5
168. Kammanadiminti SJ, Chadeem K. Suppression of NF-kappaB activation by *Entamoeba histolytica* in intestinal epithelial cells is mediated by heat shock protein 27. *J Biol Chem* (2006) **281**:26112–20. doi:10.1074/jbc.M601988200
169. Cho KN, Becker SM, Houpt ER. The NF-kappaB p50 subunit is protective during intestinal *Entamoeba histolytica* infection of 129 and C57BL/6 mice. *Infect Immun* (2010) **78**:1475–81. doi:10.1128/IAI.00669-09
170. Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* (2007) **446**:557–61. doi:10.1038/nature05698
171. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* (2008) **8**:411–20. doi:10.1038/nri2316
172. Ivory C, Kammanadiminti S, Chadee K. Innate resistance to *Entamoeba histolytica* in murine models. *Trends Parasitol* (2007) **23**:46–8. doi:10.1016/j.pt.2006.12.006
173. Dey I, Chadee K. Prostaglandin E2 produced by *Entamoeba histolytica* binds to EP4 receptors and stimulates interleukin-8 production in human colonic cells. *Infect Immun* (2008) **6**:5158–63. doi:10.1128/IAI.00645-08
174. Lejeune M, Moreau F, Chadee K. Prostaglandin E2 produced by *Entamoeba histolytica* signals via EP4 receptor and alters claudin-4 to increase ion permeability of tight junctions. *Am J Pathol* (2011) **179**:807–18. doi:10.1016/j.ajpath.2011.05.001
175. Belley A, Chadee K. Prostaglandin E(2) stimulates rat and human colonic mucin exocytosis via the EP(4) receptor. *Gastroenterology* (1999) **117**:1352–62. doi:10.1016/S0016-5085(99)70285-4
176. Moncada D, Keller K, Chadee K. *Entamoeba histolytica* cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. *Infect Immun* (2003) **71**:838–44. doi:10.1128/IAI.71.2.838-844.2003
177. Wang W, Keller K, Chadee K. *Entamoeba histolytica* modulates the nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxicity against amoebae and tumour cells. *Immunology* (1994) **83**:601–10.
178. Wang W, Chadee K. *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatibility complex Ia molecule and I-Aβ mRNA expression by a prostaglandin E2-dependent mechanism. *Infect Immun* (1995) **63**:1089–94.
179. Verma AK, Verma R, Ahuja V, Paul J. Real-time analysis of gut flora in *Entamoeba histolytica* infected patients of northern India. *BMC Microbiol* (2012) **12**:183. doi:10.1186/1471-2180-12-183
180. Bracha R, Kobiler D, Mirelman D. Attachment and ingestion of bacteria by trophozoites of *Entamoeba histolytica*. *Infect Immun* (1982) **36**:396–406.
181. Cobo ER, He C, Hirata K, Hwang G, Tran U, Eckmann L, et al. *Entamoeba histolytica* induces intestinal cathelicidins but is resistant to cathelicidin-mediated killing. *Infect Immun* (2012) **80**:143–9. doi:10.1128/IAI.05029-11
182. Burgess SL, Buonomo E, Carey M, Cowardin C, Naylor C, Noor Z, et al. Bone marrow dendritic cells from mice with an altered microbiota provide interleukin 17A-dependent protection against *Entamoeba histolytica* colitis. *MBio* (2014) **5**:e01817. doi:10.1128/mBio.01817-14
183. Band RN, Cirrito H. Growth response of axenic *Entamoeba histolytica* to hydrogen, carbon dioxide, and oxygen. *J Protozool* (1979) **26**:282–6. doi:10.1111/j.1550-7408.1979.tb02781.x
184. Mehlotra RK. Antioxidant defense mechanisms in parasitic protozoa. *Crit Rev Microbiol* (1996) **22**:295–314. doi:10.3109/10408419609105484
185. Weinbach EC, Diamond LS. *Entamoeba histolytica*. I. Aerobic metabolism. *Exp Parasitol* (1974) **35**:232–43. doi:10.1016/0014-4894(74)90027-7
186. Vicente JB, Ehrenkaufer GM, Saraiva LM, Teixeira M, Singh U. *Entamoeba histolytica* modulates a complex repertoire of novel genes in response to oxidative and nitrosative stresses: implications for amebic pathogenesis. *Cell Microbiol* (2009) **11**:51–69. doi:10.1111/j.1462-5822.2008.01236.x
187. Rastew E, Vicente JB, Singh U. Oxidative stress resistance genes contribute to the pathogenic potential of the anaerobic protozoan parasite, *Entamoeba histolytica*. *Int J Parasitol* (2012) **42**:1007–15. doi:10.1016/j.ijpara.2012.08.006
188. Schlosser S, Leitschm D, Duchêne M. *Entamoeba histolytica*: identification of thioredoxin-targeted proteins and analysis of serine acetyltransferase-1 as a prototype example. *Biochem J* (2013) **451**:277–88. doi:10.1042/BJ20121798
189. Debnath A, Parsonage D, Andrade RM, He C, Cobo ER, Hirata K, et al. A high-throughput drug screen for *Entamoeba histolytica* identifies a new lead and target. *Nat Med* (2012) **18**:956–60. doi:10.1038/nm.2758
190. Leitsch D, Kolarich D, Wilson IB, Altmann F, Duchêne M. Nitroimidazole action in *Entamoeba histolytica*: a central role for thioredoxin reductase. *PLoS Biol* (2007) **5**:e211. doi:10.1371/journal.pbio.0050211
191. Husain A, Sato D, Jeelani G, Soga T, Nozaki T. Dramatic increase in glycerol biosynthesis upon oxidative stress in the anaerobic protozoan parasite *Entamoeba histolytica*. *PLoS Negl Trop Dis* (2012) **6**:e1831. doi:10.1371/journal.pntd.0001831
192. Jeelani G, Nozaki T. Entamoeba thiol-based redox metabolism: a potential target for drug development. *Mol Biochem Parasitol* (2016). doi:10.1016/j.molbiopara.2016.01.004

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MINI REVIEW
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Evasion and Immuno-Endocrine Regulation in Parasite Infection: Two Sides of the Same Coin in Chagas Disease?

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Chagas disease is a serious illness caused by the protozoan parasite *Trypanosoma cruzi*. Nearly 30% of chronically infected people develop cardiac, digestive, or mixed alterations, suggesting a broad range of host-parasite interactions that finally impact upon chronic disease outcome. The ability of *T. cruzi* to persist and cause pathology seems to depend on diverse factors like *T. cruzi* strains, the infective load and the route of infection, presence of virulence factors, the parasite capacity to avoid protective immune response, the strength and type of host defense mechanisms and the genetic background of the host. The host-parasite interaction is subject to a constant neuro-endocrine regulation that is thought to influence the adaptive immune system, and as the infection proceeds it can lead to a broad range of outcomes, ranging from pathogen elimination to its continued persistence in the host. In this context, *T. cruzi* evasion strategies and host defense mechanisms can be envisioned as two sides of the same coin, influencing parasite persistence and different outcomes observed in Chagas disease. Understanding how *T. cruzi* evade host's innate and adaptive immune response will provide important clues to better dissect mechanisms underlying the pathophysiology of Chagas disease.

Keywords: evasion strategies, persistence, Chagas disease, immunoendocrine, thymus, virulence factors

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INTRODUCTION

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease (WHO, 2015). Nearly 30% of chronically infected people develop cardiomyopathy, megacolon, and megaesophagus or a mixed of these alterations, suggesting a broad range of host-parasite interactions that finally impact upon chronic disease outcome (Rassi et al., 2010). Different and not mutually exclusive hypotheses have been considered for the pathogenesis of chronic Chagas disease, including autoimmunity by molecular mimicry, microvascular (Ramos and Rossi, 1999), and autonomic dysfunction (Dávila et al., 2004), and tissue damage by parasite persistence (Gironès et al., 2005; Gutierrez et al., 2009; Cunha-Neto et al., 2011). The parasite persistence hypothesis predicts a chronic inflammatory reactivity as result of a failure in parasite burden control, thus promoting the development of disease pathology (Tarleton, 2001). In addition, a subpatent parasite-induced cell lysis as consequence of amastigote differentiation into trypomastigotes

(Bonney and Engman, 2008) might fuel inflammation. The presence of parasites (Añez et al., 1999; Buckner et al., 1999) or their products, such as DNA, in blood and myocardium of chronic infected hosts is well documented (Añez et al., 1999; Zhang and Tarleton, 1999; Salomone et al., 2000; Elias et al., 2003). *T. cruzi* reactivation in HIV co-infected, transplanted or immunocompromised chronic chagasic patients provides convincing evidence of parasite persistence (Tarleton, 2001; Andrade et al., 2014), reinforcing the view that disease pathology and its severity are directly related to *T. cruzi* presence within the affected tissue (Tarleton, 2001). In this review, we examined the complexity of cellular, molecular and physiologic factors involved in *T. cruzi* evasion and persistence in the light of current data.

PARASITE EVASION INVOLVE DIRECT HOST IMMUNE REGULATION AND LATENCY ESTABLISHMENT

T. cruzi has a complex biological cycle involving mammals and insect vectors. The strategies that *T. cruzi* employs to guarantee its long-term survival within mammalian hosts include evasion from phagolysosome, expression of virulence factors, direct immunomodulation and the establishment of latency sites (**Figure 1**). Trypomastigotes can invade nucleated cells through different mechanisms depending on whether the target cell is phagocytic or nonphagocytic (**Figure 2A**; Romano et al., 2012). Macrophages are the most important innate effector cells in the fight against *T. cruzi*, but when subverted in the infection they can be also exploited by the parasite as its primary niche, thus avoiding cell-mediated immunity. Protective classically activated (M1) macrophages are activated by IFN-γ, increasing the expression of nitric oxide synthase (iNOS) and nitric oxide (NO) production favoring the parasite killing. In contrast, parasite clearance is prevented when macrophages acquire an alternatively activated (M2) phenotype, with reduced NO production thus increasing the parasite persistence (Sizirensen et al., 1994; Desjardins and Descoteaux, 1997; Paulnock and Coller, 2001; Stempin et al., 2002; Martinez and Gordon, 2014). Unlike other parasites that prevent phagolysosome maturation (David Sibley, 2011), *T. cruzi* evades macrophage microbicidal activity by escaping from phagolysosome to cytoplasm, an event that is mediated by the cytolitic activity of parasite's C9 cross-reactive protein (Tc-Tox; Andrews et al., 1990; Bogdan and Röllinghoff, 1999). Once inside the cytoplasm, *T. cruzi* parasites promote STAT1 dephosphorylation, thus interfering with the transcription of IL-12 and TNF-α (De Diego et al., 1997) that ultimately abrogate IFN-γ-mediated microbicidal responses (Gazzinelli et al., 1992; Stahl et al., 2014). In addition, parasite-derived proteases shutdown IL-12 expression by interrupting the NF-κB signaling pathway (Doyle et al., 2011). Furthermore, *T. cruzi* stimulate the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β that impair the development of protective immune responses hence favoring the spread of infection and parasite persistence in the host (Silva et al., 1991; Hunter et al., 1997; Freire-de-Lima et al., 2000). *T. cruzi* can also disrupt the classical and alternative complement pathways: parasite CRP and T-DAF proteins bind to C3b and C4b fragments, inhibiting the assembly of C3 and C5 convertase on the parasite membrane (Joiner et al., 1986; Norris et al., 1991; Tambourgi et al., 1993; Zambrano-Villa et al., 2002).

The acute phase of infection is marked by a transient state of immunosuppression (Liew et al., 1987; Kierszenbaum et al., 1999, 2002; Van Overtvelt et al., 2002; Alcaide and Fresno, 2004; Gutierrez et al., 2009; Padilla et al., 2009; DosReis, 2011; Oladiran and Belosevic, 2012; Pinazo et al., 2013) involving, among other things, a strong polyclonal B cell stimulation which restricts the development of antigen-specific lymphocytes, promoting apoptosis and cell cycle arrest (Ortiz-Ortiz et al., 1980; Maleckar and Kierszenbaum, 1983; Zuñiga et al., 2000). In fact *T. cruzi* provides a good example of such immunosuppression strategy: T cells from infected mice respond poorly to mitogens (Kierszenbaum et al., 1999, 2002; Alcaide and Fresno, 2004) and they also undergo enhanced apoptosis upon stimulation of T cell receptor (TCR), increasing the unresponsiveness of host immunity (Abrahamsohn and Coffman, 1995; Martins et al., 1998; Nunes et al., 1998). Studies have supported that IL-2 deficiency is one of the hallmarks of the disease-induced T cell immunosuppression (Abrahamsohn and Coffman, 1995). The T cell unresponsiveness in Chagas disease is also the result of a direct downregulation of IL-2 receptor by the parasite glycoprotein AGC10 (Kierszenbaum et al., 1999). Recently, a novel immunosuppressive mechanism was described, which implies the IFN-γ-dependent NO secretion by immature myeloid cells (Goñi et al., 2002).

ADIPOSE TISSUE ACTS AS A RESERVOIR OF *TRYPANOSOMA CRUZI*

Another adaptive strategy displayed by *T. cruzi* parasites to optimize its persistence in the host consists in targeting tissues with particular characteristics. Some studies have shown that adipose tissue (AT) might serve as a reservoir where parasite could persist in a latent state to avoid the host-defense mechanisms, acting as a possible site of reactivation, similarly to that observed for other intracellular pathogens (**Figure 2B**; Neyrolles et al., 2006; Bechah et al., 2010, 2014). Adipocytes could harbor a significant number of parasites even in the chronic phase of infection (Combs et al., 2005). Recently, more robust evidence that AT can act as a reservoir of *T. cruzi* have arisen from experiments in which infected mice were subsequently treated with an anti-parasitic drug and afterwards immunosuppressed. Intriguingly, in a significant number of animals, the AT was the major site of parasite recrudescence (Fortes Francisco et al., 2015). Moreover, studies carried out in patients with chronic chagasic cardiomyopathy have revealed the presence of parasite-derived DNA in AT (Ferreira et al., 2011). In this sense, AT may be a reservoir from which reactivation of infection may occur, especially during periods of immunosuppression, as observed in chagasic patients co-infected with HIV, transplanted or undergoing immunosuppressive therapies (Almeida et al., 1996; Sartori et al., 1998; Campos et al., 2008; Cordova et al., 2008; Pinazo et al., 2013). Moreover, in HIV co-infected chagasic

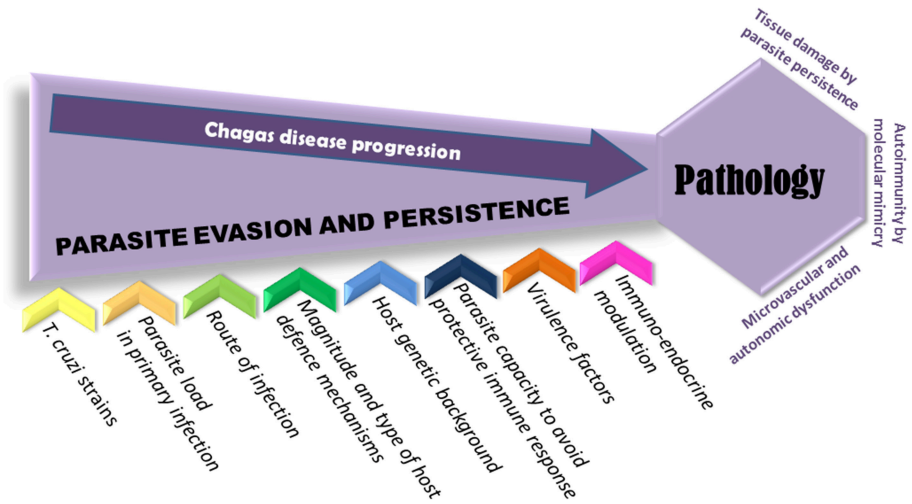

FIGURE 1 | Pathogenesis of *Trypanosoma cruzi* infection. Several hypotheses have been considered for the pathogenesis of chronic Chagas disease, comprising tissue damage by parasite persistence, autoimmunity, microvascular injury, and autonomic dysfunction. Since diverse factors are involved in parasite evasion and persistence, most of all may influence the infection outcome and the development of pathology in almost 30% of infected individuals. The ability of *T. cruzi* to evade immune system seems to depend on diverse factors like *T. cruzi* strains, the infective load and the route of infection and the presence of virulence factors; but also can be determined by the type and strength of host defense mechanisms and the genetic background of the host.

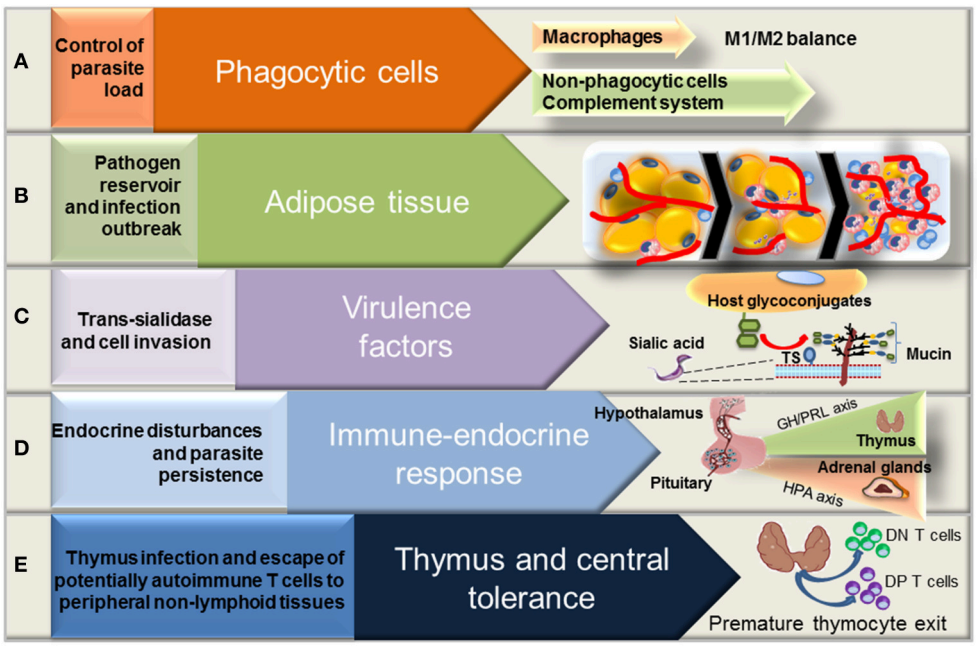

FIGURE 2 | Aspects of *Trypanosoma cruzi* evasion and persistence in the vertebrate host. *T. cruzi* parasites develop different strategies to evade the host defenses and establish a persistent infection. *T. cruzi* parasites evade the host innate immune responses associated with macrophage and complement system **(A)**. The trans-sialidase (TS), a *T. cruzi*-derived virulence factor, can also overcome the host resistance responses to optimize the invasion and parasite persistence in chronic infection **(B)**. The development of anti-parasite immune response is coupled with the activation of neuroendocrine axes that may affect the course of disease **(C)**. Adipose tissue can be considered as a parasite reservoir and may contribute to the establishment of persistent infections, playing a major role in *T. cruzi* burst during immunosuppression periods **(D)**. The recognition of *T. cruzi*-derived antigens in the thymus may restrict the central tolerance to parasite infection, while the release of immature and potentially autoimmune T cells to the peripheral non-lymphoid tissues may be related with disease pathology in the chronic phase **(E)**.

patients, periods of lipoatrophy may result in the release of parasites into the circulation (Ferreira et al., 2011). It remains to be investigated why *T. cruzi* persists in the AT. Possible reasons could be the special metabolic conditions that *T. cruzi* finds inside the adipocyte and the slow turnover of these cells. After infection, there is an influx of inflammatory cells to AT, accompanied by an *in situ* upregulation of both TNF-α and IL-6, concomitantly to a diminution of adipocytokine levels (Desjardins and Descoteaux, 1997; Chandrasekar et al., 2000; Manarin et al., 2013). Moreover, some studies suggested that metabolic alterations induced by *T. cruzi* persistence in AT may increase the risk of diabetes, metabolic syndrome and cardiovascular disease (Chandrasekar et al., 2000; Nagajyothi et al., 2009; Manarin et al., 2013).

TRYPANOSOMA CRUZI VIRULENCE FACTORS OVERCOME THE HOST RESISTANCE RESPONSE TO ESTABLISH PERSISTENT INFECTIONS

The ability of *T. cruzi* parasites to persist and cause pathology partially depends on factors such as the parasite strain, the genetic background of the host (Andrade et al., 2002; Savino et al., 2007) and the route of infection (Barreto-de-Albuquerque et al., 2015). *T. cruzi* species display a broad range of biological, biochemical, molecular and genetic characteristics, being grouped in six discrete typing units (DTUs; Macedo et al., 2004; Zingales et al., 2009). The parasite immune modulatory effects seems to be strain-dependent, a feature that might influence parasite-host interactions (Lauria-Pires, 1996; Briones et al., 1999). Different parasite strains coexist dynamically in natural reservoirs and combinations of them have been found in triatomine bugs from domestic and peridomestic areas (Noireau et al., 2009), as well as in infected patients (Cura et al., 2015; Monje-Rumi et al., 2015).

The damping of host immune response during the acute phase of Chagas disease is partially caused by *T. cruzi*-derived virulence factors (**Figure 2C**; DosReis et al., 2005; Gutierrez et al., 2009; Nagajyothi et al., 2012). One of the hallmarks of parasite's cell membrane composition is the presence of mucin-like molecules presenting sialic acid residues attached to their terminal β-galactosyl residues. These residues are transferred from host glycoconjugates by the action of parasite trans-sialidase (Schenkman et al., 1991, 1994; Previato et al., 1995; Eugenia Giorgi and De Lederkremer, 2011). Parasite mucins are encoded by more than 800 genes comprising ∼1% of the parasite genome, represented as O-glycosylated Thr/Ser/Pro-rich glycoproteins (Di Noia et al., 1995; Buscaglia et al., 2006; Mendonça-Previato et al., 2013). The *T. cruzi*-derived mucin molecules are determinant in the host-parasite interplay, since they mediate processes related to invasion of the vertebrate cells as well as subvert the host immune system. The sialylated forms of mucin-like molecules protect the parasite antigenic determinants from host humoral responses, avoiding the humoral attack mediated by anti-galactosyl antibodies and complement factor B (Kipnis et al., 1981; Joiner et al., 1986; Gazzinelli et al., 1991; Pereira-Chioccola et al., 2000). Moreover, it has been shown that once sialylated, mucin-like molecules are able to impair the host dendritic cell function through inhibition of the IL-12 expression (Erdmann et al., 2009), possibly at transcriptional level as described for IL-2 gene (Kierszenbaum et al., 1999, 2002). Furthermore, the parasite sialoglycoproteins are able to inhibit tyrosine phosphorylation of the adapter protein SLP-76 and tyrosine kinase ZAP-70, both involved in the early events of T cell activation (Alcaide and Fresno, 2004).

Recently, it has been shown that *in vivo* exposure to *T. cruzi* mucins enhances the host susceptibility, as seen by the increased parasitemia and heart tissue damage. These effects were associated with a reduction in Th1 and Th2 cytokine production, together with decreased levels in the frequency of IFN-γ producing $CD4^+$ T cells in the spleen of mice treated with parasite mucins in comparison with untreated controls (Nunes et al., 2013). Interestingly, it has been shown that the binding of acid-binding Ig-like lectin Siglec-E (CD33) by *T. cruzi* mucins inhibits the mitogenic responses of $CD4^+$ T cells. Studies conducted to address the molecular mechanisms underlying these effects have shown that the impairment of TCR/CD3-mediated activation of $CD4^+$ T cells by *T. cruzi*-derived mucins was correlated with induction of G1-phase cell cycle arrest. Importantly, it has been demonstrated that interactions of the terminal sialyl residues of *T. cruzi* mucins with $CD4^+$ T cells led to the induction of the cell cycle regulator p27/Kip1 responsible to block the transition from G1 to S phase of mytosis, thus preventing the proliferative responses (Nunes et al., 2013).

Interestingly, the limited T cell responses observed in *T. cruzi* infection contrast with the large polyclonal expansion of B lymphocytes seen in the acute phase (Ortiz-Ortiz et al., 1980), as demonstrated by the increased frequency of IgG2a and IgG2b secreting B cells in peripheral lymphoid organs of infected mice. This phenomenon results in high frequency of nonspecific antibodies with low affinity for *T. cruzi* antigens (Ouaissi et al., 2001), some of them cross-reacting with heart and neural autoantigens (Acosta and Santos-Buch, 1985; Kierszenbaum, 1999; Engman and Leon, 2002). The auto-reactive B cell responses are thought to play secondary roles in the pathogenesis of Chagas disease. The extensive polyclonal expansion of the B cells could partly affect lymphoid compartments by increasing the competition for activation and survival signals needed to promote the generation of antigen-specific lymphocyte responses against *T. cruzi* (Freitas and Rocha, 2000; Montaudouin et al., 2013).

In addition, parasite-derived glycol-inositol-phospholipids (GIPLs), which are components of the dense glycolipid layer covering the parasite cell surface, also promote alterations in the B cell compartment. These molecules work as TLR4 agonists, mediating pro-inflammatory effects (Oliveira et al., 2004). Another virulence factor encoded by *T. cruzi* that target the B cell compartment is the proline racemase, which participates in arginine and proline metabolism, acting as a potent mitogen for B cells. Shortly, *T. cruzi* -derived virulence factors are active players in the subversion of the host immune system and are determinant for the establishment of chronic persistent infection (Reina-San-Martín et al., 2000; Chamond et al., 2003).

THE IMMUNE-ENDOCRINE IMBALANCE IS A KEY DETERMINANT OF PARASITE PERSISTENCE

Immune and neuro-endocrine systems are integrated through a complex network of mediators, involving cytokines, adipocytokines, hormones, and neuropeptides that collectively act to maintain homeostasis (Besedovsky and del Rey, 1996; Fantuzzi, 2005). However, when vertebrate hosts are challenged by infectious pathogens, acute and short-term stress signals are delivered by this network to initiate and build global host mechanisms of defense (Besedovsky and del Rey, 1996). In parallel, pathogens could interfere with this neuro-endocrine response at several levels. Thus, a race between pathogen-mediated evasion mechanisms and host immune response will determine whether the microorganisms will be rapidly eliminated or persist in the host (**Figure 2D**). In mice, the anti-*T. cruzi* immune response is associated with the activation of neuro-endocrine circuitries, mainly the hypothalamic-pituitary-adrenocortical (HPA) axis (Roggero et al., 2006; Corrêa-De-Santana et al., 2006b). In this scenario, pro-inflammatory cytokines released during infection activate the HPA axis, leading to production of glucocorticoids (GC), crucial for host survival. Evidently, the neuro-endocrine circutries initiates an anti-inflammatory response attempting to minimize the infection-induced collateral tissue damage. However its immunoregulatory effect ultimately favor the parasitism and establishment of persistent infection. Comparative studies between susceptible and resistant experimental mice models have indicated that the course of *T. cruzi* infection strongly depends on the appropriate timing and magnitude of the immune-endocrine response (Roggero et al., 2006). Susceptible animals succumb as consequence of increased inflammatory response poorly counteracted by the HPA axis, while resistant animals develop a more balanced immune-endocrine response that lead to the establishment of a chronic infection and mild pathology. Moreover, when GC signaling was abrogated by adrenalectomy or treatment with GC receptor antagonist RU486, the severity of infection increased dramatically as a result of an augmented inflammation-based immunopathology (Roggero et al., 2006; Pérez et al., 2007). These findings indicate that a delicate balance between the immune and endocrine systems play a role in the establishment of chronic infections. Additionally, the activation of HPA axis leads to secretion of other adrenal steroids, such as dehydroepiandrosterone (DHEA). In this regard, the increased vulnerability of *T. cruzi* infected young animals was associated with a high corticosterone/DHEA-sulfate ratio as compared to the adult counterparts (Pérez et al., 2011). Similarly, patients with severe chronic chagasic myocarditis also revealed a disruption in the activation of HPA axis as characterized by decreased concentrations of DHEA-sulfate and unbalanced cortisol/DHEA-sulfate ratio in comparison to asymptomatic or healthy individuals (Pérez et al., 2011). Overall, these findings reinforce the view that during *T. cruzi* human infection there are endocrine disturbances that might favor parasite persistence, thus influencing the disease pathology.

Moreover, pro-inflammatory cytokines associated to *T. cruzi* infection such as TNF-α, IL-6, or IL-1β could affect the release of hypothalamic, pituitary or adrenal hormones by their direct action on the endocrine glands (Kanczkowski et al., 2013, 2015; Hueston and Deak, 2014). During experimental *T. cruzi* infection, TNF-α has been implicated in the HPA activation at central level (Roggero et al., 2006; Pérez et al., 2007), although inhibitory actions at adrenal level has been also observed (Villar et al., 2013). Acutely infected TNF-R1 knock-out mice showed an enhanced transcription of adrenal steroidogenic proteins StAR, CYP11A1, CYP11B1 and 11β-HSD1 as compared to wild type mice, suggesting that GC secretion can be down regulated by TNF-α *in situ*, independently of the signaling pathway induced by adrenocorticotropic hormone (ACTH; Corrêa-De-Santana et al., 2006a; Villar et al., 2013). Since both parasites and their antigens had been detected within adrenal glands (Corrêa-De-Santana et al., 2006a; Villar et al., 2013), their presence might induce *in situ* the release of TNF-α, with the consequent modulation of GC secretion. In addition, IL-6 has also been associated with enhanced activity of the HPA axis during experimental *T. cruzi* infection. In this regard, supernatants of adenopituitary cell cultures challenged with the parasite contained more IL-6, while infected mice also showed augmented circulating levels of this cytokine systemically (Corrêa-De-Santana et al., 2006a). The activation of hypothalamus-pituitary unit also results in the release of both growth hormone (GH) and prolactin (PRL), which are capable of improving the immune response, counteracting the GC-driven immunosuppression. *T. cruzi* infection appears to directly modulate the secretion of both hormones, since *in vitro* infection of mammosomatotrophic cell line diminished GH and PRL secretion, similarly to observed in the pituitary glands of infected mice (Corrêa-De-Santana et al., 2009). The modulation of GH and PRL secretion by diminishing the Pit-1 gene expression, a major transcription factor for both hormone genes (Corrêa-De-Santana et al., 2009). Moreover, the downregulation of these hormones during the infection might be also related to the presence of parasites or their antigens in the glands, favoring T cell and macrophage infiltration, vascular stasis along with increased depots of extracellular matrix proteins (Corrêa-De-Santana et al., 2006b, 2009). The downmodulation of GH and PRL hormones is also observed in African trypanosomiasis and may illustrates a common modulatory mechanism (Radomski et al., 1994, 1996). Moreover, there is a bulk of evidence indicating that sex steroid hormones might influence the development and course of diverse parasitic infections (Romano et al., 2015). Particularly, it has been shown that *T. cruzi* parasites have the capacity to metabolize steroid hormones (Vacchina et al., 2008), suggesting a possible role of this mechanism in the host-parasite interplay.

TRYPANOSOMA CRUZI INFECTION MAY INFLUENCE CENTRAL TOLERANCE

Several alterations in the thymic environment occur in infectious diseases (Watson et al., 1983, 1984; Savino et al., 1986; Leite de Moraes et al., 1991; Godfraind et al., 1995; Brito et al., 2003;

Chen et al., 2005; Seixas and Ostler, 2005; Suzuki et al., 2005). The most evident alteration is the atrophy of the thymus due to the apoptotic death of differentiating thymocytes (Savino, 2006). In *T. cruzi* infected mice present a marked imbalance between intrathymic and systemic stress-related endocrine circuits, where the rise of intrathymic levels of GC affect the viability of double positive $CD4^+CD8^+$ (DP) cells, double-negative $CD4^-CD8^-$ (DN) and simple positive (SP) thymocytes (Roggero et al., 2006; Pérez et al., 2007). The induction of GC-driven apoptosis of DP cells is clearly associated with the activation of caspases 8 and 9 (Farias-de-Oliveira et al., 2013). In mice, the thymic atrophy is also influenced by the premature export of immature DP and DN thymocytes to the periphery, exhibiting a pro-inflammatory profile (**Figure 2E**; Leite-de-Moraes et al., 1992; De Meis et al., 2009; Morrot et al., 2011). Interestingly, increased numbers of circulating undifferentiated DP T lymphocytes was observed in patients with cardiac forms of chronic Chagas disease (Lepletier et al., 2014). Studies have identified a potential role for sphingosine-1-phosphate receptor-1 in this abnormal exit of undifferentiated thymocytes to the periphery in Chagas disease (Lepletier et al., 2014).

The *T. cruzi* infected thymus undergoing atrophy is still able to carry out negative selection, remaining important considerations in the context of host-pathogen interactions (Mendes-da-Cruz et al., 2003; Morrot et al., 2011). *T. cruzi* parasites also colonize the thymus (Savino et al., 1989), so their antigens may be presented to recirculating parasite-specific memory T cells migrating from the periphery to the thymic microenvironment. Alternatively, the parasite colonization of thymus could lead to the generation of *T. cruzi*-specific Tregs with high affinity TCR (Pacholczyk and Kern, 2008), thus promoting the host tolerance to persistent infection. Interestingly, it has been observed that chagasic patients in the indeterminate phase shown high frequencies of circulating Tregs as compared to chronic cardiac ones (De Araujo et al., 2011), suggesting a beneficial role of Tregs in suppressing the pathology associated to disease progression. In contrast, in experimental lethal models of Chagas disease with highly Th1-polarized inflammatory responses, the expansion of Tregs is clearly restricted (González et al., 2015).

CONCLUDING REMARKS

Recent studies suggest that the immuno-endocrine host response may favor *T. cruzi* chronic persistence. Future studies attempting to understand how *T. cruzi* evade host immune response or the extent by which parasite persistence might be favored by immune-neuro-endocrine regulation will provide important clues to better dissect mechanisms underlying the pathophysiology of Chagas disease.

AUTHOR CONTRIBUTIONS

AM, FBG, SV and AP wrote the paper. All authors read and approved the final version of the manuscript.

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REFERENCES

Abrahamsohn, I. A., and Coffman, R. L. (1995). Cytokine and nitric oxide regulation of the immunosuppression in *Trypanosoma cruzi* infection. *J. Immunol.* 155, 3955–3963.

Acosta, A. M., and Santos-Buch, C. A. (1985). Autoimmune myocarditis induced by *Trypanosoma cruzi*. *Circulation* 71, 1255–1261. doi: 10.1161/01.CIR.71.6.1255

Alcaide, P., and Fresno, M. (2004). The *Trypanosoma cruzi* membrane mucin AgC10 inhibits T cell activation and IL-2 transcription through L-selectin. *Int. Immunol.* 16, 1365–1375. doi: 10.1093/intimm/dxh138

Almeida, D. R., Carvalho, A. C., Branco, J. N., Pereira, A. P., Correa, L., Vianna, P. V., et al. (1996). Chagas' disease reactivation after heart transplantation: efficacy of allopurinol treatment. *J. Heart Lung Transplant.* 15, 988–992.

Andrade, D. V., Gollob, K. *J., and Dutra, W. O.* (2014). Acute chagas disease: new global challenges for an old neglected disease. PLoS Negl. Trop. Dis. 8:e3010. doi: 10.1371/journal.pntd.0003010

Andrade, L. O., Machado, C. R. S., Chiari, E., Pena, S. D. J., and Macedo, A. M. (2002). *Trypanosoma cruzi*: role of host genetic background in the differential tissue distribution of parasite clonal populations. *Exp. Parasitol.* 100, 269–275. doi: 10.1016/S0014-4894(02)00024-3

Andrews, N. W., Abrams, C. K., Slatin, S. L., and Griffiths, G. (1990). A *T. cruzi*-secreted protein immunologically related to the complement component C9: evidence for membrane pore-forming activity at low pH. *Cell* 61, 1277–1287. doi: 10.1016/0092-8674(90)90692-8

Añez, N., Carrasco, H., Parada, H., Crisante, G., Rojas, A., Fuenmayor, C., et al. (1999). Myocardial parasite persistence in chronic chagasic patients. *Am. J. Trop. Med. Hyg.* 60, 726–732.

Barreto-de-Albuquerque, J., Silva-dos-Santos, D., Pérez, A. R., Berbert, L. R., Santana-van-Vliet, E., De Farias-de-Oliveira, D. A., et al. (2015). *Trypanosoma cruzi* Infection through the oral route promotes a severe infection in mice: new disease form from an old infection? *PLoS Negl. Trop. Dis.* 9:e0003849. doi: 10.1371/journal.pntd.0003849

Bechah, Y., Paddock, C. D., Capo, C., Mege, J. L., and Raoult, D. (2010). Adipose tissue serves as a reservoir for recrudescent *Rickettsia prowazekii* infection in a mouse model. *PLoS ONE* 5:e8547. doi: 10.1371/journal.pone.0008547

Bechah, Y., Verneau, J., Amara, A., Ben, B.arry, A. O., Lépolard, C., et al. (2014). Persistence of *Coxiella burnetii*, the agent of Q fever, in murine adipose tissue. *PLoS ONE* 9:e97503. doi: 10.1371/journal.pone.0097503

Besedovsky, H. O., and del Rey, A. (1996). Immune-neuro-endocrine interations: facts and hypotheses. *Endocr. Rev.* 17, 64–102. doi: 10.1210/edrv-17-1-64

Bogdan, C., and Röllinghoff, M. (1999). How do protozoan parasites survive inside macrophages? *Parasitol. Today* 15, 22–28. doi: 10.1016/S0169-4758(98)01362-3

Bonney, K. M., and Engman, D. M. (2008). Chagas heart disease pathogenesis: one mechanism or many? *Curr. Mol. Med.* 8, 510–518. doi: 10.2174/156652408785748004

Briones, M. R. S., Souto, R. P., Stolf, B. S., and Zingales, B. (1999). The evolution of two *Trypanosoma cruzi* subgroups inferred from rRNA genes can be correlated with the interchange of American mammalian faunas in the Cenozoic and has

implications to pathogenicity and host specificity. *Mol. Biochem. Parasitol.* 104, 219–232. doi: 10.1016/S0166-6851(99)00155-3

Brito, V. N., Souto, P. C. S., Cruz-Höfling, M. A., Ricci, L. C., and Verinaud, L. (2003). Thymus invasion and atrophy induced by *Paracoccidioides brasiliensis* in BALB/c mice. *Med. Mycol.* 41, 83–87. doi: 10.1080/714043902

Buckner, F. S., Wilson, A. J., and Van Voorhis, W. C. (1999). Detection of live *Trypanosoma cruzi* in tissues of Infected mice by using histochemical stain for β-galactosidase. *Infect Immun.* 67, 403–409.

Buscaglia, C. A., Campo, V. A., Frasch, A. C. C., and Di Noia, J. M. (2006). *Trypanosoma cruzi* surface mucins: host-dependent coat diversity. *Nat. Rev. Microbiol.* 4, 229–236. doi: 10.1038/nrmicro1351

Campos, S. V., Strabelli, T. M. V., Amato Neto, V., Silva, C. P., Bacal, F., Bocchi, E. A., et al. (2008). Risk factors for chagas' disease reactivation after heart transplantation. *J. Hear Lung Transplant.* 27, 597–602. doi: 10.1016/j.healun.2008.02.017

Chamond, N., Grégoire, C., Coatnoan, N., Rougeot, C., Freitas-Junior, L. H., Da Silveira, J. F., et al. (2003). Biochemical characterization of proline racemases from the human protozoan parasite *Trypanosoma cruzi* and definition of putative protein signatures. *J. Biol. Chem.* 278, 15484–15494. doi: 10.1074/jbc.M210830200

Chandrasekar, B., Melby, P. C., Troyer, D. A., and Freeman, G. L. (2000). Differential regulation of nitric oxide synthase isoforms in experimental acute Chagasic cardiomyopathy. *Clin. Exp. Immunol.* 121, 112–119. doi: 10.1046/j.1365-2249.2000.01258.x

Chen, W., Kuolee, R., Austin, J. W., Shen, H., Che, Y., and Conlan, J. W. (2005). Low dose aerosol infection of mice with virulent type A Francisella tularensis induces severe thymus atrophy and CD4+CD8+ thymocyte depletion. *Microb. Pathog.* 39, 189–196. doi: 10.1016/j.micpath.2005.08.005

Combs, T. P., Nagajyothi, M. S., De Almeida, C. J. G., Jelicks, L. A., Schubert, W., Lin, Y., et al. (2005). The adipocyte as an important target cell for *Trypanosoma cruzi* infection. *J. Biol. Chem.* 280, 24085–24094. doi: 10.1074/jbc.M412802200

Cordova, E., Boschi, A., Ambrosioni, J., Cudos, C., and Corti, M. (2008). Reactivation of Chagas disease with central nervous system involvement in HIV-infected patients in Argentina, 1992-2007. *Int. J. Infect. Dis.* 12, 587–592. doi: 10.1016/j.ijid.2007.12.007

Corrêa-De-Santana, E., Paez-Pereda, M., Theodoropoulou, M., Kenji Nihei, O., Gruebler, Y., Bozza, M., et al. (2006a). Hypothalamus-pituitary-adrenal axis during *Trypanosoma cruzi* acute infection in mice. *J. Neuroimmunol.* 173, 12–22. doi: 10.1016/j.jneuroim.2005.08.015

Corrêa-De-Santana, E., Paez-Pereda, M., Theodoropoulou, M., Renner, U., Stalla, J., Stalla, G. K., et al. (2009). Modulation of growth hormone and prolactin secretion in *Trypanosoma cruzi* -infected mammosomatotrophic cells. *Neuroimmunomodulation* 16, 208–212. doi: 10.1159/000205513

Corrêa-De-Santana, E., Pinto-Mariz, F., and Savino, W. (2006b). Immunoneuroendocrine interactions in Chagas disease. *Ann. N.Y. Acad. Sci.* 1088, 274–283. doi: 10.1196/annals.1366.005

Cunha-Neto, E., Teixeira, P. C., Nogueira, L. G., and Kalil, J. (2011). Autoimmunity. *Adv. Parasitol.* 76, 129–152. doi: 10.1016/B978-0-12-385895-5.00006-2

Cura, C. I., Duffy, T., Lucero, R. H., Bisio, M., Péneau, J., Jimenez-Coello, M., et al. (2015). Multiplex real-time PCR assay using TaqMan probes for the identification of *Trypanosoma cruzi* DTUs in biological and clinical samples. *PLoS Negl. Trop. Dis.* 9:e0003765. doi: 10.1371/journal.pntd.0003765

David Sibley, L. (2011). Invasion and intracellular survival by protozoan parasites. *Immunol. Rev.* 240, 72–91. doi: 10.1111/j.1600-065X.2010.00990.x

Dávila, D. F., Donis, J. H., Torres, A., and Ferrer, J. A. (2004). A modified and unifying neurogenic hypothesis can explain the natural history of chronic Chagas heart disease. *Int. J. Cardiol.* 96, 191–195. doi: 10.1016/j.ijcard.2003.06.015

De Araujo, F. F., Vitelli-Avelar, D. M., Teixeira-Carvalho, A., Renato Zuquim Antas, P., Assis Silva Gomes, J., Sathler-Avelar, R., et al. (2011). Regulatory T cells phenotype in different clinical forms of chagas' disease. *PLoS Negl. Trop. Dis.* 5:e992. doi: 10.1371/journal.pntd.0000992

De Diego, J., Punzón, C., Duarte, M., and Fresno, M. (1997). Alteration of macrophage function by a *Trypanosoma cruzi* membrane mucin. *J. Immunol.* 159, 4983–4989.

De Meis, J., Morrot, A., Farias-de-Oliveira, D. A., Villa-Verde, D. M. S., and Savino, W. (2009). Differential regional immune response in Chagas disease. *PLoS Negl. Trop. Dis.* 3:e417. doi: 10.1371/journal.pntd.0000417

Desjardins, M., and Descoteaux, A. (1997). Inhibition of phagolysosomal biogenesis by the Leishmania lipophosphoglycan. *J. Exp. Med.* 185, 2061–2068.

Di Noia, J. M., Sanchez, D. O., and Frasch, A. C. C. (1995). The protozoan *Trypanosoma cruzi* has a family of genes resembling the mucin genes of mammalian cells. *J. Biol. Chem.* 270, 24146–24149. doi: 10.1074/jbc.270.41.24146

DosReis, G. A. (2011). Evasion of immune responses by *Trypanosoma cruzi*, the etiological agent of Chagas disease. *Braz. J. Med. Biol. Res.* 44, 84–90. doi: 10.1590/S0100-879X2011007500005

DosReis, G. A., Freire-De-Lima, C. G., Nunes, M. P., and Lopes, M. F. (2005). The importance of aberrant T-cell responses in Chagas disease. *Trends Parasitol.* 21, 237–243. doi: 10.1016/j.pt.2005.03.008

Doyle, P. S., Zhou, Y. M., Hsieh, I., Greenbaum, D. C., McKerrow, J. H., and Engel, J. C. (2011). The *Trypanosoma cruzi* protease cruzain mediates immune evasion. *PLoS Pathog.* 7:e1002139. doi: 10.1371/journal.ppat.1002139

Elias, F. E., Vigliano, C. A., Laguens, R. P., Levin, M. J., and Berek, C. (2003). Analysis of the presence of *Trypanosoma cruzi* in the heart tissue of three patients with chronic Chagas' heart disease. *Am. J. Trop. Med. Hyg.* 68, 242–247.

Engman, D. M., and Leon, J. S. (2002). Pathogenesis of Chagas heart disease: role of autoimmunity. *Acta Trop.* 81, 123–132. doi: 10.1016/S0001-706X(01)00202-9

Erdmann, H., Steeg, C., Koch-Nolte, F., Fleischer, B., and Jacobs, T. (2009). Sialylated ligands on pathogenic *Trypanosoma cruzi* interact with Siglec-E (sialic acid-binding Ig-like lectin-E). *Cell. Microbiol.* 11, 1600–1611. doi: 10.1111/j.1462-5822.2009.01350.x

Eugenia Giorgi, M., and De Lederkremer, R. M. (2011). Trans-sialidase and mucins of *Trypanosoma cruzi*: an important interplay for the parasite. *Carbohydr. Res.* 346, 1389–1393. doi: 10.1016/j.carres.2011.04.006

Fantuzzi, G. (2005). Adipose tissue, adipokines, and inflammation. *J. Allergy Clin. Immunol.* 115, 911–920. doi: 10.1016/j.jaci.2005.02.023

Farias-de-Oliveira, D. A., Villa-Verde, D. M. S., Nunes Panzenhagen, P. H., Silva dos Santos, D., Berbert, L. R., Savino, W., et al. (2013). Caspase-8 and caspase-9 mediate thymocyte apoptosis in *Trypanosoma cruzi* acutely infected mice. *J. Leukoc. Biol.* 93, 227–234. doi: 10.1189/jlb.1211589

Ferreira, A. V. M., Segatto, M., Menezes, Z., Macedo, A. M., Gelape, C., de Oliveira Andrade, L., et al. (2011). Evidence for *Trypanosoma cruzi* in adipose tissue in human chronic Chagas disease. *Microbes Infect.* 13, 1002–1005. doi: 10.1016/j.micinf.2011.06.002

Fortes Francisco, A., Lewis, M. D., Jayawardhana, S., Taylor, M. C., Chatelain, E., and Kelly, J. M. (2015). The limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi* infections revealed by highly sensitive *in vivo* imaging. *Antimicrob. Agents Chemother.* 59, 4653–4661. doi: 10.1128/AAC.00520-15

Freire-de-Lima, C. G., Nascimento, D. O., Soares, M. B., Bozza, P. T., Castro-Faria-Neto, H. C., de Mello, F. G., et al. (2000). Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* 403, 199–203. doi: 10.1038/35003208

Freitas, A. A., and Rocha, B. (2000). Population biology of lymphocytes: the flight for survival. *Annu. Rev. Immunol.* 18, 83–111. doi: 10.1146/annurev.immunol.18.1.83

Gazzinelli, R. T., Oswald, I. P., Hieny, S., James, S. L., and Sher, A. (1992). The microbicidal activity of interferon-gamma-treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. *Eur. J. Immunol.* 22, 2501–2506. doi: 10.1002/eji.1830221006

Gazzinelli, R. T., Pereira, M. E., Romanha, A., Gazzinelli, G., and Brener, Z. (1991). Direct lysis of *Trypanosoma cruzi*: a novel effector mechanism of protection mediated by human anti-gal antibodies. *Parasite Immunol.* 13, 345–356. doi: 10.1111/j.1365-3024.1991.tb00288.x

Gironès, N., Cuervo, H., and Fresno, M. (2005). *Trypanosoma cruzi* -induced molecular mimicry and Chagas' disease. *Curr. Top. Microbiol. Immunol.* 296, 89–123. doi: 10.1007/3-540-30791-5_6

Godfraind, C., Holmes, K. V., and Coutelier, J. P. (1995). Thymus involution induced by mouse hepatitis virus A59 in BALB/c mice. *J. Virol.* 69, 6541–6547.

Goñi, O., Alcaide, P., and Fresno, M. (2002). Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G (Gr1(+))CD11b(+)immature myeloid suppressor cells. *Int. Immunol.* 14, 1125–1134. doi: 10.1093/intimm/dxf076

González, F. B., Villar, S. R., Fernández, R., Martin, G. H., Pérol, L., Manarin, R., et al. (2015). Immunoendocrine dysbalance during uncontrolled *T. cruzi* infection is associated with the acquisition of a Th-1-like phenotype. *Brain Behav. Immun.* 45, 219–232. doi: 10.1016/j.bbi.2014.11.016

Gutierrez, F. R. S., Guedes, P. M. M., Gazzinelli, R. T., and Silva, J. S. (2009). The role of parasite persistence in pathogenesis of Chagas heart disease. *Parasite Immunol.* 31, 673–685. doi: 10.1111/j.1365-3024.2009.01108.x

Hueston, C. M., and Deak, T. (2014). The inflamed axis: The interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic-pituitary-adrenal axis. *Physiol. Behav.* 124, 77–91. doi: 10.1016/j.physbeh.2013.10.035

Hunter, C. A., Ellis-Neyes, L. A., Slifer, T., Kanaly, S., Grünig, G., Fort, M., et al. (1997). IL-10 is required to prevent immune hyperactivity during infection with *Trypanosoma cruzi*. *J. Immunol.* 158, 3311–3316.

Joiner, K., Sher, A., Gaither, T., and Hammer, C. (1986). Evasion of alternative complement pathway by *Trypanosoma cruzi* results from inefficient binding of factor B. *Proc. Natl. Acad. Sci. U.S.A.* 83, 6593–6597. doi: 10.1073/pnas.83.17.6593

Kanczkowski, W., Alexaki, V.-I., Tran, N., Großklaus, S., Zacharowski, K., Martinez, A., et al. (2013). Hypothalamo-pituitary and immune-dependent adrenal regulation during systemic inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14801–14806. doi: 10.1073/pnas.1313945110

Kanczkowski, W., Sue, M., Zacharowski, K., Reincke, M., and Bornstein, S. R. (2015). The role of adrenal gland microenvironment in the HPA axis function and dysfunction during sepsis. *Mol. Cell. Endocrinol.* 408, 241–248. doi: 10.1016/j.mce.2014.12.019

Kierszenbaum, F. (1999). Chagas' disease and the autoimmunity hypothesis. *Clin. Microbiol. Rev.* 12, 210–223.

Kierszenbaum, F., De Diego, J. L., Fresno, M., and Sztein, M. B. (1999). Inhibitory effects of the *Trypanosoma cruzi* membrane glycoprotein AGC10 on the expression of IL-2 receptor chains and secretion of cytokines by subpopulations of activated human T lymphocytes. *Eur. J. Immunol.* 29, 1684–1691.

Kierszenbaum, F., Fresno, M., and Sztein, M. B. (2002). The *Trypanosoma cruzi* membrane glycoprotein AGC10 inhibits human lymphocyte activation by a mechanism preceding translation of both, interleukin-2 and its high-affinity receptor subunits. *Mol. Biochem. Parasitol.* 125, 91–101. doi: 10.1016/S0166-6851(02)00217-7

Kipnis, T. L., David, J. R., Alper, C. A., Sher, A., and da Silva, W. D. (1981). Enzymatic treatment transforms trypomastigotes of *Trypanosoma cruzi* into activators of alternative complement pathway and potentiates their uptake by macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 78, 602–605. doi: 10.1073/pnas.78.1.602

Lauria-Pires, L. T. A. (1996). Virulence and pathogenicity associated with diversity of *Trypanosoma cruzi* stocks and clones derived from Chagas' disease patients. *Am. J. Trop. Med. Hyg.* 55, 304–310.

Leite de Moraes, M. C., Hontebeyrie-Joskowicz, M., Leboulenger, F., Savino, W., Dardenne, M., and Lepault, F. (1991). Studies on the thymus in Chagas' disease. II. Thymocyte subset fluctuations in *Trypanosoma cruzi*-infected mice: relationship to stress. *Scand. J. Immunol.* 33, 267–275. doi: 10.1111/j.1365-3083.1991.tb01772.x

Leite-de-Moraes, M. C., Hontebeyrie-Joskowicz, M., Dardenne, M., and Savino, W. (1992). Modulation of thymocyte subsets during acute and chronic phases of experimental *Trypanosoma cruzi* infection. *Immunology* 77, 95–98.

Lepletier, A., de Almeida, L., Santos, L., da Silva Sampaio, L., Paredes, B., González, F. B., et al. (2014). Early double-negative thymocyte export in *Trypanosoma cruzi* infection is restricted by sphingosine receptors and associated with human chagas disease. *PLoS Negl. Trop. Dis.* 8:e3203. doi: 10.1371/journal.pntd.0003203

Liew, F. Y., Scott, M. T., Liu, D. S., and Croft, S. L. (1987). Suppressive substance produced by T cells from mice chronically infected with *Trypanosoma cruzi*. I. Preferential inhibition of the induction of delayed-type hypersensitivity. *J. Immunol.* 139, 2452–2457.

Macedo, A. M., Machado, C. R., Oliveira, R. P., and Pena, S. D. J. (2004). *Trypanosoma cruzi*: genetic structure of populations and relevance of genetic variability to the pathogenesis of chagas disease. *Mem. Inst. Oswaldo Cruz* 99, 1–12. doi: 10.1590/S0074-02762004000100001

Maleckar, J. R., and Kierszenbaum, F. (1983). Inhibition of mitogen-induced proliferation of mouse T and B lymphocytes by bloodstream forms of *Trypanosoma cruzi*. *J. Immunol.* 130, 908–911.

Manarin, R., Villar, S. R., Bussy, R. F., González, F. B., Deschutter, E. V., Bonantini, A. P., et al. (2013). Reciprocal influences between leptin and glucocorticoids during acute *Trypanosoma cruzi* infection. *Med. Microbiol. Immunol.* 202, 339–352. doi: 10.1007/s00430-013-0294-1

Martinez, F. O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6:13. doi: 10.12703/P6-13

Martins, G. A., Cardoso, M. A., Aliberti, J. C., and Silva, J. S. (1998). Nitric oxide-induced apoptotic cell death in the acute phase of *Trypanosoma cruzi* infection in mice. *Immunol. Lett.* 63, 113–120. doi: 10.1016/S0165-2478(98)00066-2

Mendes-da-Cruz, D. A., de Meis, J., Cotta-de-Almeida, V., and Savino, W. (2003). Experimental *Trypanosoma cruzi* infection alters the shaping of the central and peripheral T-cell repertoire. *Microbes Infect.* 5, 825–832. doi: 10.1016/S1286-4579(03)00156-4

Mendonça-Previato, L., Penha, L., Garcez, T. C., Jones, C., and Previato, J. O. (2013). Addition of α-O-GlcNAc to threonine residues define the post-translational modification of mucin-like molecules in *Trypanosoma cruzi*. *Glycoconj. J.* 30, 659–666. doi: 10.1007/s10719-013-9469-7

Monje-Rumi, M. M., Brandán, C. P., Ragone, P. G., Tomasini, N., Lauthier, J. J., Alberti D'Amato, A. M., et al. (2015). *Trypanosoma cruzi* diversity in the Gran Chaco: mixed infections and differential host distribution of TcV and TcVI. *Infect. Genet. Evol.* 29, 53–59. doi: 10.1016/j.meegid.2014.11.001

Montaudouin, C., Anson, M., Hao, Y., Duncker, S. V., Fernandez, T., Gaudin, E. et al. (2013). Quorum sensing contributes to activated IgM-secreting B cell homeostasis. *J. Immunol.* 190, 106–114. doi: 10.4049/jimmunol.1200907

Morrot, A., Terra-Granado, E., Pérez, A. R., Silva-Barbosa, S. D., Milićević, N. M., Farias-de-Oliveira, D. A., et al. (2011). Chagasic thymic atrophy does not affect negative selection but results in the export of activated CD4+CD8+ T cells in severe forms of human disease. *PLoS Negl. Trop. Dis.* 5:e1268. doi: 10.1371/journal.pntd.0001268

Nagajyothi, F., Desruisseaux, M. S., Weiss, L. M., Chua, S., Albanese, C., Machado, F. S., et al. (2009). Chagas disease, adipose tissue and the metabolic syndrome. *Mem. Inst. Oswaldo Cruz* 104(Suppl.), 219–225. doi: 10.1590/S0074-02762009000900028

Nagajyothi, F., Machado, F. S., Burleigh, B. A., Jelicks, L. A., Scherer, P. E., Mukherjee, S., et al. (2012). Mechanisms of *Trypanosoma cruzi* persistence in Chagas disease. *Cell. Microbiol.* 14, 634–643. doi: 10.1111/j.1462-5822.2012.01764.x

Neyrolles, O., Hernández-Pando, R., Pietri-Rouxel, F., Fornès, P., Tailleux, L., Payán, J. A. B., et al. (2006). Is adipose tissue a place for *Mycobacterium tuberculosis* persistence? *PLoS ONE* 1:e43. doi: 10.1371/journal.pone.0000043

Noireau, F., Diosque, P., and Jansen, A. M. (2009). *Trypanosoma cruzi*: adaptation to its vectors and its hosts. *Vet. Res.* 40:26. doi: 10.1051/vetres/2009009

Norris, K. A., Bradt, B., Cooper, N. R., and So, M. (1991). Characterization of a *Trypanosoma cruzi* C3 binding protein with functional and genetic similarities to the human complement regulatory protein, decay-accelerating factor. *J. Immunol.* 147, 2240–2247.

Nunes, M. P., Andrade, R. M., Lopes, M. F., and DosReis, G. A. (1998). Activation-induced T cell death exacerbates *Trypanosoma cruzi* replication in macrophages cocultured with CD4+ T lymphocytes from infected hosts. *J. Immunol.* 160, 1313–1319.

Nunes, M. P., Fortes, B., Silva-Filho, J. L., Terra-Granado, E., Santos, L., Conde, L., et al. (2013). Inhibitory Effects of *Trypanosoma cruzi* Sialoglycoproteins on CD4+ T cells are associated with increased susceptibility to infection. *PLoS ONE* 8:e77568. doi: 10.1371/journal.pone.0077568

Oladiran, A., and Belosevic, M. (2012). Immune evasion strategies of trypanosomes: a review. *J. Parasitol.* 98, 284–292. doi: 10.1645/GE-2925.1

Oliveira, A.-C., Peixoto, J. R., de Arruda, L. B., Campos, M. A., Gazzinelli, R. T., Golenbock, D. T., et al. (2004). Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with T. cruzi. *J. Immunol.* 173, 5688–5696. doi: 10.4049/jimmunol.173.9.5688

Ortiz-Ortiz, L., Parks, D. E., Rodriguez, M., and Weigle, W. O. (1980). Polyclonal B lymphocyte activation during *Trypanosoma cruzi* infection. *J. Immunol.* 124, 121–126.

Ouaissi, A., Da Silva, A. C., Guevara, A. G., Borges, M., and Guilvard, E. (2001). *Trypanosoma cruzi* -induced host immune system dysfunction: a rationale for parasite immunosuppressive factor(s) encoding gene targeting. *J. Biomed. Biotechnol.* 2001, 11–17. doi: 10.1155/S1110724301000055

Pacholczyk, R., and Kern, J. (2008). The T-cell receptor repertoire of regulatory T cells. *Immunology* 125, 450–458. doi: 10.1111/j.1365-2567.2008.02992.x

Padilla, A. M., Simpson, L. J., and Tarleton, R. L. (2009). Insufficient TLR activation contributes to the slow development of CD8+ T cell responses in *Trypanosoma cruzi* infection. *J. Immunol.* 183, 1245–1252. doi: 10.4049/jimmunol.0901178

Paulnock, D. M., and Coller, S. P. (2001). Analysis of macrophage activation in African trypanosomiasis. *J. Leukoc. Biol.* 69, 685–690.

Pereira-Chioccola, V. L., Acosta-Serrano, A., Correia de Almeida, I., Ferguson, M. A., Souto-Padron, T., Rodrigues, M. M., et al. (2000). Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies. *J. Cell Sci.* 113(Pt 7), 1299–1307.

Pérez, A. R., Bertoya, A. A., Revelli, S., and García, F. (2011). A high corticosterone/DHEA-s ratio in young rats infected with *Trypanosoma cruzi* is associated with increased susceptibility. *Mem. Inst. Oswaldo Cruz* 106, 416–423. doi: 10.1590/S0074-02762011000400006

Pérez, A. R., Roggero, E., Nicora, A., Palazzi, J., Besedovsky, H. O., del Rey, A., et al. (2007). Thymus atrophy during *Trypanosoma cruzi* infection is caused by an immuno-endocrine imbalance. *Brain Behav. Immun.* 21, 890–900. doi: 10.1016/j.bbi.2007.02.004

Pérez, R., Silva-Barbosa, S. D., Berbert, L. R., Revelli, S., Beloscar, J., Savino, W., et al. (2011). Immunoneuroendocrine alterations in patients with progressive forms of chronic Chagas disease. *J. Neuroimmunol.* 235, 84–90. doi: 10.1016/j.jneuroim.2011.03.010

Pinazo, M. J., Espinosa, G., Cortes-Lletget, C., Posada Ede, J., Aldasoro, E., Oliveira, I., et al. (2013). Immunosuppression and chagas disease: a management challenge. *PLoS Negl. Trop. Dis.* 7:e1965. doi: 10.1371/journal.pntd.0001965

Previato, J. O., Jones, C., Xavier, M. T., Wait, R., Travassos, L. R., Parodi, A. J., et al. (1995). Structural characterization of the major glycosylphosphatidylinositol membrane-anchored glycoprotein from epimastigote forms of *Trypanosoma cruzi* Y-strain. *J. Biol. Chem.* 270, 7241–7250. doi: 10.1074/jbc.270.13.7241

Radomski, M. W., Buguet, A., Bogui, P., Doua, F., Lonsdorfer, A., Tapie, P., et al. (1994). Disruptions in the secretion of cortisol, prolactin, and certain cytokines in human African trypanosomiasis patients. *Bull. Soc. Pathol. Exot.* 87, 376–379.

Radomski, M. W., Buguet, A., Doua, F., Bogui, P., and Tapie, P. (1996). Relationship of plasma growth hormone to slow-wave sleep in African sleeping sickness. *J. Neuroendocrinol.* 63, 393–396. doi: 10.1159/000126980

Ramos, S. G., and Rossi, M. A. (1999). Invited review microcirculation and Chagas' disease: hypothesis and recent results. *Rev. Inst. Med. Trop. Sao Paulo* 41, 123–129. doi: 10.1590/S0036-46651999000200011

Rassi, A. Jr., Rassi, A., and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* 375, 1388–1402. doi: 10.1016/S0140-6736(10)60061-X

Reina-San-Martín, B., Degrave, W., Rougeot, C., Cosson, A., Chamond, N., Cordeiro-Da-Silva, A., et al. (2000). A B-cell mitogen from a pathogenic trypanosome is a eukaryotic proline racemase. *Nat. Med.* 6, 890–897. doi: 10.1038/78651

Roggero, E., Pérez, A. R., Tamae-Kakazu, M., Piazzon, I., Nepomnaschy, I., Basedovsky, H. O., et al. (2006). Edogenous glucocorticoids cause thymus atrophy but are protective during acute *Trypanosoma cruzi* infection. *J. Endocrinol.* 190, 495–503. doi: 10.1677/joe.1.06642

Romano, M. C., Jiménez, P., Miranda-Brito, C., Valdez, R. A. (2015). Parasites and steroid hormones: corticosteroid and sex steroid synthesis, their role in the parasite physiology and development. Front. *Neurosci.* 9:224. doi: 10.3389/fnins.2015.00224

Romano, P. S., Cueto, J. A., Casassa, A. F., Vanrell, M. C., Gottlieb, R. A., and Colombo, M. I. (2012). Molecular and cellular mechanisms involved in the *Trypanosoma cruzi* /host cell interplay. *IUBMB Life* 64, 387–396. doi: 10.1002/iub.1019

Salomone, O. A., Juri, D., Omelianiuk, M. O., Sembaj, A., Aguerri, A. M., Carriazo, C., et al. (2000). Prevalence of circulating *Trypanosoma cruzi* detected by polymerase chain reaction in patients with Chagas' cardiomyopathy. *Am. J. Cardiol.* 85, 1274–1276. doi: 10.1016/S0002-9149(00)00747-5

Sartori, A. M. C., Lopes, M. H., Benvenuti, L. A., Caramelli, B., Di Pietro, A. O., Nunes, E. V., et al. (1998). Reactivation of Chagas' disease in a human immunodeficiency virus- infected patient leading to severe heart disease with a late positive direct microscopic examination of the blood. *Am. J. Trop. Med. Hyg.* 59, 784–786.

Savino, W. (2006). The thymus is a common target organ in infectious diseases. *PLoS Pathog.* 2:e62. doi: 10.1371/journal.ppat.0020062

Savino, W., Dardenne, M., Marche, C., Trophilme, D., Dupuy, J. M., Pekovic, D., et al. (1986). Thymic epithelium in AIDS. An immunohistologic study. *Am. J. Pathol.* 122, 302–307.

Savino, W., Leite-de-Moraes, M. C., Hontebeyrie-Joskowicz, M., and Dardenne, M. (1989). Studies on the thymus in Chagas' disease. I. Changes in the thymic microenvironment in mice acutely infected with *Trypanosoma cruzi*. *Eur. J. Immunol.* 19, 1727–1733. doi: 10.1002/eji.1830190930

Savino, W., Villa-Verde, D. M. S., Mendes-da-Cruz, D. A., Silva-Monteiro, E., Perez, A. R., Aoki M del, P., et al. (2007). Cytokines and cell adhesion receptors in the regulation of immunity to *Trypanosoma cruzi*. *Cytokine Growth Factor Rev.* 18, 107–124. doi: 10.1016/j.cytogfr.2007.01.010

Schenkman, S., Eichinger, D., Pereira, M. E., and Nussenzweig, V. (1994). Structural and functional properties of Trypanosoma trans-sialidase. *Annu. Rev. Microbiol.* 48, 499–523. doi: 10.1146/annurev.mi.48.100194.002435

Schenkman, S., Jiang, M. S., Hart, G. W., and Nussenzweig, V. (1991). A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage-specific epitope required for invasion of mammalian cells. *Cell* 65, 1117–1125. doi: 10.1016/0092-8674(91)90008-M

Seixas, E., and Ostler, D. (2005). Plasmodium chabaudi chabaudi (AS): differential cellular responses to infection in resistant and susceptible mice. *Exp. Parasitol.* 110, 394–405. doi: 10.1016/j.exppara.2005.03.024

Silva, J. S., Twardzik, D. R., and Reed, S. G. (1991). Regulation of *Trypanosoma cruzi* infections *in vitro* and *in vivo* by transforming growth factor beta (TGF-β). *J. Exp. Med.* 174, 539–545. doi: 10.1084/jem.174.3.539

Sizirensen, A. L., Hey, A. S., and Kharazmi, A. (1994). Leishmania major surface protease Gp63 interferes with the function of human monocytes and neutrophils *in vitro*. *APMIS* 102, 65–271. doi: 10.1111/j.1699-0463.1994.tb04874.x

Stahl, P., Ruppert, V., Schwarz, R. T., and Meyer, T. (2014). *Trypanosoma cruzi* evades the protective role of interferon-gamma-signaling in parasite-infected cells. *PLoS ONE* 9:e110512. doi: 10.1371/journal.pone.0110512

Stempin, C., Giordanengo, L., Gea, S., and Cerbán, F. (2002). Alternative activation and increase of *Trypanosoma cruzi* survival in murine macrophages stimulated by cruzipain, a parasite antigen. *J. Leukoc. Biol.* 72,727–734.

Suzuki, H., Motohara, M., Miyake, A., Ibuki, K., Fukazawa, Y., Inaba, K., et al. (2005). Intrathymic effect of acute pathogenic SHIV infection on T-lineage cells in newborn macaques. *Microbiol. Immunol.* 49, 667–679. doi: 10.1111/j.1348-0421.2005.tb03646.x

Tambourgi, D. V., Kipnis, T. L., Da Silva, W. D., Joiner, K. A., Sher, A., Heath, S., et al. (1993). A partial cDNA clone of trypomastigote decay-accelerating factor (T-DAF), a developmentally regulated complement inhibitor of *Trypanosoma cruzi*, has genetic and functional similarities to the human complement inhibitor DAF. *Infect. Immun.* 61, 3656–3663.

Tarleton, R. L. (2001). Parasite persistence in the aetiology of Chagas disease. *Int. J. Parasitol.* 31, 550–554. doi: 10.1016/S0020-7519(01)00158-8

Vacchina, P., Valdéz, R. A., Gómez, Y., Revelli, S., and Romano, M. C. (2008). Steroidogenic capacity of *Trypanosoma cruzi* trypomastigotes. *J. Steroid Biochem. Mol. Biol.* 111, 282–286. doi: 10.1016/j.jsbmb.2008.06.016

Van Overtvelt, L., Andrieu, M., Verhasselt, V., Connan, F., Choppin, J., Vercruysse, V., et al. (2002). *Trypanosoma cruzi* down-regulates lipopolysaccharide-induced MHC class I on human dendritic cells and impairs antigen presentation to specific CD8(+) T lymphocytes. *Int. Immunol.* 14, 1135–1144. doi: 10.1093/intimm/dxf077

Villar, S. R., Ronco, M. T., Fernández Bussy, R., Roggero, E., Lepletier, A., Manarin, R., et al. (2013). Tumor necrosis factor-α regulates glucocorticoid synthesis in the adrenal glands of *Trypanosoma cruzi* acutely-infected mice. The Role of TNF-R1. *PLoS ONE* 8:e63814. doi: 10.1371/journal.pone.0063814

Watson, S. R., Miller, T. B., Redington, T. J., and Bullock, W. E. (1983). Immunoregulation in experimental disseminated histoplasmosis: flow microfluorometry (FMF) studies of the Thy and Lyt phenotypes of T lymphocytes from infected mice. *J. Immunol.* 131, 984–990.

Watson, S. R., Redington, T. J., Miller, T. B., and Bullock, W. E. (1984). Flow microfluorometry analysis of alterations in T-lymphocyte subsets during murine listeriosis. *Infect. Immun.* 45, 372–377.

WHO (2015). *Third WHO Report on Neglected Tropical Diseases. Invest to Overcome Global Impact of Neglected Tropical Disease*. Geneva.

Zambrano-Villa, S., Rosales-Borjas, D., Carrero, J. C., and Ortiz-Ortiz, L. (2002). How protozoan parasites evade the immune response. *Trends Parasitol.* 18, 272–278. doi: 10.1016/S1471-4922(02)02289-4

Zhang, L., and Tarleton, R. L. (1999). Parasite persistence correlates with disease severity and localization in chronic Chagas' disease. *J. Infect. Dis.* 180, 480–486. doi: 10.1086/314889

Zingales, B., Andrade, S. G., Briones, M. R. S., Campbell, D. A., Chiari, E., Fernandes, O., et al. (2009). A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Mem. Inst. Oswaldo Cruz* 104, 1051–1054. doi: 10.1590/S0074-02762009000700021

Zuñiga, E., Motran, C., Montes, C. L., Diaz, F. L., Bocco, J. L., and Gruppi, A. (2000). *Trypanosoma cruzi* -induced immunosuppression: B cells undergo spontaneous apoptosis and lipopolysaccharide (LPS) arrests their proliferation during acute infection. *Clin. Exp. Immunol.* 119, 507–515. doi: 10.1046/j.1365-2249.2000.01150.x

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REVIEW
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Escaping Deleterious Immune Response in Their Hosts: Lessons from Trypanosomatids

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The Trypanosomatidae family includes the genera *Trypanosoma* and *Leishmania*, protozoan parasites displaying complex digenetic life cycles requiring a vertebrate host and an insect vector. *Trypanosoma brucei gambiense*, *Trypanosoma cruzi*, and *Leishmania* spp. are important human pathogens causing human African trypanosomiasis (HAT or sleeping sickness), Chagas' disease, and various clinical forms of Leishmaniasis, respectively. They are transmitted to humans by tsetse flies, triatomine bugs, or sandflies, and affect millions of people worldwide. In humans, extracellular African trypanosomes (*T. brucei*) evade the hosts' immune defenses, allowing their transmission to the next host, via the tsetse vector. By contrast, *T. cruzi* and *Leishmania* sp. have developed a complex intracellular lifestyle, also preventing several mechanisms to circumvent the host's immune response. This review seeks to set out the immune evasion strategies developed by the different trypanosomatids resulting from parasite–host interactions and will focus on: clinical and epidemiological importance of diseases; life cycles: parasites–hosts–vectors; innate immunity: key steps for trypanosomatids in invading hosts; deregulation of antigen-presenting cells; disruption of efficient specific immunity; and the immune responses used for parasite proliferation.

Keywords: Trypanosomatidae family, parasite–host interactions, immunosuppression, *Leishmania* sp., *Trypanosoma brucei* sp., *Trypanosoma cruzi*

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CLINICAL AND EPIDEMIOLOGICAL IMPORTANCE OF NEGLECTED DISEASES

Trypanosomatid parasites interact with a wide range of insects and mammals to complete their life cycles. Some species, particularly *Trypanosoma brucei gambiense, Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, and *Leishmania* spp. are pathogenic for humans, causing, respectively, human African trypanosomiasis (HAT or sleeping sickness), Chagas' disease, and cutaneous, mucocutaneaous, and visceral Leishmaniasis (VL). These infectious eukaryotic parasites have been described and identified over a century ago; however, as of today, no vaccines are available and the availability of effective prophylactic and therapeutic drugs remains limited. It is estimated that more than 20 million people are infected and that 100,000 people die each year of trypanosomiasis or Chagas' disease (1). Annually, cutaneous leishmaniasis affects around 1 million people, whereas VL is responsible for around 500,000 cases annually resulting in over 50,000 deaths (2).

Tsetse flies (*Glossina* spp.) transmit HAT-causing trypanosomes. Regarding mortality, it ranks 9th out of 25 human infectious and parasitic diseases in Africa (3) and is estimated to cause the loss of 1.5 million disability-adjusted life years per year (4). It is responsible for major setbacks in social, agricultural, and economic development in Africa (5) and constitutes a severe burden for poor rural populations to whom healthcare access is extremely difficult (6) [reviewed in Geiger et al. (7)]. The real number of infected people is most probably underestimated as it results from a mathematical extrapolation of data recorded from only partial epidemiological surveys (5, 8). In addition, wars, social conflicts and struggles, the presence of trypanosome-infected domestic animals, and climate change are recognized as factors favoring HAT development and spread (9–11). Thus, although the number estimated cases is fewer than 10,000, this disabling and fatal disease is classified among the group of poverty-promoting infectious diseases.

Two distinct forms of HAT exist which are (a) caused by two distinct trypanosome subspecies, (b) transmitted by two distinct tsetse fly vector species, and (c) widespread in two distinct geographic areas. The chronic form, caused by *T. brucei gambiense*, is transmitted by *Glossina palpalis* sp., and distributed in western and central Africa, while the acute form, caused by *T. brucei rhodesiense* is transmitted by *Glossina morsitans* sp., and restricted to East Africa. Despite these differences, the infection caused by either the chronic or the acute forms of the disease evolve similarly in two distinct clinical phases. During the first phase (stage 1 or hemato-lymphatic stage), the trypanosomes are present and multiply in the blood and in the lymph nodes; during this phase the patients exhibit intermittent fever, headache, and joint pain. Stage 2 (meningo-encephalitic stage) begins once trypanosomes have invaded the central nervous system (CNS); it is characterized by severe neurological disorders (12) [reviewed in Ref. (13)]. The two HAT forms differ in the rapidity of their respective transition from stage 1 to stage 2: several months or even several years for the chronic form, a few months or even a few weeks for the acute form. In addition, the severity of the latter is much higher than that of the former. The disease is generally fatal when not treated. Today, despite the emergence of some new drug candidates (14, 15) or drug combinations (16), the available chemotherapy remains limited and often generates severe side effects or even the development of resistant trypanosome strains (5, 17). Also, inefficient *T. b. gambiense* case detection, chronic infections that are never treated and a long stage 1 period are important contributors for stable human to human transmission in endemic areas. In contrast, for *T. b. rhodesiense* transmission, animals are the main reservoir population, greatly affecting therapeutic effectiveness and the impact of control measures (18).

Trypanosoma cruzi causes American trypanosomiasis, also called Chagas' disease. This parasite is transmitted to humans and other mammals by "kissing bugs," hematophagous insects belonging to the genus *Triatoma* [or *Rhodnius*, depending on the geographical area where the disease occurs (19)]. In addition, transfusion of infected blood, transplantation of contaminated organs, and congenital transmission are other important modes of *T. cruzi* transmission. Chagas' disease is widespread in all South American countries affecting about 7–12 million people, and putting at risk 60–80 million others (20, 21). Three hundred thousand new cases are reported to occur each year, and 21,000 patients die annually (22). Once a host has become infected, the parasite is internalized in the cells of the innate immune system, and the infection develops progressively. Similarly to HAT, two forms of the Chagas' disease can be distinguished. The acute form is marked by (a) the presence of *T. cruzi* trypomastigotes in the blood stream, (b) high fever, and (c) a severe hepatomegaly. By contrast, in the case of the chronic form of the disease, there are far fewer parasites present in the blood stream, and the other symptoms are also less severe. The chronic form can also be "silent" that is, in the absence of any symptom, the infection may remain undiagnosed. Nevertheless, 10–20 years later, 5–10% of these people will develop anatomical and functional abnormalities at their esophagus and their colon, while ~30% will develop myocarditis, leading to heart failure or sudden death (23).

Leishmaniasis is estimated to affect 12 million people in 98 countries, while ~350 million live in disease-risk areas (24), and presents an incidence of around 2 million cases per year. Despite more than 500,000 new VL cases per year causing the death of more than 50,000 patients (24, 25), this disease is classified among the neglected tropical diseases. In 2010, WHO estimated the disease to cause the loss of around 2.4 million disability-adjusted life years per year (24) [reviewed in Geiger et al. (7)].

Leishmania spp. are transmitted by sandflies belonging to the genera *Phlebotomus* and *Lutzomyia*. They induce several forms of disease in humans, ranging from localized cutaneous lesions to VL. VL, the most severe form of Leishmaniasis, is caused by parasites of the *Leishmania donovani* complex (*Leishmania donovani, Leishmania infantum*, and *Leishmania chagasi*) [see the review by Gupta et al. (26)]. Once the mammal host is infected, the parasite differentiates intracellularly inside MFs and disseminates from the skin to the spleen, liver, and bone marrow MFs (27). Most patients infected with *L. donovani* and *L. infantum* develop asymptomatic chronic latent infections. However, ~10% of infected people develop fever, severe hepatosplenomegaly, pancytopenia, cachexia, and a hyper gamma-globulinemia leading to the death if untreated (28, 29).

In this review, the authors aimed to summarize the mechanisms trypanosomatids use to escape their host deleterious immune responses. It will focus on the aspects of the parasite–host–vector life cycle; on the host innate immunity and the key steps allowing trypanosomatids to invade their hosts; on the deregulation of antigen-presenting cells (APCs); on the disruption of specific immunity, as well as on the use of immune responses to favor parasite proliferation (**Figure 1**).

LIFE CYCLES: PARASITES–HOSTS–VECTORS, COMMON AND DIVERGENT POINTS

The parasites' life cycle can be divided into two crucial phases allowing the survival inside the hosts (vertebrate and invertebrate). Immediately after their transmission by the insect vector (**Box 1**), parasites have to resist innate immunity and

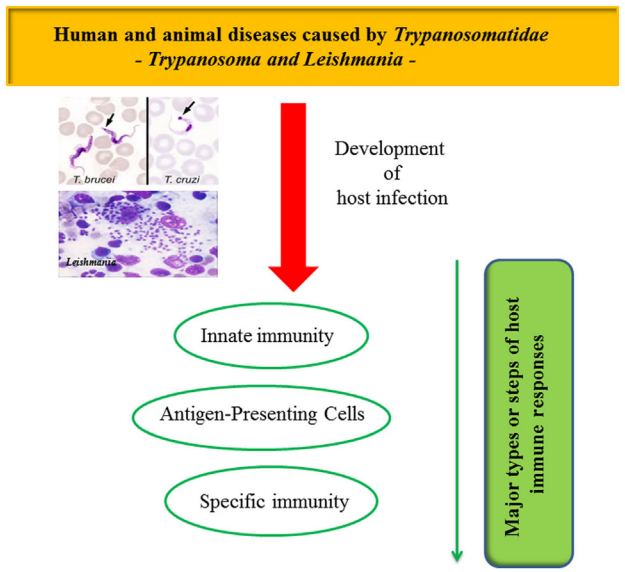

FIGURE 1 | Mechanisms used by trypanosomatids to escape their host deleterious immune response.

BOX 1 | Transmission of parasites belonging to the Trypanosomatidae family.

Sandfly: Sandflies belong to the insect order Diptera, suborder Nematocera. Within this suborder the family Psychodidae includes biting sandflies in diverse genera and non-biting owl-midges or moth flies (genus Psychoda). Among the existing phlebotomine genera, two have been proven to be vectors of one of the main zoonotic pathogens worldwide, the protozoan parasite *Leishmania*. These belong to the genera Phlebotomus in the Old World and Lutzomyia in the New World. Out of more than 800 recognized sandfly species, ~464 species are found in the New World and 375 in the Old. Among these species, only 34 are proven vectors and overall 74 species play a substantial role in *Leishmania* transmission.

Tsetse fly: Tsetse flies belong to the insect order Diptera, suborder Cyclorrhapha. They compose a family of their own, Glossinidae, which is placed within the Hippoboscoidea due to the morphological and reproductive similarities of tsetse flies to keds and other hippoboscid flies. Glossinidae includes the single genus Glossina with 23 species, 6 of which are further divided into 14 subspecies. Glossina species are arranged in three subgenera – Austenina, Nemorhina, and Glossina – which correspond roughly to groups of species found in different ecological settings.

Triatomine bugs: The members of the Triatominae belong to the insect order of the Hemiptera and the Reduvidae subfamily. Reduvidae are also known as kissing bugs, assassin bugs, or triatomines. Most of the 130 or more species of this subfamily are hematophagous and all triatomine species are potential vector of the Chagas disease parasite *Trypanosoma cruzi*. Nevertheless, only those that are well adapted to living with humans are considered important vectors (Triatoma infestans and Rhodnius prolixus).

develop either intracellularly (*Leishmania* and *T. cruzi*) where the parasites are no longer flagellated, or extracellularly in the blood flow (bloodstream forms of *T. brucei*). The diagnostic stage of the parasites relies on the presence of bloodstream forms of *T. brucei gambiense,* or amastigotes of *Leishmania* and *T. cruzi* in the vertebrate host (5, 30, 31). Parasite dissemination in their mammalian host occurs after lysis of the host cells (*Leishmania* and *T. cruzi*), then both intracellular amastigotes of *Leishmania* and bloodstream trypomastigotes of *T. cruzi* and *T. brucei* sp. are spread via blood circulation.

Transmission from the infected host to the arthropod vector occurs when sandflies, triatomine bugs, or tsetse flies take a new blood meal, ingesting either infected cells (*Leishmania*) or free-living parasites (bloodstream trypomastigotes of *T. cruzi* and *T. brucei gambiense*). After accomplishing their intravectorial differentiation, trypanosomatid parasites have the ability to colonize various parts of the arthropod vector's alimentary tract. Some of them are restricted to a single compartment of the alimentary tract, i.e., *Trypanosoma vivax* (**Figure 2**), while others have a more complex life cycle, such as *Leishmania*, which implies passage through different compartments of the alimentary tract. The transmission of Trypanosomatidae parasites by the blood feeding arthropod occurs in three ways: regurgitation (*Leishmania*/sandfly), defecation (*T. cruzi*/triatomine bugs), or injection (African Trypanosomes/Tsetse) (**Figure 2**). The first phase of arthropod colonization takes place in the probocis of the arthropod, or in the insect's midgut. Once inside the midgut of the arthropod vector, parasite movements are initially restricted by the peritrophic membrane that surrounds the bloodmeal during the digestive process.

Intracellular amastigotes of *Leishmania* are released from the host cell during the cell breakage process and then differentiate into procyclic promastigotes. Depending on the subgenus, once promastigotes have been released after the destruction of the peritrophic membrane, *Leishmania* attach to the intestinal epithelium and colonize the intestine after the pylorus or adhere to the region near the pylorus. Then, the parasites migrate forward to the stomodeal valve where they differentiate into metacyclic promastigotes ready to be transmitted during a new blood meal.

For *Trypanosoma* species, the intravectorial cycle is more complex. Trypomastigote forms of *T. cruzi* change into epimastigotes inside the triatomine vector. After this process, parasites inside the midgut of the arthropod begin to multiply concomitantly with the destruction of the peritrophic membrane. Then, a second colonization phase takes place: *T. cruzi* reaches the rectum and changes into infective metacyclic trypomastigotes that can be transmitted to a mammalian host by defecation.

Bloodstream trypomastigotes of *T. brucei* or *Trypanosoma congolense*, for example, change into procyclic trypomastigotes inside the tsetse fly. Briefly, trypanosomes of the *brucei* group (*T. b. brucei*, *T. b. rhodesiense*, or *T. b. gambiense*) are carried to the gut, later passing forward to the proboscis, from where they enter the hypopharynx and reach the salivary glands, where the infective form are produced. For the *T. vivax* type, trypanosomes migrate forward to the food canal of the proboscis where they multiply. Later, infective forms reach the hypopharynx; at this stage new hosts can be infected when tsetse flies feed.

During pathogen transmission by arthropods, immediately after vessel laceration, platelets form a plug locally and produce clotting and vasoconstrictory molecules. Because vertebrate homeostasis and inflammation is complex, the saliva of hematophagous insects adapted accordingly, containing dozens of active compounds (32, 33) [reviewed in Ribeiro et al. (34)]. The nature of arthropod feeding modes is thought to have evolved independently in several insect orders and families,

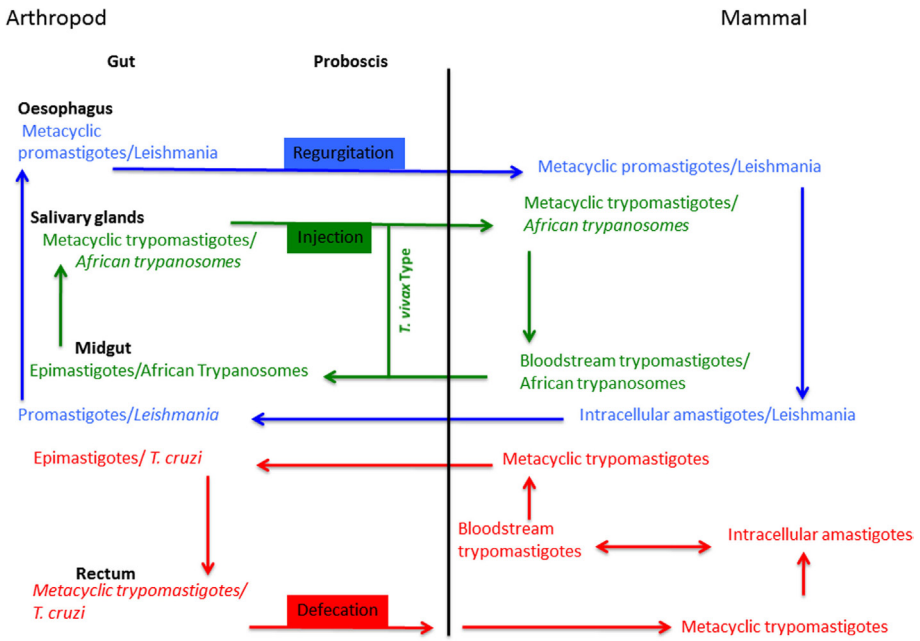

FIGURE 2 | Trypanosomatid parasites life cycles.

with the salivary composition among insects being typical of a convergent evolution scenario (35). During the transmission of Trypanosomatidae parasites by their arthropod vector, some protein and chemical components of arthropod origin are, therefore, co-transmitted to the mammalian host. They can then interfere and promote the colonization process of trypanosomatid parasites. The composition of the biological material that is co-injected (African trypanosomes and *Leishmania*) or deposited on the skin of the host (American trypanosomes) is different in its nature. If we consider the way in which *Leishmania* or African trypanosomes are transmitted, the injected cell-free biological material, along with infective parasites, contains a large amount of salivary gland proteins. In the case of *T. cruzi* transmission by triatomine, additionnal proteins, peptides, and chemicals in the feces of the bugs might also be present during the transmission of *T. cruzi*. In this particular case, the invasion of the host by infective parasites occurs later, through the bite wound or via mucosal membranes after the instinctive scratching behavior.

A brief overview of the protein salivary constitution found in the three arthropods is given in **Table 1**. Among the salivary components, only enzymes that belong to the Apyrase/5′Nucleotidase family, Protease family, various protease inhibitors, and the Antigen 5 family of proteins have been found to date to be present in the transcriptome and/or the proteome of all the arthropods involved in the transmission of trypanosomatids parasites. A second series of protein families has been commonly identified in sandflies and tsetse flies or sandflies and triatomine bugs; surprisingly none seems to be common to both tsetse flies and triatomine bugs. Lastly, many protein families are found specifically in the transcriptome and/or proteome of tsetse flies, sandflies, or triatomine bugs. For a vast majority, they play a role in vasodilatation, like the Maxalidan found in sandflies, Triafestin or Dipatelodipin found in triatomine bugs, or the PGE2 synthase found in the transcriptome of the tseste fly (see **Table 1**). In addition, proteins with anti-clotting activity are supported by different families of proteins in the three vectors of trypanosomatid parasites. Interestingly, the saliva of triatomine bugs contains a large number of proteins belonging to the lipocalin family (e.g., salivary lipocalin-5), which are described to be involved in interactions with the host's immune response (36). Interestingly, they are also found in the transcriptome of the digestive tract, more precisely in the rectum of triatomine bugs (*Rhodnius prolixus*) (37). In addition, the lipocalin signature is also found in the extracellular material of *T. cruzi* (Sereno and Mathieu-Daudet, unpublished results). Altogether, this suggests that redundant activity supported by the protein member of the lipocalin family is required to interfere with the complex immune response that is activated during *trypanosomatids* infection.

FACING INNATE IMMUNITY: KEY STEPS FOR TRYPANOSOMATID INVASION

Many protozoa cause chronic infections, most probably owing to the millenar coevolution between parasites and host immune system. The ability to escape and/or modulate both innate and adaptive immune responses is crucial for their survival (**Box 2**) [in Lopes et al. (49)]. Parasites have to manipulate host cells in order to avoid the production of antimicrobial molecules and to benefit from growth factor production. Protozoa have evolved specific mechanisms to evade these defenses.

TABLE 1 | Salivary proteins of arthropods.

Name	Function	SF	Ts	Tr	Reference
Apyrase/5′Nucleotidase	Hydrolyze ATP into ADP, which is an inducer of platellet agregation	X			(34)
			X		(38)
				X	(39)
Proteases	Hydrolysis of peptide bonds				(34)
– Metalloprotease		X	X		(39)
– Serine protease				X	(38)
Protease inhibitor domains	Interact with the proteolytic cascade of the host homeostatic and				(34)
– Serpstands for serine protease inhibitor	inflammatory processes	X			(38)
– Kazal domain				X	(40)
– Thrombinhibitor			X		(39)
– Kunitz domain			X		(39)
Antigen 5-like	Unknown	X			(41)
		X	X		(42)
		X		X	(38)
Endonuclease	Endonucleases are enzymes that cleave the phosphodiester bond with a polynucleotide chain	X	X		(34)
Hyaluronidase	Hyaluronidase hydrolyzes components of the skmatrix				(39)
Adenosine deaminase purine hydrolase	Hydrolization of adenosine into inosine and then hypoxanthine plus ribose. Adenosine	X		X	(34)
	and inosine induce mast cell degranulation and trigger itching reaction	X		X	(39)
Phospholipase	Hydrolysis the platelet agregation factor	X	X		(39)
33 kDa familly	FXa clotting inhibitor	X			(34)
Nitrophorin		X		X	
15–17 kDa familly	Unknown	X		X	(34)
					(38)
Pyrophosphatase/Phosphodiesterase	Hydrolyze dinucleotides that are important inflamatory mediators	X			(34)
Glycosydase	Carbohydrate catabolism	X			(34)
Antimicrobial peptides		X			(34)
Odorant binding protein/D7 superfamilly	Antagonize inflammation and hemostasis	X			(34)
Yellow phlebotominae family	Dopachrome convertase activity	X			(34)
41.9 kDa superfamilly	Unknown	X			(34)
Maxadilan	Vasodilatator	X			(34)
27–30 kDa	Unknown	X			(34)
Possibly multigenic Glossina-specific salivary secreted protein	Unknown		X		(39)
GE-rich salivary proteins	Unknown		X		(39)
Glycine-proline rich familly	Unknown		X		(39)
Fat body and salivary 20 kDa family	Unknown		X		(39)
3–6 kDa salivary peptide	Unknown		X		(39)
Ribonucleases	Catalyze the degradation of RNA		X		(39)
Exonucleases	Endonucleases are enzymes that cleave the phosphodiester bond at the 5′ or 3′ end of the chain		X		(39)
ProstaglandE2 Synthase	PGE2 synthesis which is a vasodilatator		X		(39)
Nitric oxyde synthase	Synthesis of nitric oxyde: vasodilatator		X		(39)
Thioester containing protein	Has a reactive cysteine that can form a thioester bond to other, Pathogen, molecules		X		(39)
Fibrinogen domacontaining/ficolproteins	Familly of proteins having the Fibrinogen C motif and Ficoli motif		X		(39)
Inositol phosphatase	Hydrolysis of inositol phosphate and phosphoinositidesubstrates involved cellular process			X	(43)
	related to signal transduction, secretion, and cytoskeletal structure			X	(38)
Peptidoglycan recognition protein	Pathogen recognition and initiation of innate defense mechanism			X	(39)
Salivary proteMYS2	Unknown			X	(38)
Lipocalin	The term lipocal means « cup of lipid »; they have the capacity to transport small hydrophobic molecules				
– RPAI	Inhibitor of platelet agregation			X	(43)
– Triplatin	Antiplatelet, vasodilatator			X	(35)
– Triafestin	Anti-clotting, antipain			X	(44)
– Pallidipin	Antiplatelet			X	(45)
– Triabin	Anti-clotting			X	(46)
– Procalin	Unknown			X	(47)
– Dipetalodipin	Antiplatelet, vasodilatator			X	(48)
– Nitrophorin	Antihistamine			X	(32)

BOX 2 | Innate immune responses.

Innate immunity is based on the recognition of pathogen-associated molecular pattern molecules (PAMPs), which are present in diverse organisms, but are absent in the host and function as an exogenous signal that alerts the host to the presence of pathogens. During infection, PAMPs are recognized by pattern-recognition receptors (PPRs) that initiate signalling cascades, which lead to the activation of transcription factors in innate immune cells and have an influence on T-lymphocyte differentiation and functions.

The survival and transmission of pathogenic protozoa depends on their ability to evade or subvert host's innate and adaptive immune responses. Evasion of innate immunity by parasitic protozoa is a critical step in their host interaction. Innate defenses include the epithelial barrier of the skin, the alternative complement cascade and other lytic serum components, lysosomal hydrolases, toxic oxygen and nitrogen metabolites of phagocytes, and immunoregulatory functions of dendritic cells (DCs). Trypanosomatids have evolved specific mechanisms to evade these defenses. The ability to avoid attack by soluble antibodies that neutralize the invasion and opsonize parasites for phagocytosis is of particular importance to extracellular parasites, such as African trypanosomes. The major strategy for evading antibody responses is the antigenic variation that protects African trypanosomes from immune recognition. The adoption of an intracellular life style, as is done by *Leishmania* and *Trypanosoma cruzi*, is the simplest way of evading humoral response. Intracellular protozoa have a remarkable adaptative capacity as they are able to resist killing by remodelling the phagosomal compartments where they reside and by interfering with the signalling pathway that leads to cellular activation. In addition, there is abundant evidence that these protozoan infections actively regulate adaptative T-cell responses, resulting in suppressed effector functions.

A great challenge to research in immunology and parasitology is the development of strategies that foster immunity against protozoan parasites and prevent their evasion, chronic or recurrent infections, and associated pathologies. A better understanding of the evasion mechanisms employed by the parasite is necessary. In the near future, a combination of strategies aimed at both early killing of parasites and neutralizing suppressive mechanisms could be necessary for effective therapies and vaccines.

Evasion of Innate Immunity

After entering a susceptible mammalian host, protozoan parasites are targeted by pre-existing soluble factors that can potentially recognize and destroy invading parasites or target them for killing by effector cells. Serum components, such as the complement system activation, provide the first line of defense. Alternative complement activation is stimulated by non-self surfaces, such as those of pathogens, wherein the activation of C3 molecules occurs through a proteolytic cleavage promoted by C3 convertases, producing C3b molecules that bind covalently to the activator surface. These molecules subsequently promote the assembly of the membrane attack complex (MAC), which is responsible for membrane lysis (50). *Leishmania* procyclic promastigotes or *T. cruzi* epimastigotes are highly susceptible to complement action, whereas the infective metacyclic and bloodstream stages are resistant (51, 52).

Leishmania can evade lysis by complement by targeting host cells through complement activation. Expression of a modified surface lipophosphoglycan (LPG) (53) was found to enhance the synthesis of surface proteinase gp63 (54) and PSA-2 (55) preventing insertion or deposition of the lytic C5b-C9 complex, thereby enhancing tolerance of complement-mediated lysis (CML). Some mutants of *Leishmania major* (null-mutants for the referred molecules) were shown to have less virulence in BALB/c mice and high susceptibility to complement lysis (56, 57).

Trypanosoma cruzi blood forms can also survive complement activation as they express glycoproteins such as gp160, gp58/68, and T-DAF. These proteins can bind to C3b and C4b, which allow evasion of complement (58–60).

In humans, only *T. brucei gambiense* and *T. b. rhodesiense* can develop infection, as other trypanosomes are susceptible to two serum complexes with a lytic activity against trypanosomes (TLF 1 and TLF 2) (61). Despite their differences, both complexes contain apolipoprotein L1 (APOL1) (62). APO L1 in TLF1 is taken up through endocytosis via the haptoglobin–hemoglobin parasite surface receptor. *T. b. gambiense* and *T. b. rhodesiense* escape APOL1 trypanolysis by expressing distinct resistance proteins (63). The *T. b. gambiense*-specific gene, *TgsGP,* is essential for human serum resistance as deletion of *TgsGP* in *T. b. gambiense* renders the parasites susceptible to human serum and recombinant APOL1. Reintroducing *TgsGP* into knockout parasite lines restores resistance (64). Protozoa must also evade other mediators of innate immunity besides to the complement.

Evasion of Cellular Innate Immunity

Remodeling Host Cell Compartments by Intracellular Parasites

Trypanosoma cruzi surface proteins, such as gp82 and gp35/50, first adhere to host cell surface receptors inducing calcium-mediated signaling (65, 66). Afterwards, *T. cruzi* trypomastigotes actively invade mammalian cells and their survival is dependent on their ability to subvert a calcium-regulated lysosomal exocytic pathway (67). They escape to the cytoplasm after a short period in the parasitophorous vacuole, which is necessary for the differentiation of trypomastigotes into amastigotes, triggered by the low vacuole pH (68). *T. cruzi* growth and development cannot be sustained within the parasitophorous vacuole. However, vacuole lysis and escape into the cytosol require exposure to this acidic environment, which is essential for the activity of Tc-TOX, a molecule secreted by the parasite. This molecule is active at acidic pH and forms a membrane pore, an activity which is facilitated by a trans-sialidase present on the trypomastigotes' surface (69, 70). Another lysosome-independent route of host cell invasion has been described using the PI3K-dependent pathway (66, 71).

The initial binding and internalization of *Leishmania* promastigotes by MFs (72) is associated with/implicates the receptor-mediated classical endocytic pathway. This pathway involves a wide diversity of receptors, opsonic or pattern-recognition, such as CR3, CR1, Fc receptors, or lectin receptors such as the mannose fucose receptor [mannan-binding protein (MBP)] and the integrin family (73, 74). LPG, the main promastigote glycoconjugate, plays an essential role in promastigote adhesion to MFs, rapidly fusing with lysosomes, transiently inhibiting phagosome maturation (75) and generating a parasitophorus vacuole that maintains an acidic pH and hydrolytic activity. This delay provides enough time for promastigotes to differentiate into more hydrolase-resistant amastigotes. The replicating amastigotes ultimately survive and reside within phagolysosomes by producing glycoconjugates that are secreted or linked to surface of cell, such as GIPLS and proteophosphoglycan (PPG). These proteins protect parasites from proteolytic damage (76).

A recent study shows that interaction between *Leishmania* and MFs depends on the polarization of the MF and on the CLR protein family (77).

African trypanosomes, by opposition to other protozoan parasites, never enter the cells of the host but live extracellularly in its fluids. These parasites are constantly exposed to the host's immune monitoring so they have developed the antigenic variation mechanism, wherein they change their surface proteins to prevent elimination (78). This surface coat is made of a densely packed array of GPI-anchored variable surface glycoproteins (VSG). GPI anchors are cleaved by parasite phospholipase C (PLC) (79), resulting in the release of surface VSGs and induction of a pro-inflammatory response in cells playing a major role in innate immunity (80, 81). During early infection, the shedding of soluble VSG glycoproteins by PLC induces a polarized Th1 cell response and IFN-γ production; however, in later stages of infection, the prolonged release of these proteins inhibits MF intracellular signaling and activation (82). Antigenic variation exhibited by African trypanosomes remains their central immune escape mechanism developed during infection (83).

Interfering with Macrophage Functions and Host Cell Signaling Pathways

Leishmania sp. and *T. cruzi* are able to resist the antimicrobial mechanisms induced in phagocytic and even in non-phagocytic host cells.

During the acute phase of infection, *T. cruzi* replicates extensively and releases immunomodulatory molecules (GPI-mucins, trans-sialidase, glycoinositolphospholipids GPILS, the cysteine proteinase cruzipain), which play a major role in subverting the host's innate immunity. GPI-mucins are responsible for parasite surface variability, leading to differential tissue adherence and evasion of host innate immune responses. Moreover, they render DCs dysfunctional for protective responses (84). *T. cruzi* uses several other mechanisms to escape immune responses from the host. In fact, the pathogens that are unable to synthesize sialic acids might adsorb these from the host as a way to engage the inhibitory siglecs, sialic acid-binding immunoglobulin-like lectins, surface proteins present in several immune cells that bind to sialic acid promoting adhesion and signaling (85). Such sialic acid–siglec association plays an important role to subvert host's immunity [review in Khatua et al. (86)]. To escape the immune responses of the host, *T. cruzi* manipulates the CD8+ T-cell sialylation (86). When sialic acids–siglec interact, activated CD8+ T cells remain unable to kill targets that bear *T. cruzi* epitopes (87). Interestingly, recent findings propose a siglec-mediated CD33 suppression pathway of cellular function in *Leishmania* infection also (86). When, sialic acids–siglec interact, activated CD8+ T cells remain unable to kill targets which bear *T. cruzi* epitopes (87). Interestingly, recent findings propose a siglec-mediated CD33 suppression pathway of cellular function in *Leishmania* infection also (86). The evasion mechanism involving *T. cruzi* GIPLs results in the suppression of CD4+ T-lymphocyte activation (88). The cysteine proteinase cruzipain produced by *T. cruzi* is able to induce both IL-10 and TGF-β secretion and arginase expression in MFs resulting in increased replication (89). These evasion mechanisms allow the parasite to delay specific responses mediated by effector T-cells. In chronic infection, the parasite hijacks the host's TGF-β pathway and maintains, consequently, the same rate of parasite death and replication (90). In fact, the vaccine efficacy against *T. cruzi* is called into question as this parasite is able to coexist with the immune response developed by CD8+ T cells.

Persistence of *Leishmania* and infection progression are caused by the inability of phagocytes to elicit both effective innate and adaptative responses (76). *Leishmania* alters some biological functions (disruption of cholesterol dynamics, alteration of the DNA methylation status of many host genes with antimicrobial functions, and retention of intracellular iron) to promote parasite growth (91). *Leishmania*-induced MF dysfunctions are related to the loss of microbicidal (NO, oxygen intermediates) and immunological activities (IL-1, IL-12, MHC, IRF7, and TLR2) (92, 93). These dysfunctions are correlated with the alteration of several phagocyte signaling events dependent on Ca2+, protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and Janus kinase 2 (JAK2) (94). JAK2 phosphotyrosine-based signaling cascades are particularly important since tyrosine phosphorylation has been shown to play a critical role in IFNγ-inducible MF function regulation, inhibited by *Leishmania* infection [e.g., nitric oxide (NO), major histocompatibility complex (MHC) II, Interleukin-IL-12] [review in Forget (95)]. Moreover, the role of tyrosine-specific phosphatase SHP-1 in *in vivo* and *in vitro* survival of the parasite and in MF inhibition (96) was shown by the use of tyrosine kinase inhibitors (such as PTP SHP-1) that inhibit the phosphorylation mediated by the enzyme (96). However, in infected MFs, the inhibition of transcription factor STAT1α is not due to SHP-1, but probably to specific proteasomal degradation of the protein (97, 98). Another important aspect in initial establishment of infection is the presence of dead parasites in the inoculum with exposed phosphatidylserine, which facilitates uptake by phagocytes and induces TGF-beta production and TNF-alpha downregulation (99).

Like other trypanosomatids, African trypanosomes divert the MF inducible metabolism of L-arginine (100). At the beginning of infection, trypanosomes induce the arginase polyamine synthesis pathway, which decreases the production of NO, and the production of trypanothione reductase, both of which needed for host colonization and parasite growth (101). *T. brucei* releases TbKHC1, a kinesin heavy chain isoform, to stimulate the activity of arginase-1 (an IL-4Rα-independent signaling enzyme but relying on SIGN-R1-dependent IL-10 secretion) for its own growth (102). Furthermore, L-arginine depletion decreases the expression of the T-cell antigen receptor ζ chain (CD3ζ), the principal signal transduction element in this receptor, impairing T-cell functions and proliferation (103). TNF-α release by MFs exerts a trypanocidal effect (104) and is limited by the activation of trypanosome adenylate cyclase. The induction of cyclic AMP release by trypanosomes into MF and the activation of protein kinase A lead to TNF-α synthesis inhibition (105).

Lectin Pathway

Trypanosomes use several mechanisms to escape from host immunity, such as the evasion of complement through the inhibition of the classical and lectin pathways, via binding to the C3

convertase that is essential for complement lysis in all pathways and host genetic deficiencies of the complement lectin pathway (CLP) (60). Specifically, in the CLP, the host factors mannose-binding lectin (MBL) and ficolins are able to recognize and bind to parasite surface carbohydrates that lead to activation of the complement cascade (106). *T. cruzi* is able to inactivate this lectin pathway by neutralizing the binding of MBL to carbohydrate (107). MBL induces the lysis of *T. cruzi*, and a deficiency in these host molecules only moderately compromises the defense of the host against *T. cruzi* (108). The receptor C5aR or Bradikinin B2R inhibits the translocation of calreticulin to the surface of *T. cruzi* from the endoplasmic reticulum, and inhibits activation of the host CI complement component C1, thereby promoting infection by *T. cruzi* (109, 110). In this way, calreticulin acts as a virulence factor (111).

Leishmania Lectin-Complement Pathway

Leishmania promastigotes in the bloodstream are known to activate the complement system, reported to effectively eliminate the parasite. A greater resistance to CML is observed for infective promastigotes (metacyclic) due to the production of a surface metalloprotease GP63 and several kinases (26). These parasites have evolved to take advantage of receptor-mediated phagocytosis as a way of entering target cells and, simultaneously, of manipulating MF activation (112). Upon inoculation of the vertebrate host, C3b protein binds to the parasite, who alters it to an inactive form, preventing elimination. At the same time, the inactive C3b molecule at the surface now functions as an opsonin ensuring phagocytosis through complement receptor 3 (CR3), which will in turn inhibit IL-12 production, favoring parasite growth (26). This mechanism is independent of NFκB, MAPK, IRF, and ETS (113). Several other receptors have been described to be important for cell invasion, such as the MBP, that plays a role in *Leishmania* opsonization by triggering a antibody-independent complement activation mechanism on the MF surface (114). Complement activation by *Leishmania brazilensis* on the surface of MFs allows attachment to/invasion of the host cell (MF) by the complement receptor link between the MBL and a surface LPGs of *Leishmania* (115). In *Leishmania donovani*, the mannose-fucose receptor (MFR) and the CR3 MF receptor were shown to act independently in the attachment of parasites by human MFs (116, 117). In addition, macrophagic CR3 receptors differently inhibit *Leishmania* promastigote binding during their growth phase. Lastly, other receptors may be involved in MF infection by *Leishmania*, including MR, TLRs, and FcgRS. The infection of DCs by *Leishmania* involves several receptor/ligand interactions on the cell surface, such as antibodies FcR, a component from complement/CR and proteoglycans/heparin-binding proteins (118).

The role of polymorphonuclear neutrophil granulocytes (PMN) in *Leishmania major* survival in the host cells is not fully understood (see section Control of immune cell population life and death). These cells are able to eliminate intracellular parasite quickly, except for *Leishmania major* promastigotes, which can survive inside PMN for a few days (119). *Leishmania mexicana* amastigotes residing in the phagolysosome MF produce a large quantity of PPG, which can be secreted into the tissue after the rupture of infected cells. This PPG interacts with the complement system resulting in a decrease in the hemolytic activity of serum. It may also prevent the opsonization of *Leishmania* amastigote. PPG stimulates the C cascade by the MBP pathway. Consequently, PPG induces complement activation and, thus, contributes to the pathology of *Leishmaniasis* (120).

DEREGULATION OF ANTIGEN-PRESENTING CELLS: PREVENTING ADAPTATIVE IMMUNITY

The main host cells targeted by all three Trypanosomatidae are MFs and DCs, both of which play a capital role in the response of the immunitary system as they are specialized APC (**Table 2**). In addition, the normal functions of T cells, B cells, and T-helper cells (Th1 and Th2) involved in host immune responses, may also be modulated, more or less specifically, by the parasites.

Trypanosoma brucei sp./*T. congolense,* and the Human/Animal African Trypanosomiasis

These *Trypanosoma* species, causing either human or animal African Trypanosomiases, induce a global MF and T-cell-mediated immunosuppression, as well as the development of suppressive cell phenotypes, in infected cattle or mice (125–128). In the case of MFs, both classically and alternatively activated cells may develop such suppressive phenotypes (129). They are antagonistically regulated and their development is modulated by the cytokine environment. So, while classically activated MF are induced by type I cytokines (TNF-α, IL-12, IFN-γ) and inhibited by type II cytokines (IL-4, IL-10, IL-13, TGF-β), the reverse is true for alternatively activated MF (130).

The MF and DC immune response function includes: (a) processing of parasite antigens in the endocytic pathway and (b) co-stimulation and presentation to T-helper cells (Th cells) of trypanosome immunogenic peptides (121). In early infection by *T. b. rhodesiense* (clone LouTat 1), naive VSG-specific Th cells were more activated by DCs than MF, which resulted in Th1-mediated protective responses (121). Then, the specific Th cells secrete molecules that activate both immune systems, innate and adaptive, with the aim to destroy the infecting trypanosomes. Thus, MF and DCs modulate the adaptive anti-trypanosomal immunity by controlling antigen presentation. According to Dagenais et al. (121), trypanosomes may have evolved so as to alter antigen presentation for their own survival as a consequence of the pressure exerted by the immune system. Depletion of DCs and splenic MFs co-stimulatory molecules downregulation by *T. b. rhodesiense* contributed to inability of both DCs and MFs to generate an efficient T-cell response specific to VSG. The ability to modulate MF and DC antigen presentation functions allows the parasite to escape killing by immune cells and may contribute to the overall immunosuppression occurring during trypanosomiasis (131).

Murine *T. brucei* infection was reported to affect the co-expression of processed antigens and MHC class II molecules on the plasma membranes of MFs with the consequence of a reduced ability of these cells to present antigen in *T. brucei*-infected

TABLE 2 | Effects of either *Trypanosoma brucei sp.*, *Trypanosoma cruzi* or *Leishmania* sp on host immune responsive cells [for *T. brucei*: Dagenais et al. (121); Vincendeau and Bouteille (122); for *T. cruzi*: Chaussabel et al. (84); Flávia Nardy et al. (123); for *Leishmania*: Bogdan et al. (124)].

	T. brucei	*T. cruzi*	*Leishmania*
Macrophages			
– Faulty Ag processing and inability to present Ag to T cells	+		+
– Faulty epitope association with MHC-II	+		+
– Decrease in T-cell responses	+		+
– Th2 response → NOS inhibition and activation of arginase production	+		+
– Production of NO, PG, IFN-γ, and TGF-β	+		+
TGF-β inhibits IL-4, IL-5, and IL-6 → inhibition of B cells differentiation and proliferation			
– Inhibition of caspase-3 production by DC → inhibition of DC apoptosis			+
Dendritic cells			
– Inhibition of MHC-II, CD40, CD80, CD86 expression and inhibition of TFN-α, IL-6, IL-10 production:	+	+	+
Inhibition of DC maturation			
No differentiation of naive T-CD4+ lymphocytes into			
Th1 (producing: IFN-γ, IL-2, TFN-α)			
Th2 (producing: IL-4, IL-5, IL-10)			
– DCs depletion	+		+
– Inhibition of caspase-3 production → inhibition of DC apoptosis			+
– Production of IL-4 → activation of Th2 response (Th2 secretes IL-4, IL-13)	+		+
→ activation of alternative pathway of macrophage → *Leishmania* survival			
TH1/TH2			
– Th2 cellular response activation; production of IL-4, IL-10, IL-13, and TGF-β	+	+	+
→ inhibition of Th1 responses			
– Th2 responses	+		+
Inhibition of macrophages NOS production			
Activation of arginase (L-arginine → L-ornithine biosynthesis)			
Biosynthesis of polyamines and trypanothione			
Favors parasite development, macrophage infection, and parasite survival			

animals (132). The permanent contact of the trypanosomes with the host immune system may have induced in some *T. brucei* strains the ability to modulate MF antigen presentation process. This process involves peptide loading onto MHC class II molecules and/or [MHC class II-peptide (pMHC)] complex translocation to the cell surface for presentation to antigen-specific Th cells (132). It was also found that suppressive MFs inhibit the proliferation of lymphocytes responding to mitogens and antigens and, thus, reduces the proliferative cytokine IL-2 secretion by T-cells (133). Moreover, the levels of IL-2 receptors on the surface of these T cells were lowered (134). However, it was also reported similar levels of immunosuppression in infected animals that are both susceptible and resistant; thus, the real efficiency of this mechanism to ensure parasite survival in its host remains questionable (135, 136).

The secretome (total excreted-secreted proteins) of *T. b. gambiense* was shown to impair the lipopolysaccharide (LPS)-induced maturation of murine DCs (131). When DCs are stimulated by LPS, MHC class II, CD40, CD80, and CD86 molecules are upregulated, and cytokines, such as tumor necrosis factor alpha, interleukin-10 (IL-10), and IL-6 are released at high levels. In *T. b. gambiense* secretome-stimulated DCs, upregulation and secretion of the previous molecules is significantly reduced. Moreover, the inhibition of DC maturation resulted in the loss of their allostimulatory capacity, leading to a dramatic decrease in Th1/Th2 cytokine production by co-cultured lymphocytes. These results provide new insights into a novel efficient immunosuppressive mechanism directly involving the alteration of DC function, which might be used by *T. b. gambiense* to interfere with the host immune responses in HAT and promote the infection process [review in Garzón et al. (131)].

Trypanosoma cruzi and Chagas' Disease

A number of reports support the idea that, during the infection process, *Trypanosoma cruzi* is able to elicit severe autoimmune responses in the host, which contributes significantly to the development of the pathogenic process of the Chagas' disease. *T. cruzi* amastigotes escape host humoral immune responses by developing inside host cells. The parasite surface is covered with mucin glycoproteins that prevent *T. cruzi* from being recognized by the host immune system, thus favoring its establishment in its host and disease onset. These mucins are receptors of residues of sialic acid, which are detached by trans-sialidase from glycoconjugates of the host; this chemical modification is of interest as the modified mucins are even more efficient than the non-sialised glycoproteins were.

In addition, *T. cruzi* produces several complement regulatory molecules, which allow the parasite to prevent complement activation, thus allowing it to evade CML (58, 66, 137).

Moreover, *T. cruzi* induces the production of both Th1 and Th2 cytokines in infected individuals, and high expression levels have been reported for Th1 cytokines IFN-γ and IL-2, as well as for Th2 cytokines IL-4 and lL-10 (138). In *T. cruzi*-infected individuals, IL-10 gene expression is actively upregulated as indicated by the presence of significantly increased levels of the corresponding mRNA. This observation suggests that high levels of IL-10 may contribute to parasite persistence, as IL-10 is known to inhibit host protective Th1 immune responses (139). Thus, the induction

of IL-10 biosynthesis may be crucial for the parasite's survival in its host. By contrast, elimination of the parasite is largely under the control of Th1-specific cytokine production (IL-12, IFN-γ, TNF-α) (140). IFN-γ produced by NK cells during *T. cruzi* infection will activate phagocytic cells, which in turn will produce toxic reactive nitrogen intermediates that, ultimately, will kill internalized parasites (141). Despite the fact that the induction of cytokines leads to both cell-mediated and humoral response and, thus, suggested to be important for the development of effective immune responses, the susceptibility or resistance of mice to *T. cruzi* infections seems not to be related to a given cytokine response (142). Lastly, the ability of *T. cruzi* to infect a host, to survive and develop, and to cause Chagas' disease depends on a complex balance between Th1 and Th2 cytokine production, as they display antagonistic effects, the former being protective for the host, the latter for the parasite.

Leishmania sp and Leishmaniasis

Basically, the evasion strategies of *Leishmania* involve diverse mechanisms, including the capacity to survive within MFs, especially by inhibiting the oxidative burst occurring in activated MFs, and to modulate the T-cell immune response. Fixation of the complement C3 protein and the subsequent binding to CR1/CR3 is essential for the initial intracellular survival of infective-stage promastigotes (143). Once *Leishmania* are located intracellularly, after receptor-mediated endocytosis, they downregulate the active oxygen-dependent killing mechanisms of activated MFs (144) [review in Bogdan et al. (145)]. The total cellular and membrane acid phosphatase activity correlates with parasite virulence (146). LPG is another potent inhibitor of oxidative burst. It works by inhibiting PKC (147), which is the enzyme involved in the production of oxidative metabolites. *Leishmania* produces substances with the ability to scavenge the effect of oxidative metabolites. Furthermore, *Leishmania* amastigotes display high activity for enzymes that are known to degrade these toxic MF products (148). In addition, antiparasitic processes dependent on oxygen, phago-lysosomal processes, physical (low pH, osmotic stress), or biological factors (lytic enzymes) contribute to the MF antiparasitic activity. Hence, the importance of LPG, which is directed against the antiparasite effectors produced by the host MF (26, 99, 149, 150).

The activation of type 1 T-helper lymphocytes (Thl) by APCs requires surface expression of MHC class II, interaction with costimulatory receptor–ligand pairs (B7/CD28, CD40/CD40L, MHC class II/CD4), and peptide presentation by MHC class II [reviewed in Kaye (151)]. *In vitro* studies demonstrated the implementation of various mechanisms *Leishmania* uses to impede T-helper cell responses. It was first demonstrated that *L. donovani* amastigotes interfere, at the transcriptome level, with the MHC class II upregulation by IFN-γ [reviewed in Reiner (152)]. In addition, *Leishmania* was shown to be able to downregulate MHC class II expression (153). In contrast to other intracellular microorganisms (such as *Listeria monocytogenes*), *L. donovani* does not upregulate the production of B7-1 costimulatory molecules and the heat-stable antigen (HSA) in MFs. The MFs were not susceptible to stimuli, that normally upregulate B7-1 or the HSA, as does *Listeria* infection or the administration of LPS, of IFN-γ or of a crude mixture of mitogen-activated T-cells cytokines (154). Another critical aspect for Th-cell activation, in addition to the presence of MHC class II and costimulatory molecules, is the availability of parasite-derived peptides for loading onto the MHC molecules (155) [review in Bogdan et al. (145)].

Although *Leishmania* parasites interact with multiple cell types, MFs and DCs are clearly the most important cells influencing the infection progression and outcome. Interleukin 12 (IL-12) is a critical cytokine necessary for CD4+ Th1 development and IFN-γ production (156) [review in Dong and Uzonna (157)]. Although MFs are able to phagocytize *Leishmania* efficiently, their ability to produce IL-12 is selectively impaired by the parasites (158). In addition, infection of MFs by *Leishmania* also leads to the production of immunoregulatory cytokines, such as IL-10 and TGF-β, which are known for their ability to inhibit or deactivate MF functions (see Evasion of Innate Immunity) (159).

Several reports show that DCs, highly efficient APCs, play a central role in orchestrating immune responses in leishmanial disease (160). Although MFs are also specialized APCs, the main host cell for *Leishmania*, as well as the most efficient parasite-killing effector, infected MFs do not secrete IL-12 (161); hence, they are unable to stimulate an antigen-specific CD4+ Th1 cell response (162) [review in Dong and Uzonna (157)]. In *L. major* infection, Ritter et al. (163) demonstrated that CD8α and langerin-negative DCs are the principal APCs. They express dermal markers of DC (MHC II^{high}, $CD11c^{+}$, $CD11b^{+}$, $CD8\alpha^{-}$, and $CD205^{low}$) (163) and overcome the induction of CD4+ T-cell response. In addition, Kautz-Neu et al. (164) reported that, in the case of low dose infection, DCs may play a role in the cutaneous leishmaniasis/*L. major* pathogenesis via the induction and expansion of regulatory T cells (164–166). In this context, the production of IL-12 by APCs is of critical importance as it is able to polarize naive T cells into Th1 subset, and, subsequently, to induce IFN-γ production (161).

Lastly, intracellular signaling activation cascades that lead to the production of effector molecules are important for an effective control of pathogens in infected host cells. A number of pathogens are able to modulate signal transduction pathways to favor their survival (167). Since *Leishmania* are obligate intracellular parasites, their survival inside mammalian host cells is critically dependent on their ability to successfully disrupt host cell signaling events [review in Dong and Uzonna (157)], which would otherwise lead to the generation of killing effector molecules. To avoid killing, the parasites must be actively involved in almost every aspect/or all aspects of host cell signaling, manipulating/inhibiting from the production of microbicide molecules to the elicitation of protective cytokines.

DISRUPTION OF EFFICIENT SPECIFIC IMMUNITY

Although mammalian hosts have developed several immunological mechanisms to eliminate both intracellular and

extracellular parasites, trypanosomatids parasites have in turn developed strategies to escape the host immune system, which enable their survival and replication. The most striking observation during infections by trypanosomatids is that specific immune responses do exist but they are completely inappropriate and ineffective, or are even responsible for immunopathological processes (168). Trypanosomatids exhibit different cellular differentiation stages and different strategies to interact with their host, but in addition to the specificity associated within the genera *Leishmania* or *Trypanosoma*, these parasites have developed common features in order to subvert their hosts' immune system and ensure successful transmission (99, 123, 169) (**Figure 3**).

Control of Immune Cell Population Life and Death

Apoptosis, a major representation of programed cell death, is an essential physiological process in maintaining cellular homeostasis, particularly in the immune system, where it participates in both eliminating autoreactive or failed cells and controlling a proliferative response (170). Trypanosomatids have developed refined mechanisms for inducing or preventing the apoptotic cell death of their hosts' immune cells. For instance, *Leishmania* and *T. cruzi*, additionally to their use of apoptosis mimicry to invade MFs (171, 172), are able to regulate apoptosis of target cells positively or negatively. This is well illustrated for *Leishmania* with neutrophils, whose lifespan can be either extended by the parasite to benefit potentially from the protection of a safe intracellular niche (173) or reduced after parasite intake, both to limit antiparasite response (174) and fuel parasite growth (175). Neutrophils are the first phagocytes recruited to the inoculation site (176) and take part in the "Trojan horse" MF invasion, where infected apoptotic neutrophils shuttle parasites silently (i.e., without inflammatory signal) via phagocytosis to their primary host cell; *Leishmania* is able to delay neutrophil apoptosis, allowing the release of chemotactic molecules and consequent recruitment of DCs and MFs, as well as the production of TGF-beta that will contribute for the silent entry into MFs (99, 177, 178). The opposite neutrophil modulations are linked to different parameters, such as the genetic background of the animal model used or the parasite inoculation route (179), but also the molecular pathways modified, such as inhibition of procaspase-3 processing (173), or activation of the survival pathway involving extracellular kinases (ERK1/2) (180).

Although they all subvert MF activity during infection, targeting the core cell type of the mammalian host immune system, trypanosomatids behave differently in managing MF cell death depending on the genus considered. *Leishmania* parasites clearly prevent MF apoptosis either when directly housed by this cell type (181), or when using the above-mentioned "Trojan horse" strategy (177, 178). *T. cruzi* also uses phagocytosis of apoptotic cells by infected MFs to promote parasite growth, upon synthesis of transforming growth factor β (TGF-β), prostaglandins, and polyamines similarly to *Leishmania* (87). As they are exclusively extracellular, African trypanosomes are constantly exposed to the hostile host environment and have, in particular, developed mechanisms to cope with the microbicidal action of MFs, without specially inducing or repressing apoptosis, but through fast induction of the alternative arginine pathway leading to polyamines production (80, 182).

One very common feature developed by trypanosomatids to ensure immune evasion consists in destroying the lymphoid lineage by parasite-driven cell death. Apoptosis of T lymphocytes during the contraction phase of an immune response occurs

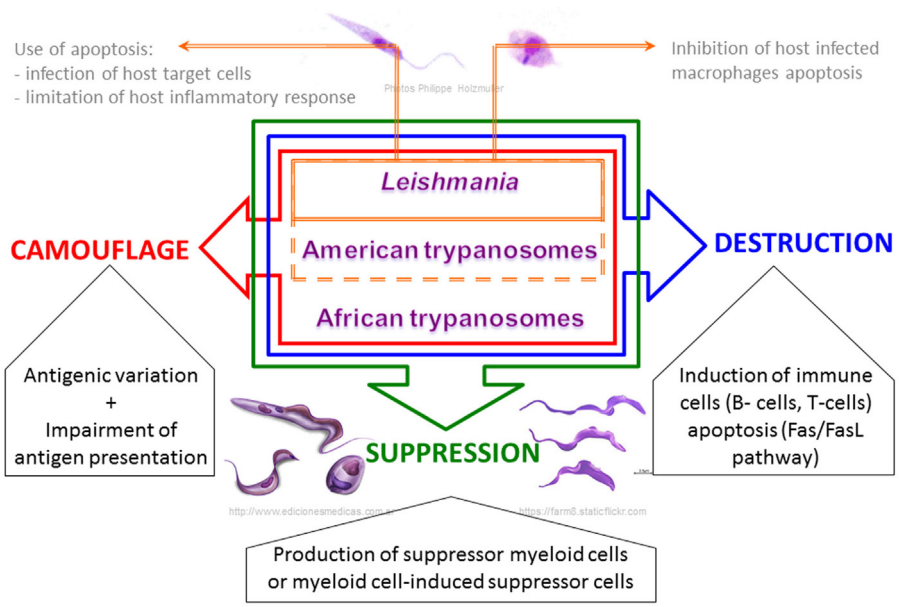

FIGURE 3 | Schematic view of the common strategic arsenal developed by trypanosomatids to interrupt effective host immunity, from immune evasion to immunosuppression.

through re-stimulation of activated T cells in a process termed activation-induced cell death (AICD), or results from the lack of survival factors, commonly referred to as activated T-cell autonomous death (ACAD) or death by neglect (183). Using the murine infection model for *T. cruzi*, splenic CD4+ and CD8+ T-cells were shown to express CD95 (Fas/Fas ligand apoptotic pathway) 2–3 weeks post infection. This observation is in accordance with their death by AICD (184). *Leishmania* parasites use the same strategy to eliminate both CD4+ and CD8+ T-cells, as observed in active human cutaneous Leishmaniasis (185), with more apoptotic spleen and peripheral blood T-lymphocytes in infected dogs compared to control animals (186). However, the molecular mechanisms are not as well defined: expression of Fas and FasL is increased in splenic human lymphocytes in acute disease (187), and Bim, a member of the Bcl-2 family, could also be involved in the apoptosis of T-cell in mouse models infected with different *Leishmania* species (188), which can otherwise be related to downregulation of kinase activities by Ser/Thr phosphatase (189). Extracellular African trypanosomes have also developed paracrine mediators able to induce not only CD45-dependent T-cell death (190) but also memory B-cells apoptosis (191, 192), which dramatically impairs the ability of infected hosts to develop adaptative immunity. In the same way, *T. cruzi* modulates the death of both IgG(+) B cells reactive to the parasite through B-cell–B-cell killing (193) and also induces a marked loss of immature B cells in the bone marrow through myeloid cells secreting a cyclooxygenase pathway product (194), thus limiting host defense and disabling B-cell generation to favor its chronic establishment.

The above paragraphs show one of the keypoints/key aspects/key mechanisms, leading to a successful infection by trypanosomatids: the ability to subtly modulate the life and death of immune cells when interacting with the host immune system.

Abolition of Efficient Specific Immunity

Molecular Camouflage and Altruistic Behavior

The most well-known system for evading the host's specific immune response is probably the antigenic variation developed by African trypanosomes. They have a "repertoire" of variable antigenic types (VATs), trypanosome variants in a given population, and they can change this surface coating by controlling variant-specific surface glycoproteins (VSGs) gene expression. When infecting the hosts, the immune system targets the major VATs; thus, the parasites with non-targeted or new VATs evade destruction. This antigenic variation developed by the trypanosomes (several species, subspecies, types, and strains) explains why they can escape from an effective immune response developed by livestock and human populations in different geographical areas (195). Indeed, 10 million identical VSGs cover the surface of the trypanosome at any given time. On the one hand, they are highly antigenic to focus the host humoral immune response; on the other hand, they make it possible to circumvent the immune destruction of the parasite by sacrificing the majority population while maintaining an untargeted population (78). Actually, specific immunity against the trypanosome's VSGs is effective, but delayed in time, which unfortunately allows the parasite to produce its other immunomodulatory effect on the host response. Although antigenic variation is a hallmark in African trypanosomes, other Trypanosomatidae also use this molecular mechanism to evade the host response. In *Leishmania*, for instance, the central repetitive domains of the hydrophilic acylated surface proteins (HASPs) are highly variant in their amino acid sequences, both within and between species, and clearly play a role in immune recognition in the host, albeit not yet fully resolved (196). In the same way, antigenic variation in *T. cruzi* led to the question as to whether *Trypanosoma cruzi* should be called the "cruzi" complex, as the parasite's diversity is substantial not only among strains but also because the interaction of the different infecting clones in the host will determine the severity of the infection (197).

Disability of Antigen Presentation (See Also Section III APCs)

Trypanosomatids, through their complex life cycles and different parasitic stages, have also developed sophisticated strategies for interfere with antigen presentation, by decreasing the expression of MHC molecules, by inhibiting the costimulatory molecules CD80 or CD86, or the synthesis of IL-1. Accordingly, specific T-cells are less stimulated and become anergic, leading to a non-efficient or an inadequate immune response. During progressive illness caused by *Leishmania*, two concomitant phenomena have been observed: an inability of APCs to process antigens properly, and the generation of a non-functional T-cell response to the processed antigens, despite functional signaling of human leukocyte antigen HLA/MHC class II molecules to T-cell receptors (TCR) (154, 198, 199). Moreover, the inability of APCs to process antigens properly has been linked to the inhibition phagolysosome biogenesis after *Leishmania* phagocytosis. In fact, the *Leishmania* surface metalloprotease GP63 cleaves a subset of soluble receptors, *N*-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs), consequently inhibiting the MHC class I presentation of *Leishmania* exogenous antigens, resulting in reduced T-cell activation (92). The strategy developed by *T. cruzi* in restricting antigen presentation is a little different, with hyperpolarization of the presented antigen repertoire (immunodominance), avoiding complete pathogen elimination by host effector cells, and thus favoring host parasitism (200). A hallmark of African trypanosome infection is that APCs functions are substantially altered, but the weight of antigen presentation in the balance between immunosusceptibility and immunotolerance appears to be more complex than for other trypanosomatids (121). Early studies in mouse models supported reduced presentation of non-parasitic exogenous antigens to T cells, presumably due to the altered display of antigenic peptide–MHC class II complexes (127, 132, 201). However, it remains unclear how the ability to present antigen is modulated among the APCs subsets and to what extent it could affect the infection outcome. This was illustrated in resistant mice infected with *T. congolense*, which were able to control infection in an MHC class II-restricted immune response manner, but only when the IL-10 function was not impaired (202), suggesting

precarious effectiveness of antigen presentation in response to African trypanosomes.

Trypanosomatid-Induced Imbalance of T-Cell Populations

In addition to the apoptotic cell death occurring in immune cell populations, the loss in number and functionality of T- and B-lymphocytes during trypanosomatid-induced diseases is a paradigm referred to as "exhaustion" (203, 204). In *T. cruzi* infections, the repertoire of CD8 (+) T-cells is dramatically restricted, which is a particular phenomenon known as immunodominance. The latter, despite targeting a different lymphocyte population, can be related to the response to a VAT-specific VSG during African trypanosome infections (78). Interestingly, mice that developed immune responses against subdominant/cryptic CD8 T-cell epitopes corresponding to the immunodominant antigen were significantly protected against *T. cruzi* infection (205). In the same way, exhaustion of cross-reactive responses to subdominant invariant epitopes by antigenic variation of the dominant antigens from African trypanosomes could explain the inefficiency of the selected lymphocyte populations, but at the same time question on the possibility of restoring protective cross-reactive immunity (206). In fact, in terms of lymphocyte populations, an increase in the CD4+:CD8+ T-cell ratio and IgG1 could be associated with self-cure in African trypanosome-infected natural host, whereas a decrease in the CD4+:CD8+ T-cell ratio and IgM could lead to disease development (207). Regarding *Leishmania* infections, susceptibility or resistance were associated long ago with a dichotomy in the development of immune response dominated by T-helper 2 (Th2) versus Th1, respectively. This was based on experimental data from mouse models infected with *L. major*, but it does not seem to be generalized to all *Leishmania* species, as complex early events shape the immune response (208), and especially as polarization is not observable in the natural host (human) where Th cells and CD4+:CD8+ ratio are either associated with the healing process or the development of the different clinical forms (209). Additionally, using mutant mouse models, it has been possible to explain more clearly the non-cure arising in resistant mice, which was due to a Treg cell imbalance (T-regulator cells), whose primary function is to suppress ongoing Th1 responses as to control tissue damage, and that functions as a suppressive pathway contributing to parasite persistence (210).

USE OF IMMUNE RESPONSES FOR PARASITE PROLIFERATION

To escape from host immunity, trypanosomatids interfere with the physiological function of various molecules of their host, such as arginine and calcium.

Arginine and Trypanosomatidae

To survive and multiply in their host, Trypanosomatidae have the possibility of exploiting the host metabolic machinery (**Figure 4**). The mechanisms used are diversified. Several strategies have been developed by Trypanosomatidae to escape host immunity. They have the potential to act upon the defense mechanism of the host, either to create a bypass of the host's defense mechanism, such as the arginine and lectin pathway, which is a mechanism of complement evasion, or to scavenge elements produced by the host, such as calcium.

Arginine is an amino acid with a basic fundamental role in the animal kingdom as a precursor of protein synthesis. It is also the substrate for enzymes leading to the production of NO or of polyamines (211). Polyamines are known growth factor promoters, and NO is a highly potent antimicrobial molecule. Physiologically, arginine is metabolized via two pathways,

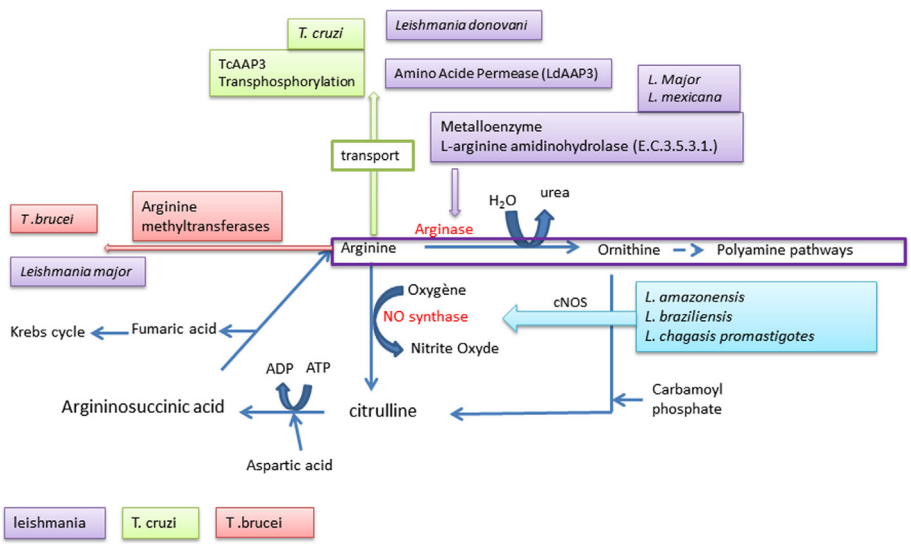

FIGURE 4 | Arginine used by Trypanosomatidae.

including a catabolic pathway involving its degradation by arginase to generate urea and ornithine, a precursor of the polyamine pathway. These products of metabolic degradation are known to promote *Trypanosomatidae* growth; on the other hand, NO exerts a broad spectrum of antimicrobial activity and it is highly toxic for *Trypanosomatidae*. The parasites *Trypanosoma brucei* (212) or *Leishmania major* (213) interfere with these processes at various steps.

They are able to promote the arginine methylation via methyltransferases. In *Trypanosoma cruzi* (214), a transphosphorylase (TcAAP3) acts on the phosphorylation level of the host arginine, while *L. donovani* scavenges the arginine synthesized by the host through an amino acid permease (LdAAP3) (215). In addition to these processes another system is exploited by Trypanosomatidae, which consists in using the host arginine for itself. *Leishmania* can hydrolyze the L-arginine of the host by an L-arginine aminohydrolase (E.C.3.5.3.1.) allowing the parasite to escape the production of host microbicidal NO (216). A few species, such as *Leishmania amazonensis*, *Leishmania braziliensis*, and *Leishmania chagasis* promastigotes, have a significant effect on this NO production using an irreversible inhibitor of nitric oxide synthase (iNOS) (217).

Free Calcium in Trypanosoma

The free calcium ion is important for trypanosomatid survival and multiplication (**Figure 5**). These pathogens are able to scavenge the ion either from the host cell intracellular stock or from the host extracellular stock. In *Trypanosoma*, intracellular calcium is stocked in a peculiar cell structure called the acidocalcisome (218). Cleavage activation of the *Trypanosoma cruzi* trypomastigote factor (PGFT) activates the PLC pathway that induces the release of intracellular free calcium via inositol, 1,4,5-triphosphate (IP3) sensitive intracellular channels. Calcium release induced the reorganization of host cell microfilaments, which play a crucial role in mammalian host cell invasion by *Trypanosoma cruzi* (219). Calcium is taken up from the surrounding environment of the parasite through the activity of a Ca^{2+}-ATPase (220). In *Trypanosoma*, the intracellular stock of free calcium, pyrophosphates, and polyphosphates is stored in specialized organites called the acidocalsisome (221). Ca^{2+} entry is regulated by PLA2 and activated by arachidonic acid and Ca^{2+} itself (222). Arachidonic acid appears to play a major role in calcium release from the cellular acidocalcisome (223). Arachidonic acid and the melittin peptide, of amphiphilic nature, induce an increase in intracellular calcium concentration in procyclic *Trypanosoma brucei*, *Leishmania donovani* promastigotes, and *Trypanosoma cruzi* amastigotes. In *Trypanosoma cruzi*, calcium plays a role in flagella motility via the flagellar calcium-binding protein (FCaBP), in all stages of development. This calcium-binding protein is localized in the flagellar membrane and acts in a calcium dose-dependent manner for its activity. In *Trypanosoma brucei* and *Trypanosoma cruzi*, calcium is carried by vacuolar transporter chaperone 4 (224). In *Trypanosoma brucei*, calcium release from intracellular storage acts through the activation of phosphoinositide phospholipase C (PI-PLC), which hydrolyzes phosphatidylinositol (PI) and PI 4,5-biphosphate (PIP2), involving the inositol 1,4,5-triphosphate (IP3)/diacylglycerol (DAG) pathway for this activation (225).

Calcium Ion in *Leishmania*

In *Leishmania*, calcium is stored in two cellular compartments: vesicles in mitochondria (226) and in the acidocalcisome (218) (**Figure 6**). The osmotic regulation of intracellular calcium is crucial for parasite survival and involves a set of ATPases whose location varies from the plasma membrane to the sarco/endoplasmic reticulum (227).

During infection of the MF, a burst occurs in the calcium steady-state concentration (228). During this process, free calcium from vesicle storage is released by the action of IP3 receptors, ryanodine receptor channels (RyRs), two-pore Ca(2+) channels (TPCs), and intracellular transient receptor potential (Trp) channels, which are mammalian channel homologs (229). This disturbance in intracellular calcium concentration has several consequences: (i) activation of guanylyl cyclase, which

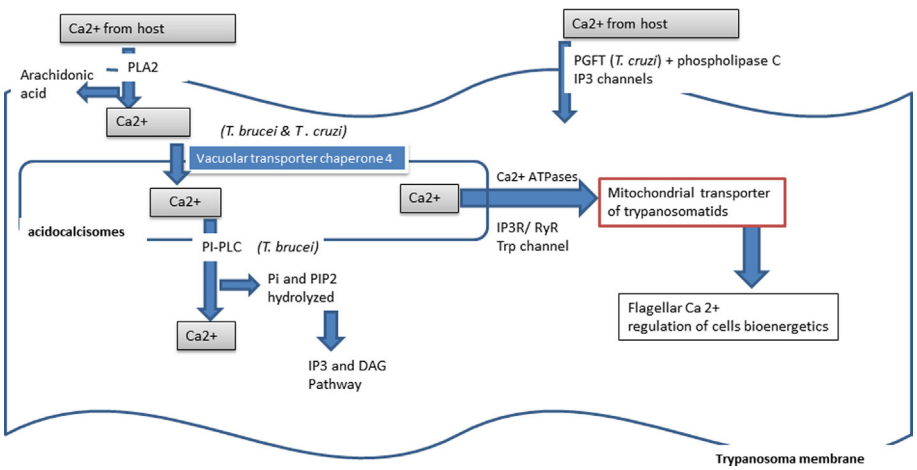

FIGURE 5 | Calcium used by *Trypanosoma*.

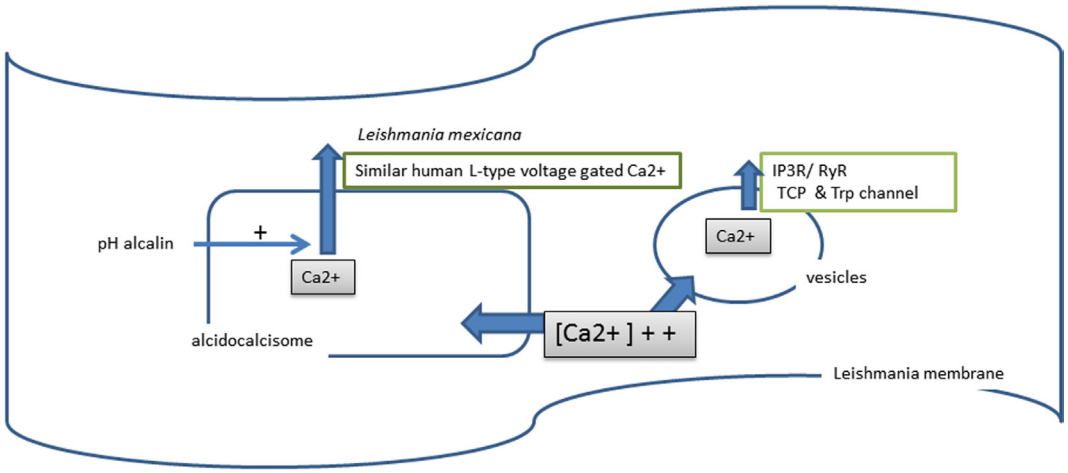

FIGURE 6 | **Calcium used by *Leishmania*.**

increases parasite infectivity (230); (ii) the release of calcium from the acidocalcisome, due to alkalization of the cytoplasmic pH (231); (iii) depolarization of the mitochondrial membrane potential and induced ATP loss, generating *Leishmania* cell death by apoptosis (232). Calcium channel blockers and nucleotides (UTP) possess anti-*Leishmania* activity (233, 234).

CONCLUSION

In conclusion, *Trypanosomatidae* parasites are multistage organisms that require a vertebrate host and an insect vector, in which they undergo many cell differentiations. Designing drugs that persistently interrupt the life cycles of these parasites requires a comprehensive understanding of their biology and the mechanisms involving host–vector–parasite interactions. Owing to the difficulties in controlling diseases caused by trypanosomatids, many studies have been focused on the strategies developed by the different parasites to escape host immune defenses, with a view to characterizing weaknesses in their escape processes that could be used to fight them. The goal of our review is to focus on these strategies developed by the different parasites to escape host immune defenses to improve knowledge of these interactions in order to initiate novel strategies for controlling the diseases caused by *Trypanosomatidae* parasites.

AUTHOR CONTRIBUTIONS

AG, GB, DS, JP, J-LL, PV, and PH wrote the review manuscript, read, and approved the final manuscript.

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REFERENCES

1. Stuart K, Brun R, Croft S, Fairlamb A, Gurtler RE, McKerrow J, et al. Kinetoplastids: related protozoan pathogens, different diseases. *J Clin Invest* (2008) 118:1301–10. doi:10.1172/JCI33945
2. WHO. *WHO Technical Report Series Control of the Leishmaniases*. (2010). Avaialble from: http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf
3. Welburn SC, Maudlin I, Simarro PP. Controlling sleeping sickness-a review. *Parasitology* (2009) 136:1943–9. doi:10.1017/S0031182009006416
4. Hotez PJ, Fenwick A, Savioli L, Molyneux DH. Rescuing the bottom billion through control of neglected tropical diseases. *Lancet* (2009) 373:1570–5. doi:10.1016/S0140-6736(09)60233-6
5. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: the way forward. *PLoS Negl Trop Dis* (2011) 5:e1007. doi:10.1371/journal.pntd.0001007
6. Odiit M, Shaw A, Welburn SC, Fèvre EM, Coleman PG, McDermott JJ. Assessing the patterns of health-seeking behaviour and awareness among sleeping-sickness patients in eastern Uganda. *Ann Trop Med Parasitol* (2004) 98:339–48. doi:10.1179/000349804225003389
7. Geiger A, Ponton F, Simo G. Adult blood-feeding tsetse flies, trypanosomes, microbiota and the fluctuating environment in sub-Saharan Africa. *ISME J* (2014) 9:1496–507. doi:10.1038/ismej.2014.236
8. Chappuis F, Lima MA, Flevaud L, Ritmeijer K. Human African trypanosomiasis in areas without surveillance. *Emerg Infect Dis* (2010) 16:354–6. doi:10.3201/eid1602.090967
9. Berrang-Ford L, Lundine J, Breau S. Conflict and human African trypanosomiasis. *Soc Sci Med* (2011) 72:398–407. doi:10.1016/j.socscimed.2010.06.006
10. Njiokou F, Nimpaye H, Simo G, Njitchouang GR, Asonganyi T, Cuny G, et al. Domestic animals as potential reservoir hosts of *Trypanosoma brucei* gambiense in sleeping sickness foci in Cameroon. *Parasite* (2010) 17:61–6. doi:10.1051/parasite/2010171061

11. Moore S, Shrestha S, Tomlinson KW, Vuong H. Predicting the effect of climate change on African trypanosomiasis: integrating epidemiology with parasite and vector biology. *J R Soc Interface* (2012) 9:817–30. doi:10.1098/rsif.2011.0654
12. Dumas M, Bouteille B. Current status of trypanosomiasis. *Med Trop* (1997) 57:65–9.
13. Holzmuller P, Grébaut P, Semballa S, Gonzatti MI, Geiger A. Proteomics: a new way to improve human African trypanosomiasis diagnosis? *Expert Rev Proteomics* (2013) 10:289–301. doi:10.1586/epr.13.14
14. Jacobs RT, Plattner JJ, Nare B, Wring SA, Chen D, Freund Y, et al. Benzoxaboroles: a new class of potential drugs for human African trypanosomiasis. *Future Med Chem* (2011) 3:1259–78. doi:10.4155/fmc.11.80
15. Torreele E, Bourdin Trunz B, Tweats D, Kaiser M, Brun R, Mazué G, et al. Fexinidazole – a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. *PLoS Negl Trop Dis* (2010) 4:e923. doi:10.1371/journal.pntd.0000923
16. Barrett MP. Potential new drugs for human African trypanosomiasis: some progress at last. *Curr Opin Infect Dis* (2010) 23:603–8. doi:10.1097/QCO.0b013e32833f9fd0
17. Bacchi CJ. Chemotherapy of human african trypanosomiasis. *Interdiscip Perspect Infect Dis* (2009) 2009:195040. doi:10.1155/2009/195040
18. Welburn SC, Maudlin I. Chapter 4 – Priorities for the elimination of Sleeping Sickness. In *Advances in Parasitology*, editors D. Rollinson, S.I. Hay. Oxford: Elsevier Ltd. (2012). 79:299–337. doi:10.1016/B978-0-12-398457-9.00004-4
19. Teixeira SM, Vieira LQ, Gazzinelli RT. Genomics, pathogenesis and control of infection with protozoan parasites. *Trends Parasitol* (2002) 18:52–4. doi:10.1016/S1471-4922(01)02208-5
20. Carabarin-Lima A, Gonzalez-Vazquez MC, Rodriguez-Morales O, Baylon-Pacheco L, Rosales-Encina JL, Reyes-Lopez PA, et al. Chagas disease (American trypanosomiasis) in Mexico: an update. *Acta Trop* (2013) 127:126–35. doi:10.1016/j.actatropica.2013.04.007
21. Barrias ES, de Carvalho TM, De Souza W. *Trypanosoma cruzi*: entry into mammalian host cells and parasitophorous vacuole formation. *Front Immunol* (2013) 4:186. doi:10.3389/fimmu.2013.00186
22. WHO. *Control of Chagas' Disease*. Technical Report Series No. 881, WHO Technical Report Series No. 905. Geneva, Switzerland: World Health Organization (2002). 113 p
23. Junqueira C, Caetano B, Bartholomeu DC, Melo MB, Ropert C, Rodrigues MM, et al. The endless race between *Trypanosoma cruzi* and host immunity: lessons for and beyond Chagas disease. *Expert Rev Mol Med* (2010) 15(12):e29. doi:10.1017/S1462399410001560
24. WHO. (2010). Avaialble from: http://www.who.int/leishmaniasis/en/
25. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* (2007) 5:873–82. doi:10.1038/nrmicro1748
26. Gupta G, Oghumu S, Satoskar AR. Mechanisms of immune evasion in leishmaniasis. *Adv Appl Microbiol* (2013) 82:155–84. doi:10.1016/B978-0-12-407679-2.00005-3
27. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) 7:e35671. doi:10.1371/journal.pone.0035671
28. Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, et al. Incidence of symptomatic and asymptomatic *Leishmania donovani* infections in high-endemic foci in India and Nepal: a prospective study. *PLoS Negl Trop Dis* (2011) 5:e1284. doi:10.1371/journal.pntd.0001284
29. Silveira FT, Lainson R, Crescente JA, de Souza AA, Campos MB, Gomes CM, et al. A prospective study on the dynamics of the clinical and immunological evolution of human *Leishmania (L.) infantum chagasi* infection in the Brazilian Amazon region. *Trans R Soc Trop Med Hyg* (2010) 104:529–35. doi:10.1016/j.trstmh.2010.05.002
30. Koltas IS, Eroglu F, Uzun S, Alabaz D. A comparative analysis of different molecular targets using PCR for diagnosis of old world leishmaniasis. *Exp Parasitol* (2016) 164:43–8. doi:10.1016/j.exppara.2016.02.007
31. Margioto Teston AP, Paula de Abreu A, Gruendling AP, Bahia MT, Gomes ML, Marques de Araújo S, et al. Differential parasitological, molecular, and serological detection of *Trypanosoma cruzi* I, II, and IV in blood of experimentally infected mice. *Exp Parasitol* (2016) 166:44–50. doi:10.1016/j.exppara.2016.03.013
32. Ribeiro JM, Walker FA. High affinity histamine-binding and antihistaminic activity of the salivary nitric oxide-carrying heme protein (nitrophorin) of *Rhodnius prolixus*. *J Exp Med* (1994) 180:2251–7. doi:10.1084/jem.180.6.2251
33. Ribeiro JMC, Assumpcao TCF, Pham VM, Fracischetti IMB, Reiseman CE. An insight into the sialotranscriptome of *Triatoma rubida* (Hemiptera: Heteroptera). *J Med Entomol* (2012) 49:563–72. doi:10.1603/ME11243
34. Ribeiro JM, Mans BJ, Arcà B. An insight into the sialome of blood-feeding Nematocera. *Insect Biochem Mol Biol* (2010) 40:767–84. doi:10.1016/j.ibmb.2010.08.002
35. Francischetti IM, Ribeiro JM, Champagne D, Andersen J. Purification, cloning, expression, and mechanism of action of a novel platelet aggregation inhibitorfrom the salivary gland of the blood-sucking bug, *Rhodnius prolixus*. *J Biol Chem* (2000) 275:12639–50. doi:10.1074/jbc.275.17.12639
36. Montandon CE, Barros E, Vidigal PM, Mendes MT, Anhê AC, de Oliveira Ramos HJ, et al. Comparative proteomic analysis of the saliva of the *Rhodnius prolixus*, *Triatoma lecticularia* and *Panstrongylus herreri* triatomines reveals a high interespecific functional biodiversity. *Insect Biochem Mol Biol* (2016) 71:83–90. doi:10.1016/j.ibmb.2016.02.009
37. Ribeiro JM, Genta FA, Sorgine MH, Logullo R, Mesquita RD, Paiva-Silva GO, et al. An insight into the transcriptome of the digestive tract of the bloodsucking bug, *Rhodnius prolixus*. *PLoS Negl Trop Dis* (2014) 8:e2594. doi:10.1371/journal.pntd.0002594
38. Santos A, Ribeiro JMC, Lehane MJ, Gontijo NF, Veloso AB, Sant'Anna MRV, et al. The sialotranscriptome of the blood-sucking bug *Triatoma brasiliensis* (Hemiptera, Triatomine). *Insect Mol Biol* (2007) 37:7012–7012. doi:10.1016/j.ibmb.2007.04.004
39. Alves-silva J, Ribeiro JMC, Van den Abbeele J, Attardo G, Hao Z, Haines LR, et al. An insight into the sialome of *Glossina morsitans morsitans*. *BMC Genomics* (2013) 11:213. doi:10.1186/1471-2164-11-213
40. Cappello M, Li S, Chen X, Li CB, Harrison L, Narashimhan S, et al. Tsetse thrombin inhibitor: bloodmeal-induced expression of an anticoagulant in salivary glands and gut tissue of *Glossina morsitans morsitans*. *Proc Natl Acad Sci U S A* (1998) 95:14290–5. doi:10.1073/pnas.95.24.14290
41. Charlab R, Valenzuela JG, Rowton ED, Ribeiro JMC. Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly *Lutzomyia longipalpis*. *Proc Natl Acad Sci U S A* (1999) 96:15155–60. doi:10.1073/pnas.96.26.15155
42. Li S, Kwon J, Askoy S. Characterization of genes expressed in the salivary glands of the tse tse fly, *Glossina morsitans morsitans*. *Insect Mol Biol* (2001) 10:69–76. doi:10.1046/j.1365-2583.2001.00240.x
43. Andersen JF, Ribeiro JM. A secreted salivary inositol polyphosphate 5-phosphatase from a blood-feeding insect: allosteric activation by soluble phosphoinositides and phosphatidylserine. *Biochemistry* (2006) 45:5450–7. doi:10.1021/bi052444j
44. Morita T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. *Toxicon* (2005) 45:1099–114. doi:10.1016/j.toxicon.2005.02.021
45. Noeske-Jungblut C, Krätzschmar J, Haendler B, Alagon A, Possani L, Verhallen P, et al. An inhibitor of collagen-induced platelet agregation from the saliva of *Triatoma pallidipennis*. *J Biol Chem* (1994) 269:5050–3.
46. Noeske-Jungblut C, Haendler B, Donner P, Alagon A, Possani L, Schleuning WD. Triabin, a highly potent exosite inhibitor of thrombin. *J Biol Chem* (1995) 270:28629–34. doi:10.1074/jbc.270.48.28629
47. Paddock CD, McKerrow JH, Hansell E, Foreman KW, Hsieh I, Marshall N. Identification, cloning, and recombinant expression of procalin, a major triatomine allergen. *J Immunol* (2001) 167:2694–9. doi:10.4049/jimmunol.167.5.2694
48. Assumpção TC, Alvarenga PH, Ribeiro JM, Andersen JF, Francischetti IM. Dipetalodipin, a novel multifunctional salivary lipocalin that inhibits platelet aggregation, vasoconstriction, and angiogenesis through unique binding specificity for TXA2, PGF2alpha, and 15(S)-HETE. *J Biol Chem* (2010) 285:39001–12. doi:10.1074/jbc.M110.152835
49. Lopes MF, Zamboni DS, Lujan HD, Rodrigues MM. Immunity to protozoan parasites. *J Parasitol Res* (2012) 2012:250793. doi:10.1155/2012/250793
50. Mendes-Sousa AF, Nascimento AA, Queiroz DC, Vale VF, Fujiwara RT, Araújo RN, et al. Different host complement systems and their interactions with saliva from *Lutzomyia longipalpis* (Diptera, Psychodidae) and

Leishmania infantum promastigotes. *PLoS One* (2013) 8:e79787. doi:10.1371/journal.pone.0079787
51. Joiner KA. Complement evasion by bacteria and parasites. *Annu Rev Microbiol* (1988) 42:201–30. doi:10.1146/annurev.mi.42.100188.001221
52. Puentes SM, Dwyer DM, Bates PA, Joiner KA. Binding and release of C3 from *Leishmania donovani* promastigotes during incubation in normal human serum. *J Immunol* (1989) 143:3743–9.
53. McConville MJ, Ralton JE. Developmentally regulated changes in the cell surface architecture of *Leishmania* parasites. *Behring Inst Mitt* (1997) 99:34–43.
54. Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, Mosser DM. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *J Immunol* (1995) 155:3102–11.
55. Lincoln LM, Ozaki M, Donelson JE, Beetham JK. Genetic complementation of *Leishmania* deficient in PSA (GP46) restores their resistance to lysis by complement. *Mol Biochem Parasitol* (2004) 137:185–9. doi:10.1016/j.molbiopara.2004.05.004
56. Späth GF, Epstein L, Leader B, Singer SM, Avila HA, Turco SJ, et al. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. *Proc Natl Acad Sci U S A* (2000) 97:9258–63. doi:10.1073/pnas.160257897
57. Bhandari V, Kumar D, Verma S, Srividya G, Negi NS, Singh R, et al. Increased parasite surface antigen-2 expression in clinical isolates of *Leishmania donovani* augments antimony resistance. *Biochem Biophys Res Commun* (2013) 440:646–51. doi:10.1016/j.bbrc.2013.09.113
58. Norris KA, Bradt B, Cooper NR, So M. Characterization of a *Trypanosoma cruzi* C3 binding protein with functional and genetic similarities to the human complement regulatory protein, decayaccelerating factor. *J Immunol* (1991) 147:2240–7.
59. Zambrano-Villa S, Rosales-Borjas D, Carrero JC, Ortiz-Ortiz L. How protozoan parasites evade the immune response. *Trends Parasitol* (2002) 18:272–8. doi:10.1016/S1471-4922(02)02289-4
60. Cestari I, Evans-Osses I, Schlapbach LJ, de Messias-Reason I, Ramirez MI. Mechanisms of complement lectin pathway activation and resistance by trypanosomatid parasites. *Mol Immunol* (2012) 53:328–34. doi:10.1016/j.molimm.2012.08.015
61. Raper J, Portela MP, Lugli E, Frevert U, Tomlinson S. Trypanosome lytic factors: novel mediators of human innate immunity. *Curr Opin Microbiol* (2001) 4:402–8. doi:10.1016/S1369-5274(00)00226-5
62. Lecordier L, Uzureau P, Tebabi P, Pérez-Morga D, Nolan D, Schumann Burkard G, et al. Identification of *Trypanosoma brucei* components involved in trypanolysis by normal human serum. *Mol Microbiol* (2014) 94:625–36. doi:10.1111/mmi.12783
63. Pays E, Vanhollebeke B, Uzureau P, Lecordier L, Pérez-Morga D. The molecular arms race between African trypanosomes and humans. *Nat Rev Microbiol* (2014) 12:575–84. doi:10.1038/nrmicro3298
64. Capewell P, Clucas C, DeJesus E, Kieft R, Hajduk S, Veitch N, et al. The TgsGP gene is essential for resistance to human serum in *Trypanosoma brucei* gambiense. *PLoS Pathog* (2013) 9:e1003686. doi:10.1371/journal.ppat.1003686
65. Burleigh BA, Woolsey AM. Cell signalling and *Trypanosoma cruzi* invasion. *Cell Microbiol* (2002) 4:701–11. doi:10.1046/j.1462-5822.2002.00226.x
66. de Souza W, de Carvalho TM, Barrias ES. Review on *Trypanosoma cruzi*: host cell interaction. *Int J Cell Biol* (2010) 2010:295394. doi:10.1155/2010/295394
67. Sibley LD, Andrews NW. Cell invasion by un-palatable parasites. *Traffic* (2000) 1:100–6. doi:10.1034/j.1600-0854.2000.010202.x
68. Amer AO, Swanson MS. A phagosome of one's own: a microbial guide to life in the macrophage. *Curr Opin Microbiol* (2002) 5:56–61. doi:10.1016/S1369-5274(02)00286-2
69. Andrews NW, Abrams CK, Slatin SL, Griffiths G. A *T. cruzi*-secreted protein immunologically related to the complement component C9: evidence for membrane pore-forming activity at low pH. *Cell* (1990) 61:1277–87. doi:10.1016/0092-8674(90)90692-8
70. Rubin-de-Celis SS, Uemura H, Yoshida N, Schenkman S. Expression of trypomastigote trans-sialidase in metacyclic forms of *Trypanosoma cruzi* increases parasite escape from its parasitophorous vacuole. *Cell Microbiol* (2006) 8:1888–98. doi:10.1111/j.1462-5822.2006.00755.x
71. Burleigh BA. Host cell signaling and *Trypanosoma cruzi* invasion: do all roads lead to lysosomes? *Sci STKE* (2005) 2005:e36. doi:10.1126/stke.2932005pe36
72. Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. *Cell Mol Life Sci* (2014) 71(7):1245–63. doi:10.1007/s00018-013-1491-1
73. Vannier-Santos MA, Martiny A, de Souza W. Cell biology of *Leishmania* spp.: invading and evading. *Curr Pharm Des* (2002) 8:297–318. doi:10.2174/1381612023396230
74. Alexander J, Russell DG. The interaction of *Leishmania* species with macrophages. *Adv Parasitol* (1992) 31:175–254. doi:10.1016/S0065-308X(08)60022-6
75. Courret N, Fréhel C, Gouhier N, Pouchelet M, Prina E, Roux P, et al. Biogenesis of *Leishmania*-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. *J Cell Sci* (2002) 115:2303–16.
76. Freitas-Teixeira PM, Silveira-Lemos D, Giunchetti RC, Baratta-Masini A, Mayrink W, Peruhype-Magalhães V, et al. Distinct pattern of immunophenotypic features of innate and adaptive immunity as a putative signature of clinical and laboratorial status of patients with localized cutaneous leishmaniasis. *Scand J Immunol* (2012) 76:421–32. doi:10.1111/j.1365-3083.2012.02748.x
77. Terrazas C, Oghumu S, Varikuti S, Martinez-Saucedo D, Beverley SM, Satoskar AR. Uncovering *Leishmania*-macrophage interplay using imaging flow cytometry. *J Immunol Methods* (2015) 423:93–8. doi:10.1016/j.jim.2015.04.022
78. Horn D. Antigenic variation in African trypanosomes. *Mol Biochem Parasitol* (2014) 195:123–9. doi:10.1016/j.molbiopara.2014.05.001
79. Carrington M, Carnall N, Crow MS, Gaud A, Redpath MB, Wasunna CL, et al. The properties and function of the glycosylphosphatidylinositol-phospholipase C in *Trypanosoma brucei*. *Mol Biochem Parasitol* (1998) 91:153–64. doi:10.1016/S0166-6851(97)00190-4
80. Magez S, Stijlemans B, Radwanska M, Pays E, Ferguson MA, De Baetselier P. The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidyl-inositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors. *J Immunol* (1998) 160:1949–56.
81. Paulnock DM, Coller SP. Analysis of macrophage activation in African trypanosomiasis. *J Leukoc Biol* (2001) 69:685–90.
82. Mansfield JM, Paulnock DM. Genetic manipulation of African trypanosomes as a tool to dissect the immunobiology of infection. *Immunology* (2008) 30:245–53. doi:10.1111/j.1365-3024.2007.01003.x
83. Namangala B. How the African trypanosomes evade host immune killing. *Parasite Immunol* (2011) 33:430–7. doi:10.1111/j.1365-3024.2011.01280.x
84. Chaussabel D, Pajak B, Vercruysse V, Bisseyé C, Garzé V, Habib M, et al. Alteration of migration and maturation of dendritic cells and T-cell depletion in the course of experimental *Trypanosoma cruzi* infection. *Lab Invest* (2003) 83:1373–82. doi:10.1097/01.LAB.0000087587.93781.6F
85. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol* (2014) 14(10):653–66. doi:10.1038/nri3737
86. Khatua B, Roy S, Mandal C. Sialic acids siglec interaction: a unique strategy to circumvent innate immune response by pathogens. *Indian J Med Res* (2013) 138:648–62.
87. Freire-de-Lima L, Alisson-Silva F, Carvalho ST, Takiya CM, Rodrigues MM, DosReis GA, et al. *Trypanosoma cruzi* subverts host cell sialylation and may compromise antigen-specific CD8+ T cell responses. *J Biol Chem* (2010) 285:13388–96. doi:10.1074/jbc.M109.096305
88. Shrikant PA, Rao R, Li Q, Kesterson J, Eppolito C, Mischo A, et al. Regulating functional cell fates in CD8 T cells. *Immunol Res* (2010) 46:12–22. doi:10.1007/s12026-009-8130-9
89. Stempin C, Giordanengo L, Gea S, Cerbán F. Alternative activation and increase of *Trypanosoma cruzi* survival in murine macrophages stimulated by cruzipain, a parasite antigen. *J Leukoc Biol* (2002) 72:727–34.
90. Nagajyothi F, Machado FS, Burleigh BA, Jelicks LA, Scherer PE, Mukherjee S, et al. Mechanisms of *Trypanosoma cruzi* persistence in Chagas disease. *Cell Microbiol* (2012) 14:634–43. doi:10.1111/j.1462-5822.2012.01764.x

91. Arango Duque G, Descoteaux A. *Leishmania* survival in the macrophage: where the ends justify the means. *Curr Opin Microbiol* (2015) 26:32–40. doi:10.1016/j.mib.2015.04.007
92. Matheoud D, Moradin N, Bellemare-Pelletier A, Shio MT, Hong WJ, Olivier M, et al. *Leishmania* evades host immunity by inhibiting antigen cross-presentation through direct cleavage of the SNARE VAMP8. *Cell Host Microbe* (2013) 14:15–25. doi:10.1016/j.chom.2013.06.003
93. Gurung P, Kanneganti TD. Innate immunity against *Leishmania* infections. *Cell Microbiol* (2015) 17:1286–94. doi:10.1111/cmi.12484
94. Bhardwaj N, Rosas LE, Lafuse WP, Satoskar AR. *Leishmania* inhibits STAT1-mediated IFN-gamma signaling in macrophages: increased tyrosine phosphorylation of dominant negative STAT1beta by *Leishmania mexicana*. *Int J Parasitol* (2005) 35:75–82. doi:10.1016/j.ijpara.2004.10.018
95. Forget G, Matte C, Siminovitch KA, Rivest S, Pouliot P, Olivier M. Regulation of the Leishmania-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. *Eur J Immunol* (2005) 35(6):1906–17.
96. Forget G, Gregory DJ, Whitcombe LA, Olivier M. Role of host protein tyrosine phosphatase SHP-1 in *Leishmania donovani*-induced inhibition of nitric oxide production. *Infect Immun* (2006) 74:6272–9. doi:10.1128/IAI.00853-05
97. Forget G, Matte C, Siminovitch KA, Rivest S, Pouliot P, Olivier M. Regulation of the *Leishmania* induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. *Eur J Immunol* (2005) 35:1906–17. doi:10.1002/eji.200526037
98. Forget G, Gregory DJ, Olivier M. Proteasome-mediated degradation of STAT1alpha following infection of macrophages with *Leishmania donovani*. *J Biol Chem* (2005) 280:30542–9. doi:10.1074/jbc.M414126200
99. Cecílio P, Pérez-Cabezas B, Santarém N, Maciel J, Rodrigues V, Cordeiro da Silva A. Deception and manipulation: the arms of *Leishmania*, a successful parasite. *Front Immunol* (2014) 5:480. doi:10.3389/fimmu.2014.00480
100. Vincendeau P, Gobert AP, Daulouède S, Moynet D, Mossalayi MD. Arginases in parasitic diseases. *Trends Parasitol* (2003) 19:9–12. doi:10.1016/S1471-4922(02)00010-7
101. Gobert AP, Daulouede S, Lepoivre M, Boucher JL, Bouteille B, Buguet A, et al. L-Arginine availability modulates local nitric oxide production and parasite killing in experimental trypanosomiasis. *Infect Immun* (2000) 68:4653–7. doi:10.1128/IAI.68.8.4653-4657.2000
102. De Muylder G, Daulouède S, Lecordier L, Uzureau P, Morias Y, Van Den Abbeele J, et al. A *Trypanosoma brucei* kinesin heavy chain promotes parasite growth by triggering host arginase activity. *PLoS Pathog* (2013) 9:e1003731. doi:10.1371/journal.ppat.1003731
103. Taheri F, Ochoa JB, Faghiri Z, Culotta K, Park HJ, Lan MS, et al. L-Arginine regulates the expression of the T-cell receptor zeta chain (CD3zeta) in Jurkat cells. *Clin Cancer Res* (2001) 7:958s–65s.
104. Daulouède S, Bouteille B, Moynet D, De Baetselier P, Courtois P, Lemesre JL, et al. Human macrophage tumor necrosis factor (TNF)-alpha production induced by *Trypanosoma brucei* gambiense and the role of TNF-alpha in parasite control. *J Infect Dis* (2001) 183:988–91. doi:10.1086/319257
105. Salmon D, Vanwalleghem G, Morias Y, Denoeud J, Krumbholz C, Lhommé F, et al. Adenylate cyclases of *Trypanosoma brucei* inhibit the innate immune response of the host. *Science* (2012) 337:463–6. doi:10.1126/science.1222753
106. Evans-Osses I, de Messias-Reason I, Ramirez MI. The emerging role of complement lectin pathway in trypanosomatids: molecular bases in activation, genetic deficiencies, susceptibility to infection, and complement system-based therapeutics. *ScientificWorldJournal* (2013) 2013:675898. doi:10.1155/2013/675898
107. Evans-Osses I, Mojoli A, Beltrame MH, da Costa DE, DaRocha WD, Velavan TP, et al. Differential ability to resist to complement lysis and invade host cells mediated by MBL in R4 and 860 strains of *Trypanosoma cruzi*. *FEBS Lett* (2014) 588:956–61. doi:10.1016/j.febslet.2014.01.054
108. Ribeiro CH, Lynch NJ, Stover CM, Ali YM, Valck C, Noya-Leal F, et al. Deficiency in mannose-binding lectin-associated serine protease-2 does not increase susceptibility to *Trypanosoma cruzi* infection. *Am J Trop Med Hyg* (2015) 92:320–4. doi:10.4269/ajtmh.14-0236
109. Ramirez G, Valck C, Ferreira VP, López N, Ferreira A. Extracellular *Trypanosoma cruzi* calreticulin in the host-parasite interplay. *Trends Parasitol* (2012) 27:115–22. doi:10.1016/j.pt.2010.12.007
110. Sánchez-Valdéz FJ, Pérez Brandán C, Ramírez G, Uncos AD, Zago MP, Cimino RO, et al. A monoallelic deletion of the TcCRT gene increases the attenuation of a cultured *Trypanosoma cruzi* strain, protecting against an in vivo virulent challenge. *PLoS Negl Trop Dis* (2014) 8:e2696. doi:10.1371/journal.pntd.0002696
111. Castillo C, Ramírez G, Valck C, Aguilar L, Maldonado I, Rosas C, et al. The interaction of classical complement component C1 with parasite and host calreticulin mediates *Trypanosoma cruzi* infection of human placenta. *PLoS Negl Trop Dis* (2013) 7:e2376. doi:10.1371/journal.pntd.0002376
112. Polando R, Dixit UG, Carter CR, Jones B, Whitcomb JP, Ballhorn W, et al. The roles of complement receptor 3 and Fcγ receptors during *Leishmania* phagosome maturation. *J Leukoc Biol* (2013) 93(6):921–32. doi:10.1189/jlb.0212086
113. Ricardo-Carter C, Favila M, Polando RE, Cotton RN, Bogard Horner K, Condon D, et al. *Leishmania major* inhibits IL-12 in macrophages by signalling through CR3 (CD11b/CD18) and down-regulation of ETS-mediated transcription. *Parasite Immunol* (2013) 35:409–20. doi:10.1111/pim.12049
114. Green PJ, Feizi T, Stoll MS, Thiel S, Prescott A, McConville MJ. Recognition of the major cell surface glycoconjugates of *Leishmania* parasites by the human serum mannan-binding protein. *Mol Biochem Parasitol* (1994) 66:319–28. doi:10.1016/0166-6851(94)90158-9
115. Ambrosio AR, De Messias-Reason IJ. *Leishmania (Viannia) braziliensis*: interaction of mannose-binding lectin with surface glycoconjugates and complement activation. An antibody-independent defence mechanism. *Parasite Immunol* (2005) 27:333–40. doi:10.1111/j.1365-3024.2005.00782.x
116. Wilson ME, Pearson RD. Roles of CR3 and mannose receptors in the attachment and ingestion of *Leishmania donovani* by human mononuclear phagocytes. *Infect Immun* (1988) 56:363–9.
117. Liu D, Uzonna JE. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* (2012) 2:83. doi:10.3389/fcimb.2012.00083
118. Bosetto MC, Giorgio S. *Leishmania amazonensis*: multiple receptor-ligand interactions are involved in amastigote infection of human dendritic cells. *Exp Parasitol* (2007) 116:306–10. doi:10.1016/j.exppara.2007.01.003
119. Laufs H, Müller K, Fleischer J, Reiling N, Jahnke N, Jensenius JC, et al. Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. *Infect Immun* (2002) 70:826–35. doi:10.1128/IAI.70.2.826-835.2002
120. Peters C, Kawakami M, Kaul M, Ilg T, Overath P, Aebischer T. Secreted proteophosphoglycan of *Leishmania mexicana* amastigotes activates complement by triggering the mannan binding lectin pathway. *Eur J Immunol* (1997) 27:2666–72. doi:10.1002/eji.1830271028
121. Dagenais TR, Freeman BE, Demick KP, Paulnock DM, Mansfield JM. Processing and presentation of variant surface glycoprotein molecules to T cells in African trypanosomiasis. *J Immunol* (2009) 183:3344–55. doi:10.4049/jimmunol.0802005
122. Vincendeau P, Bouteille B. Immunology and immunopathology of African trypanosomiasis. *An Acad Bras Cienc* (2006) 78:645–65. doi:10.1590/S0001-37652006000400004
123. Flávia Nardy A, Freire-de-Lima CG, Morrot A. Immune evasion strategies of *Trypanosoma cruzi*. *J Immunol Res* (2015) 2015:178947. doi:10.1155/2015/178947
124. Bogdan C, Röllinghoff M, Solbach W. Evasion strategies of *Leishmania* parasites. *Parasitol Today* (1990) 6:183–7. doi:10.1016/0169-4758(90)90350-D
125. Hudson KM, Byner C, Freeman J, Terry RJ. Immunodepression, high IgM levels and evasion of the immune response in murine trypanosomiasis. *Nature* (1976) 264:256–8. doi:10.1038/264256a0
126. Sharpe RT, Langley AM, Mowat GN, Macaskill JA, Holmes PH. Immunosuppression in bovine trypanosomiasis: response of cattle infected with *Trypanosoma congolense* to foot-and-mouth disease vaccination and subsequent live virus challenge. *Res Vet Sci* (1982) 32:289–93.
127. Namangala B, de Baetselier P, Brijs L, Stijlemans B, Noël W, Pays E, et al. Attenuation of *Trypanosoma brucei* is associated with reduced immunosuppression and concomitant production of Th2 lymphokines. *J Infect Dis* (2000) 181:1110–20. doi:10.1086/315322
128. Gomez-Rodriquez J, Stijlemans B, De Muylder G, Korf H, Brys L, Berberof M, et al. Identification of a parasitic modulatory protein

triggering the development of suppressive M1 macrophages during African trypanosomiasis. *J Infect Dis* (2009) 200:1849–60. doi:10.1086/648374
129. Stijlemans B, Vankrunkelsven A, Brys L, Raes G, Magez S, De Baetselier P. Scrutinizing the mechanisms underlying the induction of anemia of inflammation through GPI-mediated modulation of macrophage activation in a model of African trypanosomiasis. *Microbes Infect* (2010) 12:389–99. doi:10.1016/j.micinf.2010.02.006
130. Munder M, Eichmann K, Morán JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol* (1999) 163:3771–7.
131. Garzón E, Holzmuller P, Bras-Gonçalves R, Vincendeau P, Cuny G, Lemesre JL, et al. The *Trypanosoma brucei* gambiense secretome impairs lipopolysaccharide-induced maturation, cytokine production, and allostimulatory capacity of dendritic cells. *Infect Immun* (2013) 81:3300–8. doi:10.1128/IAI.00125-13
132. Namangala B, Brys L, Magez S, De Baetselier P, Beschin A. *Trypanosoma brucei* brucei infection impairs MHC class II antigen presentation capacity of macrophages. *Parasite Immunol* (2000) 22:361–70. doi:10.1046/j.1365-3024.2000.00314.x
133. De Baetselier P. T-cell subsets and cytokine interplay in infectious disease. In: Mustafa AS, Al-Attiyah RJ, Nath I, Chugh TD, editors. *Mechanisms Underlying Trypanosome Induced T-Cell Immunosuppression*. Basle: S. Karger (1996). p. 124–39.
134. Noel W, Hassanzadeh G, Raes G, Namangala B, Daems I, Brys L, et al. Infection stage-dependent modulation of macrophage activation in *Trypanosoma congolense*-resistant and -susceptible mice. *Infect Immun* (2002) 70:6180–7. doi:10.1128/IAI.70.11.6180-6187.2002
135. Taylor KA. Immune responses of cattle to African trypanosomes: protective or pathogenic? *Int J Parasitol* (1998) 28:219–40. doi:10.1016/S0020-7519(97)00154-9
136. Hertz CJ, Filutowicz H, Mansfield JM. Resistance to African tyrpanosomes is IFN-c dependent. *J Immunol* (1998) 161:6775–83.
137. Norris KA, Schrimpf JE, Szabo MJ. Identification of the gene family encoding the 160-kilodalton *Trypanosoma cruzi* complement regulatory protein. *Infect Immun* (1997) 65:349–57.
138. Samudio M, Montenegro-James S, Cabral M, Martinez J, Rojas De Arias A, James MA. Cytokine responses in *Trypanosoma cruzi*-infected children in Paraguay. *Am J Trop Med Hyg* (1998) 58:119–21.
139. Silva JS, Morrissey PJ, Grabstein KH, Moller KM, Anderson D, Reed SG. Interleukin 10 and interferon-c regulation of experimental *Trypanosoma cruzi* infection. *J Exp Med* (1992) 175:169–74. doi:10.1084/jem.175.1.169
140. Silva JS, Vespa GNR, Cardoso MAG, Aliberti JCS, Cunha FQ. Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. *Infect Immun* (1995) 63:4862–7.
141. Cardillo F, Voltarelli CJ, Reed SG, Silva JS. Regulation of *Trypanosoma cruzi* infection in mice by gamma interferon and interleukin 10: role of NK cells. *Infect Immun* (1996) 64:128–34.
142. Zhang L, Tarleton RL. Characterization of cytokine production in murine *T. cruzi* infection by in situ immunochemistry: lack of association between susceptibility and T helper type 2 cytokine production. *Eur J Immunol* (1996) 26:102–9. doi:10.1002/eji.1830260116
143. Wenzel UA, Bank E, Florian C, Förster S, Zimara N, Steinacker J, et al. *Leishmania major* parasite stage-dependent host cell invasion and immune evasion. *FASEB J* (2012) 26:29–39. doi:10.1096/fj.11-184895
144. Buchmüller-Rouiller Y, Mauël J. Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* spp. *Infect Immun* (1987) 55:587–93.
145. Bogdan C, Gessner A, Solbach W, Röllinghoff M. Invasion, control and persistence of *Leishmania* parasites. *Curr Opin Immunol* (1996) 8(4):517–25. doi:10.1016/S0952-7915(96)80040-9
146. Katakura K, Kobayashi A. Acid phosphatase activity of virulent and avirulent clones of *Leishmania donovani* promastigotes. *Infect Immun* (1988) 56:2856–60.
147. McNeely TB, Rosen G, Londner MV, Turco SJ. Inhibitory effects on protein kinase C activity by lipophosphoglycan fragments and glycosylphosphatidylinositol antigens of the protozoan parasite *Leishmania*. *Biochem J* (1989) 259:601–4. doi:10.1042/bj2590601
148. Pearson RD, Harcus JL, Roberts D, Donowitz GR. Differential survival of *Leishmania donovani* amastigotes in human monocytes. *J Immunol* (1983) 131:1994–9.
149. Handman E, Schnur LF, Spithill TW, Mitchell GF. Passive transfer of *Leishmania* lipopolysaccharide confers parasite survival in macrophages. *J Immunol* (1986) 137:3608–13.
150. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* (2011) 9(8):604–15. doi:10.1038/nrmicro2608
151. Kaye PM. Costimulation and the regulation of antimicrobial immunity. *Immunol Today* (1995) 16:423427. doi:10.1016/0167-5699(95)80018-2
152. Reiner NE. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. *Immunol Today* (1994) 15:374–81. doi:10.1016/0167-5699(94)90176-7
153. de Souza Leao S, Lang T, Prina E, Hellio R, Antoine JC. Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. *J Cell Sci* (1995) 108:3219–31.
154. Saha B, Das G, Vohra H, Ganguly NK, Mishra GC. Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on *Leishmania*-infected macrophages and its implications in the suppression of cell-mediated immunity. *Eur J Immunol* (1995) 25:2492–8. doi:10.1002/eji.1830250913
155. Carrada G, Cañeda C, Salaiza N, Delgado J, Ruiz A, Sanchez B, et al. Monocyte cytokine and costimulatory molecule expression in patients infected with *Leishmania mexicana*. *Parasite Immunol* (2007) 29:117–26. doi:10.1111/j.1365-3024.2006.00924.x
156. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* (1989) 7:145–73. doi:10.1146/annurev.iy.07.040189.001045
157. Dong L, Uzonna JE. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* (2012) 2:83. doi:10.3389/fcimb.2012.00083
158. Desjardins M, Descoteaux A. Survival strategies of *Leishmania donovani* in mammalian host macrophages. *Res Immunol* (1998) 149:689–92. doi:10.1016/S0923-2494(99)80040-6
159. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. *J Immunol* (2001) 166:1141–7. doi:10.4049/jimmunol.166.2.1141
160. León B, López-Bravo M, Ardavín C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against *Leishmania*. *Immunity* (2007) 26:519–31. doi:10.1016/j.immuni.2007.01.017
161. Von Stebut E, Belkaid Y, Jakob T, Sacks DL, Udey MC. Uptake of *Leishmania major* amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-*Leishmania* immunity. *J Exp Med* (1998) 188:1547–52. doi:10.1084/jem.188.8.1547
162. Kima PE, Soong L, Chicharro C, Ruddle NH, McMahon-Pratt D. *Leishmania*-infected macrophages sequester endogenously synthesized parasite antigens from presentation to CD4+ T cells. *Eur J Immunol* (1996) 26:3163–9. doi:10.1002/eji.1830261249
163. Ritter U, Meissner A, Scheidig C, Körner H. CD8 alpha- and Langerin-negative dendritic cells, but not Langerhans cells, act as principal antigen-presenting cells in leishmaniasis. *Eur J Immunol* (2004) 34:1542–50. doi:10.1002/eji.200324586
164. Kautz-Neu K, Noordegraaf M, Dinges S, Bennett CL, John D, Clausen BE, et al. Langerhans cells are negative regulators of the anti-*Leishmania* response. *J Exp Med* (2011) 208:885–91. doi:10.1084/jem.20102318
165. Costa DL, Guimarães LH, Cardoso TM, Queiroz A, Lago E, Roselino AM, et al. Characterization of regulatory T cell (Treg) function in patients infected with *Leishmania braziliensis*. *Hum Immunol* (2013) 74(12):1491–500. doi:10.1016/j.humimm.2013.08.269
166. Rai AK, Thakur CP, Kumar P, Mitra DK. Impaired expression of CD26 compromises T-cell recruitment in human visceral leishmaniasis. *Eur J Immunol* (2012) 42(10):2782–91. doi:10.1002/eji.201141912
167. Sibley LD. Invasion and intracellular survival by protozoan parasites. *Immunol Rev* (2011) 240:72–91. doi:10.1111/j.1600-065X.2010.00990.x
168. DosReis GA. Susceptible hosts: a resort for parasites right in the eye of the immune response. *An Acad Bras Cienc* (2000) 72:79–82. doi:10.1590/S0001-37652000000100011

169. Beschin A, Van Den Abbeele J, De Baetselier P, Pays E. African trypanosome control in the insect vector and mammalian host. *Trends Parasitol* (2014) 30:538–47. doi:10.1016/j.pt.2014.08.006
170. Feig C, Peter ME. How apoptosis got the immune system in shape. *Eur J Immunol* (2007) 37(Suppl 1):S61–70. doi:10.1002/eji.200737462
171. Gannavaram S, Debrabant A. Programmed cell death in *Leishmania*: biochemical evidence and role in parasite infectivity. *Front Cell Infect Microbiol* (2012) 2:95. doi:10.3389/fcimb.2012.00095
172. Burleigh BA, Andrews NW. Signaling and host cell invasion by *Trypanosoma cruzi*. *Curr Opin Microbiol* (1998) 1:461–5. doi:10.1016/S1369-5274(98)80066-0
173. Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Müller K, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* (2002) 169:898–905. doi:10.4049/jimmunol.169.2.898
174. Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL. Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-*Leishmania* response. *PLoS Pathog* (2012) 8:e1002536. doi:10.1371/journal.ppat.1002536
175. Ribeiro-Gomes FL, Otero AC, Gomes NA, Moniz-De-Souza MC, Cysne-Finkelstein L, Arnholdt AC, et al. Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J Immunol* (2004) 172:4454–62. doi:10.4049/jimmunol.172.7.4454
176. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* (2008) 321:970–4. doi:10.1126/science.1159194
177. Van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, et al. Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J Immunol* (2004) 173:6521–5. doi:10.4049/jimmunol.173.11.6521
178. Afonso L, Borges VM, Cruz H, Ribeiro-Gomes FL, DosReis GA, Dutra AN, et al. Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with *Leishmania amazonensis*. *J Leukoc Biol* (2008) 84:389–96. doi:10.1189/jlb.0108018
179. Allenbach C, Zufferey C, Perez C, Launois P, Mueller C, Tacchini-Cottier F. Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by *Leishmania major*. *J Immunol* (2006) 176:6656–64. doi:10.4049/jimmunol.176.11.6656
180. Kilpatrick LE, Sun S, Mackie D, Baik F, Li H, Korchak HM. Regulation of TNF mediated antiapoptotic signaling in human neutrophils: role of delta-PKC and ERK1/2. *J Leukoc Biol* (2006) 80:1512–21. doi:10.1189/jlb.0406284
181. Moore KJ, Matlashewski G. Intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis. *J Immunol* (1994) 152:2930–7.
182. Duleu S, Vincendeau P, Courtois P, Semballa S, Lagroye I, Daulouède S, et al. Mouse strain susceptibility to trypanosome infection: an arginase-dependent effect. *J Immunol* (2004) 172:6298–303. doi:10.4049/jimmunol.172.10.6298
183. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol* (2007) 7:532–42. doi:10.1038/nri2115
184. Guillermo LV, Silva EM, Ribeiro-Gomes FL, De Meis J, Pereira WF, Yagita H, et al. The Fas death pathway controls coordinated expansions of type 1 CD8 and type 2 CD4 T cells in *Trypanosoma cruzi* infection. *J Leukoc Biol* (2007) 81:942–51. doi:10.1189/jlb.1006643
185. Bertho AL, Santiago MA, Da-Cruz AM, Coutinho SG. Detection of early apoptosis and cell death in T CD4+ and CD8+ cells from lesions of patients with localized cutaneous leishmaniasis. *Braz J Med Biol Res* (2000) 33:317–25. doi:10.1590/S0100-879X2000000300010
186. De Lima VM, Fattori KR, de Souza F, Eugênio FR, dos Santos PS, Rozza DB, et al. Apoptosis in T lymphocytes from spleen tissue and peripheral blood of *L. (L.) chagasi* naturally infected dogs. *Vet Parasitol* (2012) 184:147–53. doi:10.1016/j.vetpar.2011.08.024
187. Potestio M, D'Agostino P, Romano GC, Milano S, Ferlazzo V, Aquino A, et al. CD4+ CCR5+ and CD4+CCR3+ lymphocyte subset and monocyte apoptosis in patients with acute visceral leishmaniasis. *Immunology* (2004) 113:260–8. doi:10.1111/j.1365-2567.2004.01948.x
188. Reckling S, Divanovic S, Karp CL, Wojciechowski S, Belkaid Y, Hildeman D. Proapoptotic Bcl-2 family member bim promotes persistent infection and limits protective immunity. *Infect Immun* (2008) 76:1179–85. doi:10.1128/IAI.01093-06
189. Mukherjee P, Sen PC, Ghose AC. Lymph node cells from BALB/c mice with chronic visceral leishmaniasis exhibiting cellular anergy and apoptosis: involvement of Ser/Thr phosphatase. *Apoptosis* (2006) 11:2013–29. doi:10.1007/s10495-006-0088-7
190. Antoine-Moussiaux N, Cornet A, Cornet F, Glineur S, Dermine M, Desmecht D. A non-cytosolic protein of *Trypanosoma evansi* induces CD45-dependent lymphocyte death. *PLoS One* (2009) 4:e5728. doi:10.1371/journal.pone.0005728
191. Bockstal V, Guirnalda P, Caljon G, Goenka R, Telfer JC, Frenkel D, et al. *T. brucei* infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. *PLoS Pathog* (2011) 7:e1002089. doi:10.1371/journal.ppat.1002089
192. Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S. Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. *PLoS Pathog* (2008) 4(5):e1000078. doi:10.1371/journal.ppat.1000078
193. Zuñiga E, Motran CC, Montes CL, Yagita H, Gruppi A. *Trypanosoma cruzi* infection selectively renders parasite-specific IgG+ B lymphocytes susceptible to Fas/Fas ligand-mediated fratricide. *J Immunol* (2002) 168:3965–73. doi:10.4049/jimmunol.168.8.3965
194. Zuniga E, Acosta-Rodriguez E, Merino MC, Montes C, Gruppi A. Depletion of immature B cells during *Trypanosoma cruzi* infection: involvement of myeloid cells and the cyclooxygenase pathway. *Eur J Immunol* (2005) 35:1849–58. doi:10.1002/eji.200526005
195. Vickerman K. Antigenic variation in trypanosomes. *Nature* (1978) 273:613–7. doi:10.1038/273613a0
196. Depledge DP, MacLean LM, Hodgkinson MR, Smith BA, Jackson AP, Ma S, et al. *Leishmania*-specific surface antigens show sub-genus sequence variation and immune recognition. *PLoS Negl Trop Dis* (2010) 4:e829. doi:10.1371/journal.pntd.0000829
197. Devera R, Fernandes O, Coura JR. Should *Trypanosoma cruzi* be called "cruzi" complex? A review of the parasite diversity and the potential of selecting population after in vitro culturing and mice infection. *Mem Inst Oswaldo Cruz* (2003) 98:1–12. doi:10.1590/S0074-02762003000100001
198. Reiner NE, Ng W, McMaster WR. Parasite-accessory cell interactions in murine leishmaniasis. II. *Leishmania donovani* suppresses macrophage expression of class I and class II major histocompatibility complex gene products. *J Immunol* (1987) 138:1926–32.
199. Flora R, Aghazadeh-Dibavar S, Bandyopadhyay M, Dasgupta S. Immunosuppression during *Leishmania donovani* infection: a potential target for the development of therapy. *Ann Parasitol* (2014) 60:239–45.
200. Tzelepis F, de Alencar BC, Penido ML, Claser C, Machado AV, Bruna-Romero O, et al. Infection with *Trypanosoma cruzi* restricts the repertoire of parasite-specific CD8+ T cells leading to immunodominance. *J Immunol* (2008) 180:1737–48. doi:10.4049/jimmunol.180.3.1737
201. Paulnock DM, Smith C, Mansfield JM. *Antigen Presenting Cell Function in African Trypanosomiasis*. New York: Alan R Liss Inc (1989). p. 135–44.
202. Shi MQ, Wei GJ, Tabel H. *Trypanosoma congolense* infections: MHC class II-restricted immune responses mediate either protection or disease, depending on IL-10 function. *Parasite Immunol* (2007) 29:107–11. doi:10.1111/j.1365-3024.2006.00925.x
203. Rodrigues V, Cordeiro-da-Silva A, Laforge M, Ouaissi A, Akharid K, Silvestre R, et al. Impairment of T cell function in parasitic infections. *PLoS Negl Trop Dis* (2014) 8:e2567. doi:10.1371/journal.pntd.0002567
204. Gigley JP, Bhadra R, Moretto MM, Khan IA. T cell exhaustion in protozoan disease. *Trends Parasitol* (2012) 28:377–84. doi:10.1016/j.pt.2012.07.001
205. Dominguez MR, Silveira EL, de Vasconcelos JR, de Alencar BC, Machado AV, Bruna-Romero O, et al. Subdominant/cryptic CD8 T cell epitopes contribute to resistance against experimental infection with a human protozoan parasite. *PLoS One* (2011) 6:e22011. doi:10.1371/journal.pone.0022011
206. Johnson PL, Kochin BF, Ahmed R, Antia R. How do antigenically varying pathogens avoid cross-reactive responses to invariant antigens? *Proc Biol Sci* (2012) 279:2777–85. doi:10.1098/rspb.2012.0005
207. Onah DN, Hopkins J, Luckins AG. Changes in peripheral blood lymphocyte subpopulations and parasite-specific antibody responses in *Trypanosoma*

evansi infection of sheep. *Parasitol Res* (1999) 85:263–9. doi:10.1007/s004360050545
208. Mougneau E, Bihl F, Glaichenhaus N. Cell biology and immunology of *Leishmania*. *Immunol Rev* (2011) 240:286–96. doi:10.1111/j.1600-065X.2010.00983.x
209. Da Silva Santos C, Brodskyn CI. The role of CD4 and CD8 T cells in human cutaneous leishmaniasis. *Front Public Health* (2014) 2:165. doi:10.3389/fpubh.2014.00165
210. Sacks D, Anderson C. Re-examination of the immunosuppressive mechanisms mediating non-cure of *Leishmania* infection in mice. *Immunol Rev* (2004) 201:225–38. doi:10.1111/j.0105-2896.2004.00185.x
211. Wu G, Morris J. Arginine metabolism: nitric oxide and beyond. *Biochem J* (1998) 336:1–17. doi:10.1042/bj3360001
212. Lott K, Zhu L, Fisk JC, Tomasello DL, Read LK. Functional interplay between protein arginine methyltransferases in *Trypanosoma brucei*. *Microbiologyopen* (2014) 3:595–609. doi:10.1002/mbo3.191
213. Ferreira TR, Alves-Ferreira EV, Defina TP, Walrad P, Papadopoulou B, Cruz AK. Altered expression of an RBP-associated arginine methyltransferase 7 in *Leishmania major* affects parasite infection. *Mol Microbiol* (2014) 94(5):1085–1102. doi:10.1111/mmi.12819
214. Miranda MR, Sayé M, Bouvier LA, Cámara Mde L, Montserrat J, Pereira CA. Cationic amino acid uptake constitutes a metabolic regulation mechanism and occurs in the flagellar pocket of *Trypanosoma cruzi*. *PLoS One* (2012) 7:e32760. doi:10.1371/journal.pone.0032760
215. Prati F, Goldman-Pinkovich A, Lizzi F, Belluti F, Koren R, Zilberstein D, et al. Quinone-amino acid conjugates targeting *Leishmania* amino acid transporters. *PLoS One* (2014) 9:e107994. doi:10.1371/journal.pone.0107994
216. Da Silva MF, Brodskyn LM. Arginase in *Leishmania*. *Subcell Biochem* (2014) 74:103–17. doi:10.1007/978-94-007-7305-9_4
217. Genestra M, Cysne-Finkelstein L, Guedes-Silva D, Leon LL. Effect of L-arginine analogs and a calcium chelator on nitric oxide (NO) production by *Leishmania* sp. *J Enzyme Inhib Med Chem* (2003) 18:445–52. doi:10.1080/1475636031000138787
218. Docampo R, Moreno SN. Acidocalcisome: a novel Ca2+ storage compartment in trypanosomatids and apicomplexan parasites. *Parasitol Today* (1999) 15:443–8. doi:10.1016/S0169-4758(99)01531-8
219. Rodríguez A, Rioult MG, Ora A, Andrews NW. A trypanosome-soluble factor induces IP3 formation, intracellular Ca2+ mobilization and microfilament rearrangement in host cells. *J Cell Biol* (1995) 129:1263–73. doi:10.1083/jcb.129.5.1263
220. Docampo R, Huang G. Calcium signaling in trypanosomatid parasites. *Cell Calcium* (2015) 57:194–202. doi:10.1016/j.ceca.2014.10.015
221. Moreno SN, Docampo R. The role of acidocalcisomes in parasitic protists. *J Eukaryot Microbiol* (2009) 56:208–13. doi:10.1111/j.1550-7408.2009.00404.x
222. Eintracht J, Maathai R, Mellors A, Ruben L. Calcium entry in *Trypanosoma brucei* is regulated by phospholipase A2 and arachidonic acid. *Biochem J* (1998) 336:659–66. doi:10.1042/bj3360659
223. Catisti R, Uyemura SA, Docampo R, Vercesi AE. Calcium mobilization by arachidonic acid in trypanosomatids. *Mol Biochem Parasitol* (2000) 105:261–71. doi:10.1016/S0166-6851(99)00186-3
224. Ulrich PN, Lander N, Kurup SP, Reiss L, Brewer J, Soares Medeiros LC, et al. The acidocalcisome vacuolar transporter chaperone 4 catalyzes the synthesis of polyphosphate in insect-stages of *Trypanosoma brucei* and *T. cruzi*. *J Eukaryot Microbiol* (2014) 61:155–65. doi:10.1111/jeu.12093
225. King-Keller S, Moore CA, Docampo R, Moreno SN. Ca2+ regulation of *Trypanosoma brucei* phosphoinositide phospholipase C. *Eukaryot Cell* (2015) 14:486–94. doi:10.1128/EC.00019-15
226. Benaim G, Bermudez R, Urbina JA. Ca2+ transport in isolated mitochondrial vesicles from *Leishmania braziliensis* promastigotes. *Mol Biochem Parasitol* (1990) 39:61–8. doi:10.1016/0166-6851(90)90008-A
227. Benaim B, Garcia CR. Targeting calcium homeostasis as the therapy of Chagas' disease and leishmaniasis – a review. *Trop Biomed* (2011) 28:471–81.
228. Roy N, Chakraborty S, Paul Chowdhury B, Banerjee S, Halder K, Majumder S, et al. Regulation of PKC mediated signaling by calcium during visceral leishmaniasis. *PLoS One* (2014) 9(10):e110843. doi:10.1371/journal.pone.0110843
229. Prole DL, Taylor CW. Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites. *PLoS One* (2011) 6:e26218. doi:10.1371/journal.pone.0026218
230. Karmakar S, Ukil A, Mukherjee S, Das PK. Regulation of guanylyl cyclase by intracellular Ca2+ in relation to the infectivity of the protozoan parasite, *Leishmania donovani*. *Int J Biochem Cell Biol* (2006) 38:1277–89. doi:10.1016/j.biocel.2006.01.002
231. Gupta S, Raychaudhury B, Banerjee S, Das B, Datta SC. An intracellular calcium store is present in *Leishmania donovani* glycosomes. *Exp Parasitol* (2006) 113:161–7. doi:10.1016/j.exppara.2005.12.020
232. Dolai S, Pal S, Yadav RK, Adak S. Endoplasmic reticulum stress-induced apoptosis in *Leishmania* through Ca2+-dependent and caspase-independent mechanism. *J Biol Chem* (2011) 286:13638–46. doi:10.1074/jbc.M110.201889
233. Reimão JQ, Tempone AG. Investigation into in vitro anti-leishmanial combinations of calcium channel blockers and current anti-leishmanial drugs. *Mem Inst Oswaldo Cruz* (2011) 106:1032–8. doi:10.1590/S0074-02762011000800022
234. Marques-da-Silva C, Chaves MM, Chaves SP, Figliuolo VR, Meyer-Fernandes JR, Corte-Real S, et al. Infection with *Leishmania amazonensis* upregulates purinergic receptor expression and induces host-cell susceptibility to UTP-mediated apoptosis. *Cell Microbiol* (2011) 13:1410–28. doi:10.1111/j.1462-5822.2011.01630.x

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REVIEW
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Immune Evasion Strategies of *Trypanosoma brucei* within the Mammalian Host: Progression to Pathogenicity

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The diseases caused by African trypanosomes (AT) are of both medical and veterinary importance and have adversely influenced the economic development of sub-Saharan Africa. Moreover, so far not a single field applicable vaccine exists, and chemotherapy is the only strategy available to treat the disease. These strictly extracellular protozoan parasites are confronted with different arms of the host's immune response (cellular as well as humoral) and via an elaborate and efficient (vector)–parasite–host interplay they have evolved efficient immune escape mechanisms to evade/manipulate the entire host immune response. This is of importance, since these parasites need to survive sufficiently long in their mammalian/vector host in order to complete their life cycle/transmission. Here, we will give an overview of the different mechanisms AT (i.e. *T. brucei* as a model organism) employ, comprising both tsetse fly saliva and parasite-derived components to modulate host innate immune responses thereby sculpturing an environment that allows survival and development within the mammalian host.

Keywords: African trypanosomosis, *T. brucei*, tsetse fly, innate immune response, pathogenicity

INTRODUCTION

Trypanosomatids, which include African trypanosomes (AT), American trypanosomes (i.e. *Trypanosoma cruzi* causing Chagas' disease) and different *Leishmania* species, comprise a large group of flagellated unicellular protozoa with a parasitic and complex digenetic life cycle. These diseases, exhibiting high morbidity and mortality rates, affect millions of impoverished populations in the developing world, display a limited response to chemotherapy, and are classified as neglected tropical diseases by the World Health Organization (WHO) (1, 2). In contrast to the other two trypanosomatids, the diseases caused by AT are of both medical and veterinary importance and

Abbreviations: BBB, blood–brain barrier; DMG, dimyristoyl glycerol; GPI, glycosylphosphatidylinositol; Hb, hemoglobin; Hp, haptoglobin; HpHbR, haptoglobin–hemoglobin receptor; LS, long slender; M1 cells, classically activated myeloid cells; NHS, normal human serum; SR-A, scavenger receptor type A; SRA, serum resistance antigen; SS, short stumpy; TbAdC, *T. brucei* adenylate cyclase; TbKHC, *T. brucei* kinesin heavy chain; TgsGP, *T. b. gambiense*-specific glycoprotein; TLF, Trypanosome lytic factor; TLTF, T-lymphocyte-triggering factor; TSIF, trypanosome suppression immunomodulating factor.

adversely influence the economic development of sub-Saharan Africa. Indeed, upon transmission through the bite of their blood-feeding vector (i.e., the tsetse fly, *Glossina spp.*), these parasites can cause fatal diseases in mammals, commonly called sleeping sickness in humans [Human African Trypanosomosis (HAT)] or Nagana (AAT, Animal African Trypanosomosis) in domestic livestock. According to the WHO, from the 60 million people living in the risk areas (i.e., the "tsetse" belt), approximately 300,000 people are currently infected with trypanosomes leading to 10,000–40,000 deaths annually (3, 4). The human pathogens *Trypanosoma brucei gambiense* (accounting for over 95% of cases) and *Trypanosoma brucei rhodesiense* (accounting for the remainder of cases) do not only differ in geographical distribution but also differ biologically, clinically, therapeutically, and epidemiologically and cause separate diseases (3, 5, 6). By contrast, the animal pathogens causing either Nagana (*Trypanosoma brucei brucei*, *Trypanosoma congolense, Trypanosoma vivax*) or Surra (*Trypanosoma evansi*) or Dourine (*Trypanosoma equiperdum*), do not cause disease in humans. Of note, some atypical human infections with animal trypanosomes, such as *T. evansi*, have been reported, which relate to deficiencies in the innate resistance to these otherwise non-human pathogens (7). Yet, AAT mainly caused by *T. congolense* and to a lesser extent by *T. b. brucei* and *T. vivax* forms a major constraint on cattle production. Hence, Nagana has a great impact on the nutrition of millions of people living in the most endemic areas, and on the agriculture economics of their countries, resulting in an estimated annual economic cost of about US$ 4 billion (8). Furthermore, the lack of prospect for vaccine development against AT is reinforced by (i) the fact that pharmaceutical companies are less prone to engage/invest in drug discovery/development against diseases that affect the poorest people, (ii) the political instability of the affected regions, (iii) the fact that wild animals function as reservoir of the parasite and, therefore, hamper the control of the disease, and (iv) the inappropriate use of the available drugs resulting in the emergence of drug resistance (8–11). Nevertheless, so far chemotherapy using compounds that target unique organelles of trypanosomes (i.e., glycosomes and kinetoplast) that are absent in the mammalian host and/or trypanosome metabolic pathways that differ from the host counterparts (carbohydrate metabolism, protein and lipid modifications, programed cell death) remain the only therapeutic choice for these diseases (12–16).

In contrast to the other two trypanosomatids, AT are strictly extracellular. Hence, they have developed efficient immune escape mechanisms to evade/manipulate the entire host immune response (cellular and humoral), involving an elaborate and efficient vector–parasite–host interplay, to survive sufficiently long in their mammalian host in order to complete their life cycle/transmission. Most of the research on AT uses murine models, which are more easily amenable compared to cattle or other domestic animals. Furthermore, given that the HAT causing *T. b. rhodesiense* and *T. b. gambiense* parasites highly resemble *T. b. brucei* (a non-human pathogenic subspecies causing Nagana), and chronic murine HAT models are scarce, the majority of research uses *T. b. brucei* as a model (17, 18). In this review, we will give an overview of the immunological events occurring during the early stages of infection within the mammalian host, using *T. b. brucei* as a model organism. We will also describe the different strategies that trypanosomes developed to sequentially activate and modulate innate immune responses to successfully escape immune elimination and maintain a chronic infection. Finally, we will discuss briefly how the host innate/adaptive immune response can culminate in immunopathogenicity development in trypanosusceptible animals.

EVASION MECHANISMS OF AFRICAN TRYPANOSOMES IN THE MAMMALIAN HOST

Trypanosoma brucei parasites cycle between the alimentary tract/salivary glands of the tsetse fly vector and the blood/tissues of the mammalian host. In each host, parasites undergo many life cycle changes (i.e., in the tsetse fly as procyclic/epimastigote/metacyclic forms and in the mammalian host as bloodstream forms) with discrete/important morphological and metabolic changes, which are programed precisely to adapt to different growth conditions/nutrient availability imposed by the different hosts and microenvironments they inhabit (19–22). These include, fine-tuning of energy metabolism, organelle reorganization, and biochemical and structural remodeling, which is supported by major changes in gene expression and proliferation status to adapt/survive in the different hosts (23). Furthermore, within the mammalian host, the complex life cycle of *T. brucei* consists of a succession of proliferative [long slender (LS)] and quiescent [short stumpy (SS)] developmental forms, which vary in cell architecture and function (23). Hereby, in response to a quorum sensing mechanism involving a stumpy-inducing factor (SIF) (24, 25), the LS forms differentiate into SS forms that are pre-adapted for the next developmental transition to procyclic forms, which occurs after ingestion by a tsetse fly (26).

Due to millions of years of co-evolution, these parasites have been able to thwart host innate responses and escape early recognition, allowing the initiation of infection in their respective hosts. In this section, we will give an overview of how trypanosomes can benefit from tsetse fly saliva components to initiate infection and subsequently how trypanosomal components can dampen/sculpture distinct innate immune responses in the mammalian host, which are pivotal in allowing early parasite infection and subsequent chronic infection.

Tsetse Fly Saliva Components Sculpture an Immune-Tolerant Microenvironment to Allow Establishment of Trypanosome Infections

A typical infection in the mammalian host begins when the infective stage, i.e., the metacyclic form, is co-injected with saliva intradermally by the tsetse fly. Hereby, the skin of the vertebrate host is a crucial anatomical barrier that pathogens have to overcome in order to establish infection. Within this microenvironment, pharmacological as well as immunological processes occur aimed at preventing pathogen development, whereby cells (lymphocytes, myeloid phagocytes, keratinocytes,…) sense the presence of damage-associated molecular patterns (DAMPs) as well as pathogen-associated molecular patterns (PAMPs) via different

pattern recognition receptors (PRRs), leading to the secretion of pro-inflammatory cytokines, type-I IFN, chemokines, reactive oxygen and nitrogen species, and antimicrobial peptides (27–29). Yet, during evolution, the skin has become a key interface for arthropod-borne diseases, whereby the pathogen in concert with saliva components transforms the skin barrier into an immune-tolerant organ supporting parasite development (30–32). This was strengthened by the observations by Caljon et al. (33) that the presence of tsetse fly saliva allowed a faster onset of the disease, which was associated with a reduced induction of inflammatory mediators at the site of infection and the interference of tsetse fly saliva with host hemostatic reactions (34). Indeed, tsetse fly saliva was shown to exert a dual pharmacological role (see **Figure 1**, red panel), (i) interfere with vertebrate host responses to enable successful blood feeding via the suppression of vasoconstriction, platelet aggregation, and coagulation [involving the anti-coagulant thrombin inhibitor (TTI), a 5′Nucleotidase-related apyrase and Adenosine Deaminase-related proteins (ADA)] (35–41) and (ii) modulate the host immune environment at the bite site that impacts pathogen transmission (42–44). Saliva was also reported to be highly immunogenic/allergenic in nature, thereby promoting infection onset in saliva immunized animals (45). For instance, Tsetse Antigen5 (TAg5) was shown to sensitize mice and trigger acute hypersensitivity reactions, which in turn could allow more efficient parasite extravasation into the blood circulation (46). A recently identified immunoregulatory peptide Gloss2 in tsetse fly saliva was shown to inhibit the secretion of trypanolytic molecules, such as TNF, and other pro-inflammatory cytokines, such as IFN-γ and IL-6, which could allow parasites to avoid initial elimination (44). Yet, through the use of transcriptome analyses and the availability of a partially annotated tsetse genome, it might be expected that many more proteins will be identified in the near future (34, 47).

Within the local immune-tolerant skin microenvironment, the metacyclic parasites respond to the increased temperature and rapidly transform into blood-stage trypomastigotes (LS forms), which divide by binary fission in the interstitial spaces at the bite site. Subsequently, they disseminate via the draining lymph nodes (48, 49). The first visible sign of a trypanosome infection coincides in many but not all instances with the occurrence of a "chancre," several days after infection (48, 50, 51). This development (onset, size, and duration) of the chancre correlates with the number of metacyclic parasites inoculated into the skin and is due to a local immune response directed against the variable antigen type (VAT) of the proliferating metacyclic forms (48). This consists of buildup of metabolic waste and cell debris from apoptotic cells, mainly neutrophils, releasing their intracellular cargo [i.e., neutrophil extracellular traps (NETs), antimicrobial peptides] aiming at capturing and subsequently killing the pathogens (52). Of note, although there are so far no reports documenting the contribution of neutrophils at the early stages of AT infection, the contribution of these phagocytes are documented in many other protozoan infections, such as Leishmaniasis and Malaria (52–54). Subsequently, the apoptotic cells in concert with parasite- and vector-derived components will be internalized by myeloid phagocytic cells (MPC) and degraded/processed to initiate innate immune responses (52–54). Also $CD4^+$ T lymphocytes were shown to play a key role in chancre formation, since *in vivo* depletion of $CD4^+$ T cells before inoculation of trypanosomes via a tsetse-fly bite resulted in a significant reduction of chancre formation (50).

Trypanosome-Derived Components Allow Parasite Survival and Sculpture Host Responses

SRA and TgsGP Allow Resistance to Normal Human Serum-Mediated Lysis

An important first step in the initiation and establishment of a trypanosome infection in the mammalian host is associated with cell cycle re-entry and metabolic/morphological/structural changes (see **Figure 1**, blue panel). This is required for acquisition of nutrients (i.e., glucose/iron/heme) in order to proliferate and subsequently activate immune evasion mechanisms to establish infection (20, 48, 55). Since trypanosomes are deficient in heme biosynthesis and heme cannot diffuse through the parasites' membrane (56–58), they require uptake of exogenous heme by the haptoglobin (Hp)–hemoglobin (Hb) receptor (HpHbR) located in the parasites' flagellar pocket (59). Following release of Hb from destroyed erythrocytes, it will be complexed with Hp, forming a Hp–Hb complex, which can be recognized by the myeloid phagocyte system (MPS) via CD163 and by the trypanosomal HpHbR. This will allow parasites to acquire and incorporate heme into intracellular hemoproteins required for optimal parasite growth and resistance to the oxidative burst by host cells. However, the HpHbR is also involved in primate innate immunity against certain trypanosome species (60, 61). Indeed, the serum of catarrhine primates and humans contains two trypanolytic particles: (i) a 500 kDa high-density lipoprotein (HDL)-bound trypanosome lytic factor (TLF)-1 and (ii) a 2 mDa large lipid-poor (<2%) IgM/apolipoprotein A-1 complex called TLF-2, that harbor the trypanolytic primate-specific apolipoprotein L1 (ApoL1), ApoA1, and Hp-related protein (Hpr) (62–65). Importantly, Hpr is a gene duplication product exhibiting high homology with Hp, which interacts with Hb to form an Hpr–Hb complex on the TLF-1 particles (66, 67). Following binding of the TLF-1 particle to the HpHbR (60, 68, 69), the entire TLF-1 particle is endocytosed and targeted to the lysosome. Subsequently, ApoL1 forms a pore in the endolysosomal membrane and triggers lysosomal swelling leading to the lethal release of lysosomal content into the parasites' cytosol (70–76). In addition, it was shown that the C-terminal kinesin *TbKIFC1* is involved in ApoL1-mediated lysis, whereby it transports ApoL1 from the endolysosomal membrane to the mitochondrion, leading to mitochondrial membrane depolarization and fenestration and subsequently lysis (77). Two different models are proposed to explain TLF-2 mediated killing; (i) since both Hpr and ApoL1 are present in this particle and TLF-2 killing of *T. b. brucei* is partly dependent on the *Tb*HpHbR receptor for uptake, TLF-2 may function in a manner similar to TLF-1 (60, 69). Yet, given that TLF-2 killing was not inhibited by the addition of Hp, a potent competitive inhibitor of TLF-1 uptake, it is more likely that TLF-2 has a different mode of internalization than TLF-1 (62, 73). (ii) TLF-2 uptake may also be linked to ApoL1 interaction with the *T. b. brucei* variable surface

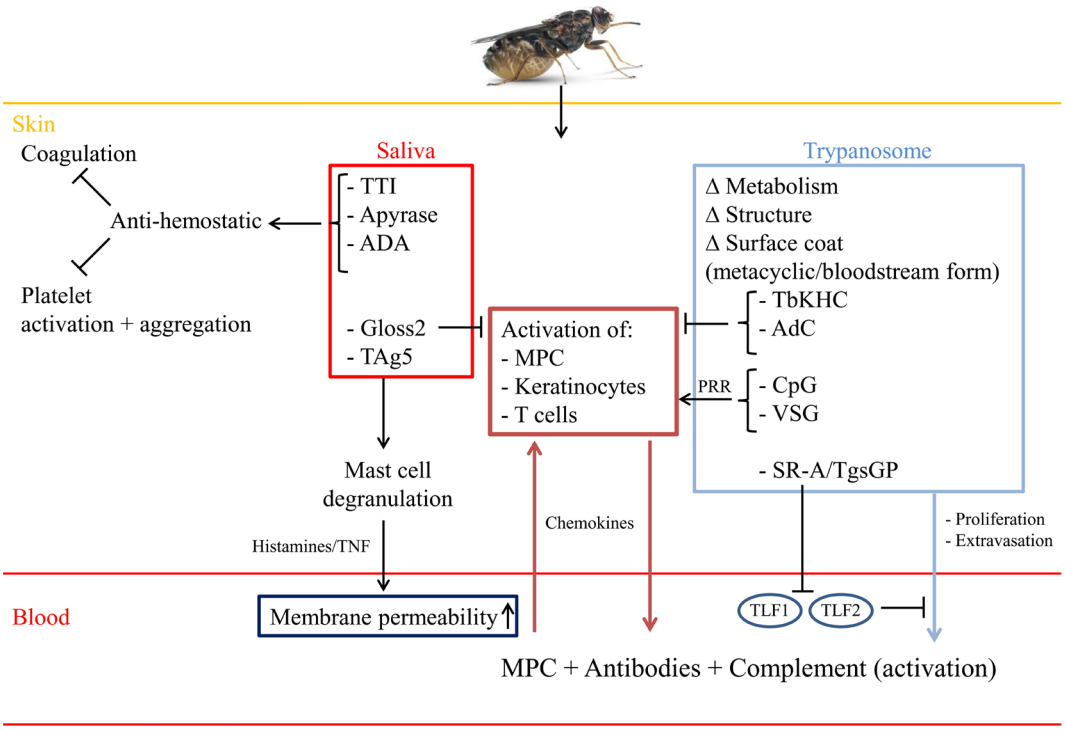

FIGURE 1 | Saliva components and parasite-derived factors sculpture the skin microenvironment. Upon the bite of a trypanosome-infected tsetse fly, trypanosomes and saliva components are inoculated intradermally leading to modulation of the skin microenvironment into a trypanosome receptive habitat. To this end, saliva components, such as TTI, a 5′Nucleotidase-related apyrase and Adenosine Deaminase-related proteins (ADA) prevent blood coagulation and platelet activation/aggregation, while the TAg5 allergen leads to activation of mast cells. Subsequently, these mast cells degranulate and release histamine and TNF, thereby increasing vasodilatation and allowing extra/intravasation of immune cells [myeloid phagocytic cells (MPC)] as well as parasites. In addition, this will allow infiltration of antibodies as well as complement factors needed for early parasite elimination. Yet, also the complement system (via C3a and C5a) can contribute to (i) increased vasopermeability and (ii) recruitment and activation of immune cells (PMN,…). By contrast, the Gloss2 peptide is able to downregulate inflammatory responses that are triggered upon breaching the skin anatomical barrier and/or encounter of metacyclic trypanosomes. Within the skin, these metacyclic parasites transform into LS bloodstream forms, which is associated with metabolic/structural/morphological changes, including switching of their metacyclic VSG into a blood-stream form VSG, required for survival within the mammalian host. The PAMPs of these pathogens (such as VSG and CpG) can be recognized by tissue-resident MPC or keratinocytes expressing PRR, leading to their activation and subsequent release of innate immune response triggering signals. For instance, release of chemokines will trigger the recruitment of MPC, which can amplify the immune response needed to eliminate skin-associated trypanosomes. Yet, trypanosomes try to dampen the initial pro-inflammatory immune response by (i) releasing TbKHC or (ii) following phagocytosis of altruistic parasites releasing TbAdC, thereby allowing the remaining parasite to survive and proliferate. Within the blood circulation, the parasites encounter the trypanolytic molecules TLF-1 and 2, leading to elimination of non-primate infecting parasites. Yet, HAT-causing parasites express SRA or TgsGP, which inactivate the (ApoL1/HpR) TLF-1/2-mediated trypanolytic effects, thereby allowing proliferation within the blood circulation.

glycoprotein (VSG) coat or TLF-2-associated IgM may bind *T. b. brucei*, as it is the only protein component that distinguishes both classes of TLF (78). Yet, so far no results supporting either mechanism of TLF-2 binding to *T. b. brucei* have been reported.

In contrast to the widespread *T. b. brucei* subspecies, which is highly infectious in many non-primate species that do not express Hpr and ApoL1 (79), the human pathogenic subspecies *T. b. rhodesiense* and *T. b. gambiense* express resistance proteins. Indeed, *T. b. rhodesiense* expresses a serum resistance antigen (SRA) and *T. b. gambiense* expresses a specific glycoprotein (TgsGP) counteracting ApoL1 activity (80–82), thereby enabling these parasites to evade the lethal action of TLF particles (see **Figure 1**, blue panel). Furthermore, *T. b. gambiense* exhibits low-level HpHbR expression and harbors an amino acid substitution (L210S) in HpHbR, leading to reduced TLF-1 uptake (76, 80–85). Recently, it was shown that SRA can be transferred from *T. b. rhodesiense* to *T. b. brucei* by membranous nanotubes that originate from the flagellar membrane and disassociate into free extracellular vesicles (EV) (86). Hence, this could result in the exchange of virulence factors that confer resistance to innate elimination.

T. brucei-Derived Kinesin Heavy Chain (TbKHC1) and Adenylate Cyclase Dampen Inflammation and Promote Parasite Growth

Besides parasite-derived factors playing a role in resistance to normal human serum (NHS), some parasite-derived molecules (see **Figure 1**, blue panel) are also able to dampen pro-inflammatory responses (TNF, NO) by classically activated macrophages (M1), needed for initial parasite control. One such important *T. brucei* protein is the Kinesin Heavy Chain 1 (TbKHC1) (87), which is released by the parasites in the environment via an unknown mechanism and sustains the development of the first (most

prominent) peak of parasitemia in the blood and its control by the host. Following binding of TbKHC1 to the SIGN-R1 molecule (i.e., a surface C-type lectin expressed mainly by marginal zone macrophages within the spleen), the arginine/NO metabolism is modulated in favor of arginase activity via an IL-10-dependent induction of arginase-1 and down-regulation of iNOS activities. In turn, this stimulates the production by the host of L-ornithine and hereby the synthesis of polyamines, which are essential nutrients for growth of trypanosomes in the host (88). Consequently, IL-10/arginase-1-producing immune cells are impaired in their capacity to destroy the parasite, thereby favoring parasite settlement. Another factor that trypanosomes use to establish infection comprises in the large family of transmembrane receptor-like adenylate cyclases (AdCs), called *T. brucei* Adenylate Cyclase (TbAdC) (89), which converts ATP into cyclic adenosine monophosphate (cAMP). During steady-state conditions, the TbAdC levels are low as is the cAMP production, yet upon stress (such as phagocytosis by M1 cells) the TbAdC levels can be elevated ~250-fold above the basal cellular content (89, 90). Subsequently, the cytoplasmic cAMP levels within the phagocytes increase, activating protein kinase A and leading to the inhibition of the synthesis of the trypanolytic cytokine TNF (91, 92). Hence, it seems that trypanosomes have developed a system whereby altruistic parasites are phagocytosed, thereby disabling the M1-mediated innate immune response required for parasite control (see **Figure 1**), and paving the way for initiation and establishment of the first wave of parasitemia.

Surface Coat Remodeling Prevents Elimination by the Humoral Immune Response

Given that AT are strictly extracellular parasites, they are confronted with the hosts' humoral immune response. Yet, one of the most fundamental changes occurring when parasites are inoculated into the mammalian host is the remodeling of the parasite cell surface (93). Indeed, within the mammalian host the metacyclic forms rapidly transform into the typical LS bloodstream forms expressing a different uniform VSG coat (94). This VSG coat consists of 5×10^6 homodimers of 50–60 kDa subunits held on the extracellular face of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (95), which consists of a ethanolaminephosphate-6-mannose-α1,2-mannose-α1,6-mannose-α1,4-glucosamine-α1,6-myo-inositol-1-phospholipid motif and a short galactose chain (96–99). Despite great variations in primary sequence, the secondary and tertiary structural features are highly conserved within the ordered coat structure (100). Although VSG molecules are free to diffuse in the plane of the membrane (101, 102), this ~15-nm-thick VSG coat has a dual role: shield off buried invariant proteins from recognition by the hosts' innate/acquired immune system and protect bloodstream parasites against complement-mediated lysis. Indeed, activation of the alternative pathway, which occurs in the absence of specific antibodies (Abs), may potentially play a crucial role in parasite clearance during the early stage of infection. Yet, it was shown by Devine et al. (103) that *T. b. gambiense* parasites, which are covered by C3, specifically inhibit the activation of the alternative pathway through their VSGs by masking sites on the plasma membrane, which are capable of promoting alternative pathway activation (104). Hence, the activation of the alternative pathway did not proceed further than the establishment of the C3 convertase, thereby impairing the generation of the terminal complex (C5–C9) which normally induces trypanolysis (103). In addition, soluble complement molecules, such as C3a and C5a, secreted during early stages of trypanosome infection, can further contribute to the initiation of the early inflammatory immune response within the chancre and may also act as (i) chemotactic agents attracting phagocytes to the site of infection and (ii) release histamine from mast cells thereby increasing microvascular permeability (105), which would allow/enable parasite extravasation into the blood circulation. Of note, the classical pathway, activated by immune complexes of trypanosome antigens and Abs, seems to contribute to trypanosome clearance through antibody-mediated trypanolysis and/or phagocytosis, which is of importance during peak parasitemia clearance (see later). Yet, also in this scenario, parasites are able to eliminate/remove surface-bound IgG (immune complexes) as well as complement through their rapid VSG recycling system and thereby prevent elimination (106). Furthermore, since complement is essential in antibody-mediated destruction of trypanosomes, by releasing vast amounts of soluble VSG (sVSG), mainly observed at the peak of parasitemia, this will scavenge complement factors and, hence, induce a state of hypocomplementemia (107, 108). This might favor the survival and escape of a minority of the parasites.

Additionally, binding of anti-VSG IgG or IgM to the trypanosome's coat results in parasite aggregation. Yet, trypanosomes are able to disaggregate in an energy-dependent manner involving protein kinase-C as part of the defense against the host humoral immune system (109). Hence, this could function as a survival strategy in the presence of antibody prior to the occurrence of VSG switching (109). The parasite's surface consisting of repetitive monotypic VSG molecules can cross-link B cell receptors (BCRs) and subsequently lead to T-cell-independent B-cell activation (110). However, during the process of antigenic variation (from metacyclic form toward trypomastigote form) mediated via changing VSG expression sites (i.e., *in situ* switching or transcriptional control) or by gene replacement resulting in a switch of the terminal telomeric VSG gene, heterologous VSG molecules are presented on the surface, thereby forming a mosaic VSG coat, which prevents direct B cell activation until a VSG uniformity is obtained (111, 112). This in turn might allow parasites to transiently escape T cell-independent B cell-mediated elimination and gives time to transform into trypomastigote forms adapted to survive in the mammalian host. Hence, this process gives the parasites an immunological advantage during the process of antigenic variation and is an efficient mechanism to escape antibody-mediated elimination during the early as well as chronic stage of infection (111).

VSG and VSG-Derived Fragments Trigger Different Cellular Innate Immune Responses

The VSG coat plays a key role in the interaction with the host, whereby it is involved in a population survival strategy through antigenic variation as well as in an individual cell survival strategy through rapid endocytosis, removal of bound antibody, and recycling back to the cell surface (106). The parasites not only use the

VSG as an efficient escape mechanism jeopardizing the induction of an effective antibody response (113–115), but also use it as means to modulate the hosts' cellular responses. Indeed, *T. brucei* parasites contain an endogenous phospholipase C (PLC) known as the GPI-PLC, which is activated upon hypotonic lysis, stress, or during antigenic variation (90, 116, 117), and shown not to be essential but rather to act as a virulence factor given that a PLC$^{-/-}$ mutant was attenuated in mice (118). Activation of the GPI-PLC hydrolyzes the GPI-anchor on the VSG (119, 120). This hydrolysis will convert the hydrophobic membrane-form VSG (mfVSG) into a water sVSG (117), thereby leaving the dimyristoyl glycerol (DMG) compound of the GPI-anchor in the membrane and releasing the glycosylinositolphosphate (GIP)-VSG part (121). Both components (DMG and GIP-VSG) exhibit distinct functions as far as activating potential of host immune cells is concerned (121, 122). Indeed, the GIP-VSG moiety is recognized by a Type A scavenger receptor expressed on myeloid cells, thereby initiating the activation of NF-κB and MAPK pathways and the expression of pro-inflammatory genes, such as TNF-α, IL-6, IL-12p40, and GM-CSF (123). This is further amplified when myeloid cells are primed with T-cell derived IFN-γ (124). Hereby, the galactose side chain of VSG is responsible for TNF-α production following activation of the protein tyrosine kinase (PTK) pathway (121, 125). However, reversing the order of exposure (i.e., exposing myeloid cells to GIP-VSG before IFN-γ stimulation) resulted in a down-regulation of IFN-γ-inducible responses, including transcription of inducible NO synthase and secretion of NO, which was associated with reduction in the level of STAT1 phosphorylation (126). This event might be of importance during the initial stage of infection, i.e., when sVSG is released from metacyclic forms during the early transition into bloodstream forms (see **Figure 2**). The GPI moiety, and in particular its DMG anchor that is released mainly during the descending phase of acute infection and during chronic infection, activates the protein kinase-C (PKC) pathway, and mediates macrophage priming/hyperactivation and LPS hyper-responsiveness in a MyD88-dependent manner (121, 122, 127, 128). Importantly, also in experimental bovine models, the DMG compound was shown to be crucial for M1 over-activation (129). In addition, the DMG compound of the mfVSG anchor seems to be crucial, via its IL-1α-inducing and -priming activity, in further fueling TNF induction (130).

CpG DNA is Used as Immunomodulatory Molecule to Trigger Macrophage Activation and Early Polyclonal B Cell Activation

Non-mammalian genomic DNA (i.e., CpG DNA) can also induce a host immune response (131). In this context, low amounts of tsetse-inoculated metacyclic parasites or SS blood-stream form parasites, continuously generated during the parasite cycle in the mammalian host, are eliminated/lysed giving rise to release of CpG DNA into circulation (see **Figure 2**). In turn, these CpG oligonucleotides trigger TLR9 signaling leading to the induction of M1 activation and polyclonal B-cell activation and subsequent isotype switching (132, 133). Importantly, CpG-mediated signaling can independently or synergistically with parasite-derived lipid or protein molecules (see further) activate the production of pro-inflammatory cytokines and NO needed for optimal peak parasitemia control. Indeed, as shown by Drennan et al. (127), during *T. brucei* infections, there is partial requirement for TLR9 signaling in the production of IFN-γ and VSG-specific IgG2a antibodies and for mammalian TLR family and MyD88 signaling in the innate immune recognition of *T. brucei*. Polyclonal B-cell activation, on the other hand, which is induced independently of BCR specificity, may play an important role in the defense against infections by enhancing natural antibody production and inducing memory B cells. Hence, polyclonal B cell activation increases the levels of natural antibodies to keep up with multiplication of the microorganisms, thus containing pathogen dissemination. Although triggering of polyclonal B cell activation is a natural innate immune response induced by many pathogens, the induction of polyclonal B cell activation (B cell expansion) might also be used as an immune evasion mechanism, whereby unselectively differentiating B cells can differentiate into short-lived plasmablasts (producing unspecific IgM), which ultimately results in apoptosis/elimination of the targeted B cell population (134, 135). In addition, regulatory B cells might also be induced and exert an immunosuppressive function by the secretion of IL-10, IL-17, IL-35, and transforming growth factor-β (TGFβ), and thereby dampen the initial pro-inflammatory immune response aimed at controlling infection (110, 136). However, so far no evidence of the occurrence of regulatory B cells is provided in this model.

TLTF Triggers IFN-γ Production by CD8$^+$ T Cells

Another trypanosome-derived factor documented to play a key role in early parasite–host interactions is the trypanosome-derived lymphocyte-triggering factor (TLTF), a secreted 185 kDa invariant glycoprotein able to trigger IFN-γ production by CD8$^+$ T cells (137–140). It was shown by Hamadien et al. (138) that early during *T. brucei* infection (day 3 p.i.) high levels of TLTF could be measured in the serum prior to IFN-γ production. Yet, later on during infection, these levels declined and coincided with increased levels of anti-TLTF antibodies. Of note, it was suggested that detection of TLTF and anti-TLTF antibodies in cerebrospinal fluid of HAT patients could be used as a tool for detection and staging of the disease (141). In addition, work from the same group and Nishimura et al. (142), revealed that IFN-γ was also able to trigger TLTF secretion in *in vitro* cultures of *T. brucei* parasites in a dose and tyrosine protein kinase-dependent manner and to stimulate parasite growth (143, 144). This suggests that TLTF and IFN-γ exert bidirectional activating signals between parasites and CD8$^+$ cells. Hence, these molecules might play a crucial regulatory function in the parasite–host interactions and influence the disease course during experimental African trypanosomosis (see **Figure 2**), whereby (i) TLTF released by *T. brucei* parasites triggers early IFN-γ production by CD8$^+$ T cells leading to the activation of M1 cells, (ii) IFN-γ triggers further secretion of TLTF by the proliferating parasites and was also suggested to be a growth factor for trypanosomes (142, 145). However, an alternative explanation for the apparent IFN-γ-mediated parasite growth effects, which cannot be excluded in *in vivo* settings, is that the early expansion of proliferating parasites (cf. ascending phase of first peak parasitemia) releases more TbKHC which in turn stimulates the synthesis of the essential nutrients, i.e., polyamines (see above) (88).

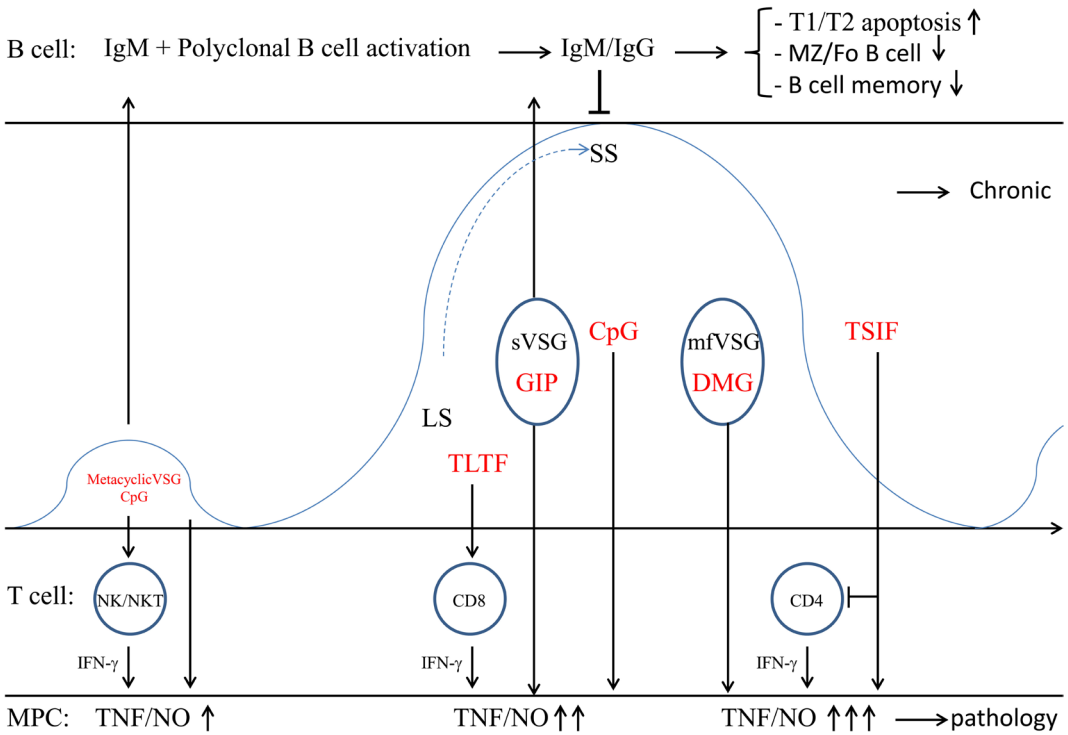

FIGURE 2 | Trypanosome establishment within the mammalian host. Within the blood circulation (several days post infection) the metacyclic trypanosomes give rise to a first small peak (which is not always observed). Subsequently, the metacyclic trypanosomes change their metacyclic VSG coat into the bloodstream VSG, thereby expressing a mosaic VSG that prevents Ab-mediated elimination. This dense VSG coat also prevents recognition of buried epitopes, including binding of complement factors (C3) to their surface. Also Ab-mediated elimination is prevented due to the rapid recycling of these VSG–Ab complexes and VSG shedding (i.e., sVSG release) that in turn scavenges circulating complement. Recognition of sVSG via SR-A on myeloid cells, in concert with CpG recognized via TLR9, results in the activation of MPC, which trigger activation of NK/NKT and T cells. In turn, these cells produce IFN-γ needed for proper activation of myeloid cells (M1 cells) and subsequent release of pro-inflammatory mediators (TNF/NO). Of note, initially, when GIP-sVSG is released via PLC activation due to stress prior to IFN-γ production, there is a weak activation of myeloid cells. Yet, triggering of PRR at the level of B cells (i.e., TLR9 via CpG) can also lead to polyclonal B-cell activation. Subsequently, parasites rapidly multiply as LS forms giving rise to the most prominent parasitemia peak. Trypanosomes also release TLTF that triggers IFN-γ production by $CD8^+$ T cells, which in turn stimulates parasite proliferation. However, IFN-γ exposure in concert with GIP-sVSG release will trigger an enhanced production of trypanolytic molecules by myeloid cells, which in concert with anti-VSG antibodies are needed for peak parasitemia control. Upon reaching the peak of parasitemia, the majority of the parasites differentiate into non-proliferative SS forms that are pre-adapted for uptake by tsetse flies, while a minority undergoes antigenic variation. Yet, in the mammalian host, these SS forms are deemed to die, thereby releasing mfVSG as well as CpG. These molecules exert dual functions; (i) the DMG of mfVSG triggers macrophage hyperactivation and LPS-hypersensitivity, while CpG further fuels polyclonal B-cell activation. These B cells can differentiate into short-lived plasmablasts (producing unspecific IgM) and ultimately results in apoptosis/elimination of all B-cell subsets and loss of B-cell memory. At this stage of infection, parasites also release TSIF that further stimulates the production of suppressive M1 and induces T-cell suppression. Once the first peak of parasitemia is controlled, the infection is established and the hosts' adaptive immune response will develop, whereby the B- and T-cell response are impaired and there is a polarized M1 activation leading the trypanosomosis-associated pathogenicity.

TbTSIF Induces M1 Cells and Triggers T-Cell Suppression

Several trypanosome components have been shown to exert a macrophage-activating potential, leading to NO-dependent suppression of T-cell proliferation (146, 147). Another parasite-derived molecule exerting the potential to modulate the host immune network is the *T. brucei*-derived Trypanosome Suppression Immunomodulating Factor (TSIF) (148). Since this molecule plays a role in triggering suppressive M1, it is most likely released during the descending phase of infection (see **Figure 2**), at the moment that M1 cells exert their most prominent effects (i.e., production of trypanolytic molecules TNF/NO). Furthermore, as shown by Gomez-Rodriguez et al. (148), this molecule is able to (i) block T-cell proliferation in a cell–cell contact and IFN-γ/NO-dependent manner and (ii) limit secretion of immune-protective IL-10 by alternatively activated macrophages (M2) required to dampen M1-mediated pathogenic effects. Hence, TbTSIF could play a dual role, i.e., contribute to initial parasite control (via TNF/NO) and fuel suppressive M1 and T-cell suppression leading to pathogenicity. However, T-cell suppression could also be a means of the parasite to negatively affect/inhibit B-cell development and thereby impair effective humoral responses (see later) and allow/guarantee parasite survival. In addition, it seems that TbTSIF is also essential for *T. brucei* development/biology since TbTSIF knock-out parasites were not viable and died within 2 days.

Host Innate/Adaptive Responses Determine Trypanosome-Associated Pathogenicity

Since AT can establish chronic infections in their mammalian host, which is associated with different forms of pathogenicity (anemia, liver injury, weight loss, neuropathology,…), it is clear that the innate response is insufficient for complete elimination of the parasites and, hence, will require the help of the adaptive immune response to combat infection. Yet, the modulation of the innate immune response might also affect the rejoinder of the adaptive immune response. In this section, we will elaborate on what is happening during the later stage of infection once the trypanosome infection is established in the mammalian host.

Trypanosome-Infections Impair B-Cell Functionality

As mentioned before, although trypanosomes use antigenic variation of their VSG coat as an efficient way to escape host humoral responses, trypanosomes also directly/indirectly affect B cell development as an additional means to escape elimination. Important to mention is that experiments in μMT (B cell deficient) and $IgM^{-/-}$ mice revealed that the initial development of peak parasitemia is independent of infection-induced anti-VSG antibodies. In addition, *in vivo* parasite VSG switching is an intrinsically programed genetic process that is independent of B cells or antibody pressure, with the function of antibodies mainly limited to the elimination of the remaining non-switched parasites (149). Studies in experimental rodent infection models have implicated T-cell-independent anti-VSG IgM responses to be the first line of host defense against proliferating parasites (150) (see before). Although B cells aid in periodically clearing circulating parasite levels by VSG-specific antibodies, they are limited by their VSG-specificity, yet they are required for long-term survival, while IgM antibodies play only a limited role in this process (149, 151–153). Importantly, similar observations were obtained in a Cape Buffalo model for natural trypanosomosis resistance (154). An additional aspect that plays a role in antibody-mediated recognition of trypanosomes is that though polyclonal antibodies are raised against different parts of the VSG molecule (155), only surface exposed regions (N-terminal more variable region) of the VSG could play a role in parasite elimination given that the buried epitopes (C-terminals more conserved region) are inaccessible for conventional antibodies (102, 156). Indeed, the VSG coat functions as a protective coat shielding of conserved buried epitopes/proteins, thereby preventing elimination of successive waves of trypanosomes expressing a different VSG coat.

The data so far indicate that *T. brucei* parasites affect B cells already early during infection (within 1 week p.i.) at different levels, resulting in the loss of humoral immune competence in trypanosusceptible hosts. This early undermining of humoral responses is important given that the production of high-affinity, antigen-specific, class-switched, antibodies takes up to 10 days after immunization (157). First, as mentioned before, there is induction of non-specific, polyclonal B-cell activation leading to clonal exhaustion (158–160). Second, there is destruction of the splenic B cell compartment that is manifested by the occurrence of marginal zone and follicular B cell (FoB) depletion. Hereby, IFN-γ was shown to play a key role in destruction of FoBs (161), which was associated with enhanced expression of the death receptor Fas, leading to loss of protective B cell memory responses against unrelated antigens. Third, it was shown that during *T. brucei* infection there is an impaired B-cell lymphopoiesis in the bone marrow and spleen already at the level of transitional B cells (159, 162). Hereby, there was massive cell death observed in transitional B cells *in vitro* through a contact-dependent mechanism, which is not dependent on TNF or prostaglandin-dependent death pathways (159). Of note, the mechanism(s) of *T. brucei*-induced transitional B-cell depletion *in vivo* remains to be fully elucidated.

Collectively, trypanosomes deliberately undermine the host's capacity to sustain antibody responses against recurring parasitemia waves by depleting transitional B cells, which in turn impairs the replenishment of the mature marginal zone and FoB populations. Since parasite-specific antibodies are essential for parasite control, inhibition of B-cell maturation at the transitional stage is an efficient evasive mechanism to prevent the buildup of protective "humoral" immunity against successive parasitemia waves. In this context, it was recently shown by De Trez et al. (163) that *T. brucei* infection is impairing the maintenance of the antigen-specific plasma B-cell pool.

Trypanosome Infections Induce Early IFN-γ-Mediated M1 Polarization that Subsequently Contributes to Pathogenicity Development

The parasite-derived components sVSG and CpG DNA that are released trigger via specific receptors (SR-A, TLR9) myeloid cell activation (121–123, 127, 164). In turn, this triggers T cell activation and the release of IFN-γ (165), which primes macrophages to become fully activated/M1 polarized thereby releasing pro-inflammatory molecules (TNF/NO) needed for parasite control (166, 167). This type 1 cytokine storm can also culminate in pathology development if maintained during later stages of infection (166–172). Yet, only animals able to produce tissue-protective IL-10 can exhibit an alleviated pathogenicity (167). Importantly, the balance of these different activation/deactivation signals may determine the outcome of infection (173, 174). Recently, it was shown that different lymphocyte populations play a role in IFN-γ production, whereby NK and NKT cells are the earliest IFN-γ producers, followed by $CD8^+$ and $CD4^+$ T cells (124). A possible explanation for this transition in different IFN-γ-producing T cells during the early stages of infection could be that: (i) initially type-I IFN released by for instance TLR9-activated myeloid cells can trigger NK/NKT-cell activation (175, 176); (ii) subsequently, parasite-derived TLTF will trigger IFN-γ production by $CD8^+$ T cells in a non-antigen-specific manner (140, 145, 177); and (iii) finally, the increased release and subsequent processing of sVSG will lead to MHC-II presentation and activation of $CD4^+$ T cells, thereby further fueling IFN-γ production and M1 polarization (178).

Whatever the source of IFN-γ may be, research so far indicates that early IFN-γ production triggers an acute inflammatory reaction resulting in acute anemia development, as witnessed by a 50% reduction in circulating red blood cells (RBC) within 2 days following peak parasitemia. After a short recovery phase,

a subsequent gradually increasing loss of RBCs occurs during the chronic infection stage (166, 167, 179). Of note, anemia development was found to be independent of antibodies and the height of the parasitemia peak, whereby the acute nature of this phenomenon implies a consumptive etiology (149, 168). IFN-γ plays also a crucial role in the recruitment and activation of erythrophagocytic myeloid cells. In addition, the work of Cnops et al. (124) indicates that the absence of NK, NKT, and $CD8^+$ T cells, but not $CD4^+$ T cells, during the early stage of infection results in a reduced anemic phenotype similar to IFNγR−/− mice. In addition, it was recently shown that trypanosomes can release extracellular vesicles (EV) that can fuse to mammalian erythrocytes thereby changing their physical properties and making them more susceptible to erythrophagocytosis (86). This in turn leads to acute anemia and could be a means of the parasites to acquire essential nutrients [hemoglobin and/or iron (see before)]. Hence, both host-induced and parasite-induced factors could account for acute anemia development. Subsequently, the hosts' ability to respond to the acute anemia will determine whether anemia persists or not during the chronic phase of infection (55, 166, 180–182).

Another pathological feature associated with *T. brucei* infections is neuropathology, whereby parasites pass the BBB and cause severe neurological complications. Interestingly, the work of Amin et al. (183) showed that *T. b. brucei* parasites penetrate the BBB very early during infection (within 2–3 days post infection), whereby they proposed that TLR9 and MyD88-mediated activation of DCs triggers via type-I IFN (IFN-α/β) T-cell activation. Subsequently, these activated T cells invade the central nervous system (CNS) in a IFN-α/β, IFN-γ and TNF-dependent manner, whereby TNF can induce the expression of adhesion molecules (ICAM-1 and VCAM-1) in brain endothelial cells in a TNFR1-dependent manner and contributes to the leakiness of inter-endothelial cell tight junctions or stimulation of matrix metalloproteases activities that open the parenchymal basement membranes (184, 185). Furthermore, the same group showed that IFN-γ, as well as the IFN-inducible chemokine CXCL10, promotes the penetration of T cells and parasites in the brain (186, 187), suggesting that parasites can also follow T cells during their brain invasion across the BBB. However, the work of Frevert et al. (188) showed, using a murine model and intravital brain imaging, that bloodstream forms of *T. b. brucei* and *T. b. rhodesiense* enter the brain parenchyma within hours post injection, before a significant level of microvascular inflammation is detectable. Yet, there are differences in the trypanosome strain used and the infection dose as well as the route of infection that could account for the different results. Collectively, it seems that whatever mechanism (host-mediated or not) parasites use to pass the BBB and infiltrate the brain, extravasations of parasites from the blood into the brain might be an alternative evasion mechanism to escape humoral responses that predominate in the blood circulation and thereby allow future transmission when parasites migrate back into the blood.

CONCLUSION AND PERSPECTIVES

Overall, it seems that trypanosomes have evolved efficient immune escape mechanisms to sculpture the hosts' innate/adaptive immune response in order to establish an environment suitable for parasite survival and transmission. This manipulation of the host response has its cost since this undermines the hosts' capacity to respond/recover following establishment of the parasites. Hereby, persistence of inflammation during the chronic stage of infection culminates into pathogenicity and subsequent death if left untreated. Hence, identification of host-derived factors playing a role in persistence of inflammation could be an alternative means to alleviate trypanosomosis-associated pathogenicity. In this context, it was recently shown that the pleiotropic host molecule macrophage migration inhibitory factor (MIF) plays a key role persistence of inflammation and infection-associated pathogenicity (180). Hence, future intervention strategies against African trypanosomosis might require a dual approach, i.e., development of efficient anti-trypanosomal agents combined with neutralization of anti-pathogenicity inducing "host" factors, which combined might allow reducing the economical losses of the affected continents.

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REFERENCES

1. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, et al. The trypanosomiases. *Lancet* (2003) 362(9394):1469–80. doi:10.1016/S0140-6736(03)14694-6
2. World Health Organization. Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis. *World Health Organ Tech Rep Ser* (2012) (975):v–xii,1–100.
3. Franco JR, Simarro PP, Diarra A, Jannin JG. Epidemiology of human African trypanosomiasis. *Clin Epidemiol* (2014) 6:257–75. doi:10.2147/CLEP.S39728
4. World Health Organization. Control and surveillance of human African trypanosomiasis. *World Health Organ Tech Rep Ser* (2013) (984):1–237.
5. Malvy D, Chappuis F. Sleeping sickness. *Clin Microbiol Infect* (2011) 17(7):986–95. doi:10.1111/j.1469-0691.2011.03536.x
6. Matthews KR, McCulloch R, Morrison LJ. The within-host dynamics of African trypanosome infections. *Philos Trans R Soc Lond B Biol Sci* (2015) 370(1675). doi:10.1098/rstb.2014.0288
7. Truc P, Buscher P, Cuny G, Gonzatti MI, Jannin J, Joshi P, et al. Atypical human infections by animal trypanosomes. *PLoS Negl Trop Dis* (2013) 7(9):e2256. doi:10.1371/journal.pntd.0002256

8. Ilemobade AA. Tsetse and trypanosomosis in Africa: the challenges, the opportunities. *Onderstepoort J Vet Res* (2009) 76(1):35–40. doi:10.4102/ojvr.v76i1.59
9. Baker N, de Koning HP, Maser P, Horn D. Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. *Trends Parasitol* (2013) 29(3):110–8. doi:10.1016/j.pt.2012.12.005
10. Jacobs RT, Nare B, Phillips MA. State of the art in African trypanosome drug discovery. *Curr Top Med Chem* (2011) 11(10):1255–74. doi:10.2174/156802611795429167
11. Barrett MP, Vincent IM, Burchmore RJ, Kazibwe AJ, Matovu E. Drug resistance in human African trypanosomiasis. *Future Microbiol* (2011) 6(9):1037–47. doi:10.2217/fmb.11.88
12. Babokhov P, Sanyaolu AO, Oyibo WA, Fagbenro-Beyioku AF, Iriemenam NC. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathog Glob Health* (2013) 107(5):242–52. doi:10.1179/2047773213Y.0000000105
13. Stein J, Mogk S, Mudogo CN, Sommer BP, Scholze M, Meiwes A, et al. Drug development against sleeping sickness: old wine in new bottles? *Curr Med Chem* (2014) 21(15):1713–27. doi:10.2174/0929867320666131119121636
14. Kroubi M, Karembe H, Betbeder D. Drug delivery systems in the treatment of African trypanosomiasis infections. *Expert Opin Drug Deliv* (2011) 8(6):735–47. doi:10.1517/17425247.2011.574122
15. Naula C, Burchmore R. A plethora of targets, a paucity of drugs: progress towards the development of novel chemotherapies for human African trypanosomiasis. *Expert Rev Anti Infect Ther* (2003) 1(1):157–65. doi:10.1586/14787210.1.1.157
16. Vincent IM, Barrett MP. Metabolomic-based strategies for anti-parasite drug discovery. *J Biomol Screen* (2015) 20(1):44–55. doi:10.1177/1087057114551519
17. Keita M, Bouteille B, Enanga B, Vallat JM, Dumas M. *Trypanosoma brucei* brucei: a long-term model of human African trypanosomiasis in mice, meningo-encephalitis, astrocytosis, and neurological disorders. *Exp Parasitol* (1997) 85(2):183–92. doi:10.1006/expr.1996.4136
18. Magez S, Caljon G. Mouse models for pathogenic African trypanosomes: unravelling the immunology of host-parasite-vector interactions. *Parasite Immunol* (2011) 33(8):423–9. doi:10.1111/j.1365-3024.2011.01293.x
19. Li Z. Regulation of the cell division cycle in *Trypanosoma brucei*. *Eukaryot Cell* (2012) 11(10):1180–90. doi:10.1128/EC.00145-12
20. Fenn K, Matthews KR. The cell biology of *Trypanosoma brucei* differentiation. *Curr Opin Microbiol* (2007) 10(6):539–46. doi:10.1016/j.mib.2007.09.014
21. Bringaud F, Riviere L, Coustou V. Energy metabolism of trypanosomatids: adaptation to available carbon sources. *Mol Biochem Parasitol* (2006) 149(1):1–9. doi:10.1016/j.molbiopara.2006.03.017
22. Rodrigues JC, Godinho JL, de Souza W. Biology of human pathogenic trypanosomatids: epidemiology, lifecycle and ultrastructure. *Subcell Biochem* (2014) 74:1–42. doi:10.1007/978-94-007-7305-9_1
23. MacGregor P, Szoor B, Savill NJ, Matthews KR. Trypanosomal immune evasion, chronicity and transmission: an elegant balancing act. *Nat Rev Microbiol* (2012) 10(6):431–8. doi:10.1038/nrmicro2779
24. Mony BM, Matthews KR. Assembling the components of the quorum sensing pathway in African trypanosomes. *Mol Microbiol* (2015) 96(2):220–32. doi:10.1111/mmi.12949
25. Mony BM, MacGregor P, Ivens A, Rojas F, Cowton A, Young J, et al. Genome-wide dissection of the quorum sensing signalling pathway in *Trypanosoma brucei*. *Nature* (2014) 505(7485):681–5. doi:10.1038/nature12864
26. Rico E, Rojas F, Mony BM, Szoor B, Macgregor P, Matthews KR. Bloodstream form pre-adaptation to the tsetse fly in *Trypanosoma brucei*. *Front Cell Infect Microbiol* (2013) 3:78. doi:10.3389/fcimb.2013.00078
27. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* (2006) 124(4):783–801. doi:10.1016/j.cell.2006.02.015
28. Kennedy-Crispin M, Billick E, Mitsui H, Gulati N, Fujita H, Gilleaudeau P, et al. Human keratinocytes' response to injury upregulates CCL20 and other genes linking innate and adaptive immunity. *J Invest Dermatol* (2012) 132(1):105–13. doi:10.1038/jid.2011.262
29. Harder J, Schroder JM, Glaser R. The skin surface as antimicrobial barrier: present concepts and future outlooks. *Exp Dermatol* (2013) 22(1):1–5. doi:10.1111/exd.12046
30. Frischknecht F. The skin as interface in the transmission of arthropod-borne pathogens. *Cell Microbiol* (2007) 9(7):1630–40. doi:10.1111/j.1462-5822.2007.00955.x
31. Bernard Q, Jaulhac B, Boulanger N. Smuggling across the border: how arthropod-borne pathogens evade and exploit the host defense system of the skin. *J Invest Dermatol* (2014) 134(5):1211–9. doi:10.1038/jid.2014.36
32. Bernard Q, Jaulhac B, Boulanger N. Skin and arthropods: an effective interaction used by pathogens in vector-borne diseases. *Eur J Dermatol* (2015) 25(Suppl 1):18–22. doi:10.1684/ejd.2015.2550
33. Caljon G, Van Den Abbeele J, Stijlemans B, Coosemans M, De Baetselier P, Magez S. Tsetse fly saliva accelerates the onset of *Trypanosoma brucei* infection in a mouse model associated with a reduced host inflammatory response. *Infect Immun* (2006) 74(11):6324–30. doi:10.1128/IAI.01046-06
34. Telleria EL, Benoit JB, Zhao X, Savage AF, Regmi S, Alves e Silva TL, et al. Insights into the trypanosome-host interactions revealed through transcriptomic analysis of parasitized tsetse fly salivary glands. *PLoS Negl Trop Dis* (2014) 8(4):e2649. doi:10.1371/journal.pntd.0002649
35. Cappello M, Li S, Chen X, Li CB, Harrison L, Narashimhan S, et al. Tsetse thrombin inhibitor: bloodmeal-induced expression of an anticoagulant in salivary glands and gut tissue of *Glossina morsitans* morsitans. *Proc Natl Acad Sci U S A* (1998) 95(24):14290–5. doi:10.1073/pnas.95.24.14290
36. Cappello M, Bergum PW, Vlasuk GP, Furmidge BA, Pritchard DI, Aksoy S. Isolation and characterization of the tsetse thrombin inhibitor: a potent antithrombotic peptide from the saliva of *Glossina morsitans* morsitans. *Am J Trop Med Hyg* (1996) 54(5):475–80.
37. Mant MJ, Parker KR. Two platelet aggregation inhibitors in tsetse (*Glossina*) saliva with studies of roles of thrombin and citrate in in vitro platelet aggregation. *Br J Haematol* (1981) 48(4):601–8. doi:10.1111/j.1365-2141.1981.00601.x
38. Alves-Silva J, Ribeiro JM, Van Den Abbeele J, Attardo G, Hao Z, Haines LR, et al. An insight into the sialome of *Glossina morsitans* morsitans. *BMC Genomics* (2010) 11:213. doi:10.1186/1471-2164-11-213
39. Caljon G, De Ridder K, De Baetselier P, Coosemans M, Van Den Abbeele J. Identification of a tsetse fly salivary protein with dual inhibitory action on human platelet aggregation. *PLoS One* (2010) 5(3):e9671. doi:10.1371/journal.pone.0009671
40. Li S, Aksoy S. A family of genes with growth factor and adenosine deaminase similarity are preferentially expressed in the salivary glands of *Glossina m.* morsitans. *Gene* (2000) 252(1–2):83–93. doi:10.1016/S0378-1119(00)00226-2
41. Zhao X, Alves e Silva TL, Cronin L, Savage AF, O'Neill M, Nerima B, et al. Immunogenicity and serological cross-reactivity of saliva proteins among different tsetse species. *PLoS Negl Trop Dis* (2015) 9(8):e0004038. doi:10.1371/journal.pntd.0004038
42. Ribeiro JM, Francischetti IM. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol* (2003) 48:73–88. doi:10.1146/annurev.ento.48.060402.102812
43. Champagne DE. Antihemostatic strategies of blood-feeding arthropods. *Curr Drug Targets Cardiovasc Haematol Disord* (2004) 4(4):375–96. doi:10.2174/1568006043335862
44. Bai X, Yao H, Du C, Chen Y, Lai R, Rong M. An immunoregulatory peptide from tsetse fly salivary glands of *Glossina morsitans* morsitans. *Biochimie* (2015) 118:123–8. doi:10.1016/j.biochi.2015.09.001
45. Caljon G, Van Den Abbeele J, Sternberg JM, Coosemans M, De Baetselier P, Magez S. Tsetse fly saliva biases the immune response to Th2 and induces anti-vector antibodies that are a useful tool for exposure assessment. *Int J Parasitol* (2006) 36(9):1025–35. doi:10.1016/j.ijpara.2006.05.002
46. Caljon G, Broos K, De Goeyse I, De Ridder K, Sternberg JM, Coosemans M, et al. Identification of a functional Antigen5-related allergen in the saliva of a blood feeding insect, the tsetse fly. *Insect Biochem Mol Biol* (2009) 39(5–6):332–41. doi:10.1016/j.ibmb.2009.01.010
47. Initiative IGG. Genome sequence of the tsetse fly (*Glossina morsitans*): vector of African trypanosomiasis. *Science* (2014) 344(6182):380–6. doi:10.1126/science.1249656
48. Barry JD, Emergy DL. Parasite development and host responses during the establishment of *Trypanosoma brucei* infection transmitted by tsetse fly. *Parasitology* (1984) 88(Pt 1):67–84. doi:10.1017/S0031182000054354
49. Luckins AG, Sutherland D, Mwangi D, Hopkins J. Early stages of infection with *Trypanosoma congolense*: parasite kinetics and expression of

metacyclic variable antigen types. *Acta Trop* (1994) 58(3–4):199–206. doi:10.1016/0001-706X(94)90014-0
50. Naessens J, Mwangi DM, Buza J, Moloo SK. Local skin reaction (chancre) induced following inoculation of metacyclic trypanosomes in cattle by tsetse flies is dependent on CD4 T lymphocytes. *Parasite Immunol* (2003) 25(8–9):413–9. doi:10.1111/j.1365-3024.2003.00649.x
51. Thuita JK, Kagira JM, Mwangangi D, Matovu E, Turner CM, Masiga D. *Trypanosoma brucei* rhodesiense transmitted by a single tsetse fly bite in vervet monkeys as a model of human African trypanosomiasis. *PLoS Negl Trop Dis* (2008) 2(5):e238. doi:10.1371/journal.pntd.0000238
52. Abi Abdallah DS, Denkers EY. Neutrophils cast extracellular traps in response to protozoan parasites. *Front Immunol* (2012) 3:382. doi:10.3389/fimmu.2012.00382
53. Ribeiro-Gomes FL, Romano A, Lee S, Roffe E, Peters NC, Debrabant A, et al. Apoptotic cell clearance of *Leishmania* major-infected neutrophils by dendritic cells inhibits CD8(+) T-cell priming in vitro by Mer tyrosine kinase-dependent signaling. *Cell Death Dis* (2015) 6:e2018. doi:10.1038/cddis.2015.351
54. Leoratti FM, Trevelin SC, Cunha FQ, Rocha BC, Costa PA, Gravina HD, et al. Neutrophil paralysis in *Plasmodium vivax* malaria. *PLoS Negl Trop Dis* (2012) 6(6):e1710. doi:10.1371/journal.pntd.0001710
55. Stijlemans B, Beschin A, Magez S, Van Ginderachter JA, De Baetselier P. Iron Homeostasis and *Trypanosoma brucei* associated immunopathogenicity development: a battle/quest for iron. *Biomed Res Int* (2015) 2015:819389. doi:10.1155/2015/819389
56. Tripodi KE, Menendez Bravo SM, Cricco JA. Role of heme and heme-proteins in trypanosomatid essential metabolic pathways. *Enzyme Res* (2011) 2011:873230. doi:10.4061/2011/873230
57. Koreny L, Obornik M, Lukes J. Make it, take it, or leave it: heme metabolism of parasites. *PLoS Pathog* (2013) 9(1):e1003088. doi:10.1371/journal.ppat.1003088
58. Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, et al. The genome of the African trypanosome *Trypanosoma brucei*. *Science* (2005) 309(5733):416–22. doi:10.1126/science.1112642
59. Higgins MK, Tkachenko O, Brown A, Reed J, Raper J, Carrington M. Structure of the trypanosome haptoglobin-hemoglobin receptor and implications for nutrient uptake and innate immunity. *Proc Natl Acad Sci U S A* (2013) 110(5):1905–10. doi:10.1073/pnas.1214943110
60. Stephens NA, Kieft R, Macleod A, Hajduk SL. Trypanosome resistance to human innate immunity: targeting Achilles' heel. *Trends Parasitol* (2012) 28(12):539–45. doi:10.1016/j.pt.2012.09.002
61. Vanhollebeke B, De Muylder G, Nielsen MJ, Pays A, Tebabi P, Dieu M, et al. A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. *Science* (2008) 320(5876):677–81. doi:10.1126/science.1156296
62. Raper J, Fung R, Ghiso J, Nussenzweig V, Tomlinson S. Characterization of a novel trypanosome lytic factor from human serum. *Infect Immun* (1999) 67(4):1910–6.
63. Lugli EB, Pouliot M, Portela Mdel P, Loomis MR, Raper J. Characterization of primate trypanosome lytic factors. *Mol Biochem Parasitol* (2004) 138(1):9–20. doi:10.1016/j.molbiopara.2004.07.004
64. Shiflett AM, Bishop JR, Pahwa A, Hajduk SL. Human high density lipoproteins are platforms for the assembly of multi-component innate immune complexes. *J Biol Chem* (2005) 280(38):32578–85. doi:10.1074/jbc.M503510200
65. Namangala B. Contribution of innate immune responses towards resistance to African trypanosome infections. *Scand J Immunol* (2012) 75(1):5–15. doi:10.1111/j.1365-3083.2011.02619.x
66. Maeda N. Nucleotide sequence of the haptoglobin and haptoglobin-related gene pair. The haptoglobin-related gene contains a retrovirus-like element. *J Biol Chem* (1985) 260(11):6698–709.
67. Widener J, Nielsen MJ, Shiflett A, Moestrup SK, Hajduk S. Hemoglobin is a co-factor of human trypanosome lytic factor. *PLoS Pathog* (2007) 3(9):1250–61. doi:10.1371/journal.ppat.0030129
68. Drain J, Bishop JR, Hajduk SL. Haptoglobin-related protein mediates trypanosome lytic factor binding to trypanosomes. *J Biol Chem* (2001) 276(32):30254–60. doi:10.1074/jbc.M010198200
69. Bullard W, Kieft R, Capewell P, Veitch NJ, Macleod A, Hajduk SL. Haptoglobin-hemoglobin receptor independent killing of African trypanosomes by human serum and trypanosome lytic factors. *Virulence* (2012) 3(1):72–6. doi:10.4161/viru.3.1.18295
70. Perez-Morga D, Vanhollebeke B, Paturiaux-Hanocq F, Nolan DP, Lins L, Homble F, et al. Apolipoprotein L-I promotes trypanosome lysis by forming pores in lysosomal membranes. *Science* (2005) 309(5733):469–72. doi:10.1126/science.1114566
71. Harrington JM, Nishanova T, Pena SR, Hess M, Scelsi CL, Widener J, et al. A retained secretory signal peptide mediates high density lipoprotein (HDL) assembly and function of haptoglobin-related protein. *J Biol Chem* (2014) 289(36):24811–20. doi:10.1074/jbc.M114.567578
72. Nielsen MJ, Moestrup SK. Receptor targeting of hemoglobin mediated by the haptoglobins: roles beyond heme scavenging. *Blood* (2009) 114(4):764–71. doi:10.1182/blood-2009-01-198309
73. Molina-Portela MP, Samanovic M, Raper J. Distinct roles of apolipoprotein components within the trypanosome lytic factor complex revealed in a novel transgenic mouse model. *J Exp Med* (2008) 205(8):1721–8. doi:10.1084/jem.20071463
74. Vanhollebeke B, Lecordier L, Perez-Morga D, Amiguet-Vercher A, Pays E. Human serum lyses *Trypanosoma brucei* by triggering uncontrolled swelling of the parasite lysosome. *J Eukaryot Microbiol* (2007) 54(5):448–51. doi:10.1111/j.1550-7408.2007.00285.x
75. Harrington JM, Howell S, Hajduk SL. Membrane permeabilization by trypanosome lytic factor, a cytolytic human high density lipoprotein. *J Biol Chem* (2009) 284(20):13505–12. doi:10.1074/jbc.M900151200
76. Vanhamme L, Paturiaux-Hanocq F, Poelvoorde P, Nolan DP, Lins L, Van Den Abbeele J, et al. Apolipoprotein L-I is the trypanosome lytic factor of human serum. *Nature* (2003) 422(6927):83–7. doi:10.1038/nature01461
77. Vanwalleghem G, Fontaine F, Lecordier L, Tebabi P, Klewe K, Nolan DP, et al. Coupling of lysosomal and mitochondrial membrane permeabilization in trypanolysis by APOL1. *Nat Commun* (2015) 6:8078. doi:10.1038/ncomms9078
78. Vanhollebeke B, Pays E. The trypanolytic factor of human serum: many ways to enter the parasite, a single way to kill. *Mol Microbiol* (2010) 76(4):806–14. doi:10.1111/j.1365-2958.2010.07156.x
79. Stodkilde K, Torvund-Jensen M, Moestrup SK, Andersen CB. Structural basis for trypanosomal haem acquisition and susceptibility to the host innate immune system. *Nat Commun* (2014) 5:5487. doi:10.1038/ncomms6487
80. Xong HV, Vanhamme L, Chamekh M, Chimfwembe CE, Van Den Abbeele J, Pays A, et al. A VSG expression site-associated gene confers resistance to human serum in *Trypanosoma rhodesiense*. *Cell* (1998) 95(6):839–46. doi:10.1016/S0092-8674(00)81706-7
81. Uzureau P, Uzureau S, Lecordier L, Fontaine F, Tebabi P, Homble F, et al. Mechanism of *Trypanosoma brucei* gambiense resistance to human serum. *Nature* (2013) 501(7467):430–4. doi:10.1038/nature12516
82. Capewell P, Clucas C, DeJesus E, Kieft R, Hajduk S, Veitch N, et al. The TgsGP gene is essential for resistance to human serum in *Trypanosoma brucei* gambiense. *PLoS Pathog* (2013) 9(10):e1003686. doi:10.1371/journal.ppat.1003686
83. Capewell P, Veitch NJ, Turner CM, Raper J, Berriman M, Hajduk SL, et al. Differences between *Trypanosoma brucei* gambiense groups 1 and 2 in their resistance to killing by trypanolytic factor 1. *PLoS Negl Trop Dis* (2011) 5(9):e1287. doi:10.1371/journal.pntd.0001287
84. Kieft R, Capewell P, Turner CM, Veitch NJ, MacLeod A, Hajduk S. Mechanism of *Trypanosoma brucei* gambiense (group 1) resistance to human trypanosome lytic factor. *Proc Natl Acad Sci U S A* (2010) 107(37):16137–41. doi:10.1073/pnas.1007074107
85. DeJesus E, Kieft R, Albright B, Stephens NA, Hajduk SL. A single amino acid substitution in the group 1 *Trypanosoma brucei* gambiense haptoglobin-hemoglobin receptor abolishes TLF-1 binding. *PLoS Pathog* (2013) 9(4):e1003317. doi:10.1371/journal.ppat.1003317
86. Szempruch AJ, Sykes SE, Kieft R, Dennison L, Becker AC, Gartrell A, et al. Extracellular vesicles from *Trypanosoma brucei* mediate virulence factor transfer and cause host anemia. *Cell* (2016) 164(1–2):246–57. doi:10.1016/j.cell.2015.11.051
87. De Muylder G, Daulouede S, Lecordier L, Uzureau P, Morias Y, Van Den Abbeele J, et al. A *Trypanosoma brucei* kinesin heavy chain promotes parasite growth by triggering host arginase activity. *PLoS Pathog* (2013) 9(10):e1003731. doi:10.1371/journal.ppat.1003731

88. Willert E, Phillips MA. Regulation and function of polyamines in African trypanosomes. *Trends Parasitol* (2012) 28(2):66–72. doi:10.1016/j.pt.2011.11.001
89. Salmon D, Vanwalleghem G, Morias Y, Denoeud J, Krumbholz C, Lhomme F, et al. Adenylate cyclases of *Trypanosoma brucei* inhibit the innate immune response of the host. *Science* (2012) 337(6093):463–6. doi:10.1126/science.1222753
90. Rolin S, Hanocq-Quertier J, Paturiaux-Hanocq F, Nolan D, Salmon D, Webb H, et al. Simultaneous but independent activation of adenylate cyclase and glycosylphosphatidylinositol-phospholipase C under stress conditions in *Trypanosoma brucei*. *J Biol Chem* (1996) 271(18):10844–52. doi:10.1074/jbc.271.18.10844
91. Magez S, Geuskens M, Beschin A, del Favero H, Verschueren H, Lucas R, et al. Specific uptake of tumor necrosis factor-alpha is involved in growth control of *Trypanosoma brucei*. *J Cell Biol* (1997) 137(3):715–27. doi:10.1083/jcb.137.3.715
92. Lucas R, Magez S, De Leys R, Fransen L, Scheerlinck JP, Rampelberg M, et al. Mapping the lectin-like activity of tumor necrosis factor. *Science* (1994) 263(5148):814–7. doi:10.1126/science.8303299
93. Shimogawa MM, Saada EA, Vashisht AA, Barshop WD, Wohlschlegel JA, Hill KL. Cell surface proteomics provides insight into stage-specific remodeling of the host-parasite interface in *Trypanosoma brucei*. *Mol Cell Proteomics* (2015) 14(7):1977–88. doi:10.1074/mcp.M114.045146
94. Van Den Abbeele J, Claes Y, van Bockstaele D, Le Ray D, Coosemans M. *Trypanosoma brucei* spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. *Parasitology* (1999) 118(Pt 5):469–78. doi:10.1017/S0031182099004217
95. Mehlert A, Bond CS, Ferguson MA. The glycoforms of a *Trypanosoma brucei* variant surface glycoprotein and molecular modeling of a glycosylated surface coat. *Glycobiology* (2002) 12(10):607–12. doi:10.1093/glycob/cwf079
96. Jones NG, Nietlispach D, Sharma R, Burke DF, Eyres I, Mues M, et al. Structure of a glycosylphosphatidylinositol-anchored domain from a trypanosome variant surface glycoprotein. *J Biol Chem* (2008) 283(6):3584–93. doi:10.1074/jbc.M706207200
97. Chattopadhyay A, Jones NG, Nietlispach D, Nielsen PR, Voorheis HP, Mott HR, et al. Structure of the C-terminal domain from *Trypanosoma brucei* variant surface glycoprotein MITat1.2. *J Biol Chem* (2005) 280(8):7228–35. doi:10.1074/jbc.M410787200
98. Ferguson MA. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J Cell Sci* (1999) 112(Pt 17):2799–809.
99. Mehlert A, Richardson JM, Ferguson MA. Structure of the glycosylphosphatidylinositol membrane anchor glycan of a class-2 variant surface glycoprotein from *Trypanosoma brucei*. *J Mol Biol* (1998) 277(2):379–92. doi:10.1006/jmbi.1997.1600
100. Blum ML, Down JA, Gurnett AM, Carrington M, Turner MJ, Wiley DC. A structural motif in the variant surface glycoproteins of *Trypanosoma brucei*. *Nature* (1993) 362(6421):603–9. doi:10.1038/362603a0
101. Bulow R, Overath P, Davoust J. Rapid lateral diffusion of the variant surface glycoprotein in the coat of *Trypanosoma brucei*. *Biochemistry* (1988) 27(7):2384–8. doi:10.1021/bi00407a020
102. Schwede A, Macleod OJ, MacGregor P, Carrington M. How does the VSG coat of bloodstream form African trypanosomes interact with external proteins? *PLoS Pathog* (2015) 11(12):e1005259. doi:10.1371/journal.ppat.1005259
103. Devine DV, Falk RJ, Balber AE. Restriction of the alternative pathway of human complement by intact *Trypanosoma brucei* subsp. gambiense. *Infect Immun* (1986) 52(1):223–9.
104. Ferrante A, Allison AC. Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat. *Parasite Immunol* (1983) 5(5):491–8. doi:10.1111/j.1365-3024.1983.tb00763.x
105. Marshall JS, Jawdat DM. Mast cells in innate immunity. *J Allergy Clin Immunol* (2004) 114(1):21–7. doi:10.1016/j.jaci.2004.04.045
106. Engstler M, Pfohl T, Herminghaus S, Boshart M, Wiegertjes G, Heddergott N, et al. Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. *Cell* (2007) 131(3):505–15. doi:10.1016/j.cell.2007.08.046
107. Balber AE, Bangs JD, Jones SM, Proia RL. Inactivation or elimination of potentially trypanolytic, complement-activating immune complexes by pathogenic trypanosomes. *Infect Immun* (1979) 24(3):617–27.
108. Musoke AJ, Barbet AF. Activation of complement by variant-specific surface antigen of *Trypanosoma brucei*. *Nature* (1977) 270(5636):438–40. doi:10.1038/270438a0
109. O'Beirne C, Lowry CM, Voorheis HP. Both IgM and IgG anti-VSG antibodies initiate a cycle of aggregation-disaggregation of bloodstream forms of *Trypanosoma brucei* without damage to the parasite. *Mol Biochem Parasitol* (1998) 91(1):165–93. doi:10.1016/S0166-6851(97)00191-6
110. Nothelfer K, Sansonetti PJ, Phalipon A. Pathogen manipulation of B cells: the best defence is a good offence. *Nat Rev Microbiol* (2015) 13(3):173–84. doi:10.1038/nrmicro3415
111. Hall JP, Wang H, Barry JD. Mosaic VSGs and the scale of *Trypanosoma brucei* antigenic variation. *PLoS Pathog* (2013) 9(7):e1003502. doi:10.1371/journal.ppat.1003502
112. Dubois ME, Demick KP, Mansfield JM. Trypanosomes expressing a mosaic variant surface glycoprotein coat escape early detection by the immune system. *Infect Immun* (2005) 73(5):2690–7. doi:10.1128/IAI.73.5.2690-2697.2005
113. Borst P. Antigenic variation and allelic exclusion. *Cell* (2002) 109(1):5–8. doi:10.1016/S0092-8674(02)00711-0
114. Kassem A, Pays E, Vanhamme L. Transcription is initiated on silent variant surface glycoprotein expression sites despite monoallelic expression in *Trypanosoma brucei*. *Proc Natl Acad Sci U S A* (2014) 111(24):8943–8. doi:10.1073/pnas.1404873111
115. Pays E, Vanhamme L, Perez-Morga D. Antigenic variation in *Trypanosoma brucei*: facts, challenges and mysteries. *Curr Opin Microbiol* (2004) 7(4):369–74. doi:10.1016/j.mib.2004.05.001
116. Hereld D, Krakow JL, Bangs JD, Hart GW, Englund PT. A phospholipase C from *Trypanosoma brucei* which selectively cleaves the glycolipid on the variant surface glycoprotein. *J Biol Chem* (1986) 261(29):13813–9.
117. Cardoso de Almeida ML, Turner MJ. The membrane form of variant surface glycoproteins of *Trypanosoma brucei*. *Nature* (1983) 302(5906):349–52. doi:10.1038/302349a0
118. Webb H, Carnall N, Vanhamme L, Rolin S, Van Den Abbeele J, Welburn S, et al. The GPI-phospholipase C of *Trypanosoma brucei* is nonessential but influences parasitemia in mice. *J Cell Biol* (1997) 139(1):103–14. doi:10.1083/jcb.139.1.103
119. Bulow R, Overath P. Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of *Trypanosoma brucei*. *J Biol Chem* (1986) 261(25):11918–23.
120. Ferguson MA, Homans SW, Dwek RA, Rademacher TW. Glycosylphosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science* (1988) 239(4841 Pt 1):753–9. doi:10.1126/science.3340856
121. Magez S, Stijlemans B, Baral T, De Baetselier P. VSG-GPI anchors of African trypanosomes: their role in macrophage activation and induction of infection-associated immunopathology. *Microbes Infect* (2002) 4(9):999–1006. doi:10.1016/S1286-4579(02)01617-9
122. Magez S, Stijlemans B, Radwanska M, Pays E, Ferguson MA, De Baetselier P. The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors. *J Immunol* (1998) 160(4):1949–56.
123. Leppert BJ, Mansfield JM, Paulnock DM. The soluble variant surface glycoprotein of African trypanosomes is recognized by a macrophage scavenger receptor and induces I kappa B alpha degradation independently of TRAF6-mediated TLR signaling. *J Immunol* (2007) 179(1):548–56. doi:10.4049/jimmunol.179.1.548
124. Cnops J, De Trez C, Stijlemans B, Keirsse J, Kauffmann F, Barkhuizen M, et al. NK-, NKT- and CD8-derived IFNgamma drives myeloid cell activation and erythrophagocytosis, resulting in trypanosomosis-associated acute anemia. *PLoS Pathog* (2015) 11(6):e1004964. doi:10.1371/journal.ppat.1004964
125. Tachado SD, Gerold P, Schwarz R, Novakovic S, McConville M, Schofield L. Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma*, and *Leishmania*: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. *Proc Natl Acad Sci U S A* (1997) 94(8):4022–7. doi:10.1073/pnas.94.8.4022
126. Coller SP, Mansfield JM, Paulnock DM. Glycosylinositolphosphate soluble variant surface glycoprotein inhibits IFN-gamma-induced nitric oxide

production via reduction in STAT1 phosphorylation in African trypanosomiasis. *J Immunol* (2003) 171(3):1466–72. doi:10.4049/jimmunol.171.3.1466
127. Drennan MB, Stijlemans B, Van den Abbeele J, Quesniaux VJ, Barkhuizen M, Brombacher F, et al. The induction of a type 1 immune response following a *Trypanosoma brucei* infection is MyD88 dependent. *J Immunol* (2005) 175(4):2501–9. doi:10.4049/jimmunol.175.4.2501
128. Tachado SD, Mazhari-Tabrizi R, Schofield L. Specificity in signal transduction among glycosylphosphatidylinositols of *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. *Parasite Immunol* (1999) 21(12):609–17. doi:10.1046/j.1365-3024.1999.00268.x
129. Sileghem M, Saya R, Grab DJ, Naessens J. An accessory role for the diacylglycerol moiety of variable surface glycoprotein of African trypanosomes in the stimulation of bovine monocytes. *Vet Immunol Immunopathol* (2001) 78(3–4):325–39. doi:10.1016/S0165-2427(01)00241-0
130. Philip R, Epstein LB. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. *Nature* (1986) 323(6083):86–9. doi:10.1038/323086a0
131. Shoda LK, Kegerreis KA, Suarez CE, Roditi I, Corral RS, Bertot GM, et al. DNA from protozoan parasites *Babesia bovis*, Trypanosoma cruzi, and T. brucei is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. *Infect Immun* (2001) 69(4):2162–71. doi:10.1128/IAI.69.4.2162-2171.2001
132. Gazzinelli RT, Camargo MM, Almeida IC, Morita YS, Giraldo M, Acosta-Serrano A, et al. Travassos and A. Sher: identification and characterization of protozoan products that trigger the synthesis of IL-12 by inflammatory macrophages. *Chem Immunol* (1997) 68:136–52. doi:10.1159/000058689
133. Sparwasser T, Hultner L, Koch ES, Luz A, Lipford GB, Wagner H. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J Immunol* (1999) 162(4):2368–74.
134. Goodyear CS, Silverman GJ. Death by a B cell superantigen: in vivo VH-targeted apoptotic supraclonal B cell deletion by a Staphylococcal Toxin. *J Exp Med* (2003) 197(9):1125–39. doi:10.1084/jem.20020552
135. Silverman GJ, Goodyear CS. Confounding B-cell defences: lessons from a staphylococcal superantigen. *Nat Rev Immunol* (2006) 6(6):465–75. doi:10.1038/nri1853
136. Parcina M, Miranda-Garcia MA, Durlanik S, Ziegler S, Over B, Georg P, et al. Pathogen-triggered activation of plasmacytoid dendritic cells induces IL-10-producing B cells in response to Staphylococcus aureus. *J Immunol* (2013) 190(4):1591–602. doi:10.4049/jimmunol.1201222
137. Hill KL, Hutchings NR, Grandgenett PM, Donelson JE. T lymphocyte-triggering factor of african trypanosomes is associated with the flagellar fraction of the cytoskeleton and represents a new family of proteins that are present in several divergent eukaryotes. *J Biol Chem* (2000) 275(50):39369–78. doi:10.1074/jbc.M006907200
138. Hamadien M, Lycke N, Bakhiet M. Induction of the trypanosome lymphocyte-triggering factor (TLTF) and neutralizing antibodies to the TLTF in experimental african trypanosomiasis. *Immunology* (1999) 96(4):606–11. doi:10.1046/j.1365-2567.1999.00730.x
139. Vaidya T, Bakhiet M, Hill KL, Olsson T, Kristensson K, Donelson JE. The gene for a T lymphocyte triggering factor from African trypanosomes. *J Exp Med* (1997) 186(3):433–8. doi:10.1084/jem.186.3.433
140. Olsson T, Bakhiet M, Hojeberg B, Ljungdahl A, Edlund C, Andersson G, et al. CD8 is critically involved in lymphocyte activation by a *T. brucei* brucei-released molecule. *Cell* (1993) 72(5):715–27. doi:10.1016/0092-8674(93)90400-K
141. Abdulla MH, Bakhiet M, Lejon V, Andersson J, McKerrow J, Al-Obeed O, et al. TLTF in cerebrospinal fluid for detection and staging of T. b. gambiense infection. *PLoS One* (2013) 8(11):e79281. doi:10.1371/journal.pone.0079281
142. Nishimura K, Hamashita K, Okamoto Y, Kawahara F, Ihara H, Kozaki S, et al. Differential effects of interferon-gamma on production of trypanosome-derived lymphocyte-triggering factor by *Trypanosoma brucei* gambiense and *Trypanosoma brucei* brucei. *J Parasitol* (2004) 90(4):740–5. doi:10.1645/GE-211R1
143. Hamadien M, Bakhiet M, Harris RA. Interferon-gamma induces secretion of trypanosome lymphocyte triggering factor via tyrosine protein kinases. *Parasitology* (2000) 120(Pt 3):281–7. doi:10.1017/S0031182099005521
144. Bakhiet M, Olsson T, Mhlanga J, Buscher P, Lycke N, van der Meide PH, et al. Human and rodent interferon-gamma as a growth factor for *Trypanosoma brucei*. *Eur J Immunol* (1996) 26(6):1359–64. doi:10.1002/eji.1830260627
145. Olsson T, Bakhiet M, Edlund C, Hojeberg B, Van der Meide PH, Kristensson K. Bidirectional activating signals between *Trypanosoma brucei* and CD8+ T cells: a trypanosome-released factor triggers interferon-gamma production that stimulates parasite growth. *Eur J Immunol* (1991) 21(10):2447–54. doi:10.1002/eji.1830211022
146. Darji A, Beschin A, Sileghem M, Heremans H, Brys L, De Baetselier P. In vitro simulation of immunosuppression caused by *Trypanosoma brucei*: active involvement of gamma interferon and tumor necrosis factor in the pathway of suppression. *Infect Immun* (1996) 64(6):1937–43.
147. Sternberg MJ, Mabbott NA. Nitric oxide-mediated suppression of T cell responses during *Trypanosoma brucei* infection: soluble trypanosome products and interferon-gamma are synergistic inducers of nitric oxide synthase. *Eur J Immunol* (1996) 26(3):539–43. doi:10.1002/eji.1830260306
148. Gomez-Rodriguez J, Stijlemans B, De Muylder G, Korf H, Brys L, Berberof M, et al. Identification of a parasitic immunomodulatory protein triggering the development of suppressive M1 macrophages during African trypanosomiasis. *J Infect Dis* (2009) 200(12):1849–60. doi:10.1086/648374
149. Magez S, Schwegmann A, Atkinson R, Claes F, Drennan M, De Baetselier P, et al. The role of B-cells and IgM antibodies in parasitemia, anemia, and VSG switching in *Trypanosoma brucei*-infected mice. *PLoS Pathog* (2008) 4(8):e1000122. doi:10.1371/journal.ppat.1000122
150. Reinitz DM, Mansfield JM. T-cell-independent and T-cell-dependent B-cell responses to exposed variant surface glycoprotein epitopes in trypanosome-infected mice. *Infect Immun* (1990) 58(7):2337–42.
151. Radwanska M, Magez S, Michel A, Stijlemans B, Geuskens M, Pays E. Comparative analysis of antibody responses against HSP60, invariant surface glycoprotein 70, and variant surface glycoprotein reveals a complex antigen-specific pattern of immunoglobulin isotype switching during infection by *Trypanosoma brucei*. *Infect Immun* (2000) 68(2):848–60. doi:10.1128/IAI.68.2.848-860.2000
152. de Gee AL, McCann PP, Mansfield JM. Role of antibody in the elimination of trypanosomes after DL-alpha-difluoromethylornithine chemotherapy. *J Parasitol* (1983) 69(5):818–22. doi:10.2307/3281038
153. Levine RF, Mansfield JM. Genetics of resistance to the African trypanosomes. III. Variant-specific antibody responses of H-2-compatible resistant and susceptible mice. *J Immunol* (1984) 133(3):1564–9.
154. Guirnalda P, Murphy NB, Nolan D, Black SJ. Anti-*Trypanosoma brucei* activity in Cape buffalo serum during the cryptic phase of parasitemia is mediated by antibodies. *Int J Parasitol* (2007) 37(12):1391–9. doi:10.1016/j.ijpara.2007.04.019
155. Reinitz DM, Mansfield JM. Independent regulation of B cell responses to surface and subsurface epitopes of African trypanosome variable surface glycoproteins. *J Immunol* (1988) 141(2):620–6.
156. Stijlemans B, Conrath K, Cortez-Retamozo V, Van Xong H, Wyns L, Senter P, et al. Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. *J Biol Chem* (2004) 279(2):1256–61. doi:10.1074/jbc.M307341200
157. Magez S, Radwanska M. African trypanosomiasis and antibodies: implications for vaccination, therapy and diagnosis. *Future Microbiol* (2009) 4(8):1075–87. doi:10.2217/fmb.09.65
158. Corsini AC, Clayton C, Askonas BA, Ogilvie BM. Suppressor cells and loss of B-cell potential in mice infected with *Trypanosoma brucei*. *Clin Exp Immunol* (1977) 29(1):122–31.
159. Bockstal V, Guirnalda P, Caljon G, Goenka R, Telfer JC, Frenkel D, et al. *T. brucei* infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. *PLoS Pathog* (2011) 7(6):e1002089. doi:10.1371/journal.ppat.1002089
160. Diffley P. Trypanosomal surface coat variant antigen causes polyclonal lymphocyte activation. *J Immunol* (1983) 131(4):1983–6.
161. Cnops J, De Trez C, Bulte D, Radwanska M, Ryffel B, Magez S. IFN-gamma mediates early B-cell loss in experimental African trypanosomosis. *Parasite Immunol* (2015) 37(9):479–84. doi:10.1111/pim.12208
162. Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S. Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. *PLoS Pathog* (2008) 4(5):e1000078. doi:10.1371/journal.ppat.1000078

163. De Trez C, Katsandegwaza B, Caljon G, Magez S. Experimental African trypanosome infection by needle passage or natural tsetse fly challenge thwarts the development of collagen-induced arthritis in DBA/1 prone mice via an impairment of antigen specific B cell autoantibody titers. *PLoS One* (2015) 10(6):e0130431. doi:10.1371/journal.pone.0130431
164. Mansfield JM, Paulnock DM. Regulation of innate and acquired immunity in African trypanosomiasis. *Parasite Immunol* (2005) 27(10–11):361–71. doi:10.1111/j.1365-3024.2005.00791.x
165. Mansfield JM. T-cell responses to the trypanosome variant surface glycoprotein: a new paradigm? *Parasitol Today* (1994) 10(7):267–70. doi:10.1016/0169-4758(94)90143-0
166. Stijlemans B, Vankrunkelsven A, Caljon G, Bockstal V, Guilliams M, Bosschaerts T, et al. The central role of macrophages in trypanosomiasis-associated anemia: rationale for therapeutical approaches. *Endocr Metab Immune Disord Drug Targets* (2010) 10(1):71–82. doi:10.2174/187153010790827966
167. Stijlemans B, Guilliams M, Raes G, Beschin A, Magez S, De Baetselier P. African trypanosomosis: from immune escape and immunopathology to immune intervention. *Vet Parasitol* (2007) 148(1):3–13. doi:10.1016/j.vetpar.2007.05.005
168. Hertz CJ, Filutowicz H, Mansfield JM. Resistance to the African trypanosomes is IFN-gamma dependent. *J Immunol* (1998) 161(12):6775–83.
169. Schopf LR, Filutowicz H, Bi XJ, Mansfield JM. Interleukin-4-dependent immunoglobulin G1 isotype switch in the presence of a polarized antigen-specific Th1-cell response to the trypanosome variant surface glycoprotein. *Infect Immun* (1998) 66(2):451–61.
170. Stijlemans B, Baral TN, Guilliams M, Brys L, Korf J, Drennan M, et al. A glycosylphosphatidylinositol-based treatment alleviates trypanosomiasis-associated immunopathology. *J Immunol* (2007) 179(6):4003–14. doi:10.4049/jimmunol.179.6.4003
171. Cnops J, Magez S, De Trez C. Escape mechanisms of African trypanosomes: why trypanosomosis is keeping us awake. *Parasitology* (2015) 142(3):417–27. doi:10.1017/S0031182014001838
172. Vincendeau P, Bouteille B. Immunology and immunopathology of African trypanosomiasis. *An Acad Bras Cienc* (2006) 78(4):645–65. doi:10.1590/S0001-37652006000400004
173. Paulnock DM, Coller SP. Analysis of macrophage activation in African trypanosomiasis. *J Leukoc Biol* (2001) 69(5):685–90.
174. Namangala B, De Baetselier P, Noel W, Brys L, Beschin A. Alternative versus classical macrophage activation during experimental African trypanosomosis. *J Leukoc Biol* (2001) 69(3):387–96. doi:10.1016/S0020-7519(01)00170-9
175. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, et al. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity* (2007) 27(4):597–609. doi:10.1016/j.immuni.2007.08.017
176. Lopez R, Demick KP, Mansfield JM, Paulnock DM. Type I IFNs play a role in early resistance, but subsequent susceptibility, to the African trypanosomes. *J Immunol* (2008) 181(7):4908–17. doi:10.4049/jimmunol.181.7.4908
177. Bakhiet M, Buscher P, Harris RA, Kristensson K, Wigzell H, Olsson T. Different trypanozoan species possess CD8 dependent lymphocyte triggering factor-like activity. *Immunol Lett* (1996) 50(1–2):71–80. doi:10.1016/0165-2478(96)02521-7
178. Schleifer KW, Filutowicz H, Schopf LR, Mansfield JM. Characterization of T helper cell responses to the trypanosome variant surface glycoprotein. *J Immunol* (1993) 150(7):2910–9.
179. Stijlemans B, Vankrunkelsven A, Brys L, Magez S, De Baetselier P. Role of iron homeostasis in trypanosomiasis-associated anemia. *Immunobiology* (2008) 213(9–10):823–35. doi:10.1016/j.imbio.2008.07.023
180. Stijlemans B, Leng L, Brys L, Sparkes A, Vansintjan L, Caljon G, et al. MIF contributes to *Trypanosoma brucei* associated immunopathogenicity development. *PLoS Pathog* (2014) 10(9):e1004414. doi:10.1371/journal.ppat.1004414
181. Vankrunkelsven A, De Ceulaer K, Hsu D, Liu FT, De Baetselier P, Stijlemans B. Lack of galectin-3 alleviates trypanosomiasis-associated anemia of inflammation. *Immunobiology* (2010) 215(9–10):833–41. doi:10.1016/j.imbio.2010.05.028
182. Stijlemans B, Vankrunkelsven A, Brys L, Raes G, Magez S, De Baetselier P. Scrutinizing the mechanisms underlying the induction of anemia of inflammation through GPI-mediated modulation of macrophage activation in a model of African trypanosomiasis. *Microbes Infect* (2010) 12(5):389–99. doi:10.1016/j.micinf.2010.02.006
183. Amin DN, Vodnala SK, Masocha W, Sun B, Kristensson K, Rottenberg ME. Distinct Toll-like receptor signals regulate cerebral parasite load and interferon alpha/beta and tumor necrosis factor alpha-dependent T-cell infiltration in the brains of *Trypanosoma brucei*-infected mice. *J Infect Dis* (2012) 205(2):320–32. doi:10.1093/infdis/jir734
184. Zeni P, Doepker E, Schulze-Topphoff U, Huewel S, Tenenbaum T, Galla HJ. MMPs contribute to TNF-alpha-induced alteration of the blood-cerebrospinal fluid barrier in vitro. *Am J Physiol Cell Physiol* (2007) 293(3):C855–64. doi:10.1152/ajpcell.00470.2006
185. Hanemaaijer R, Koolwijk P, le Clercq L, de Vree WJ, van Hinsbergh VW. Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. Effects of tumour necrosis factor alpha, interleukin 1 and phorbol ester. *Biochem J* (1993) 296(Pt 3):803–9. doi:10.1042/bj2960803
186. Masocha W, Robertson B, Rottenberg ME, Mhlanga J, Sorokin L, Kristensson K. Cerebral vessel laminins and IFN-gamma define *Trypanosoma brucei* brucei penetration of the blood-brain barrier. *J Clin Invest* (2004) 114(5):689–94. doi:10.1172/JCI22104
187. Amin DN, Rottenberg ME, Thomsen AR, Mumba D, Fenger C, Kristensson K, et al. Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. *J Infect Dis* (2009) 200(10):1556–65. doi:10.1086/644597
188. Frevert U, Movila A, Nikolskaia OV, Raper J, Mackey ZB, Abdulla M, et al. Early invasion of brain parenchyma by African trypanosomes. *PLoS One* (2012) 7(8):e43913. doi:10.1371/journal.pone.0043913

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Malaria-Cutaneous Leishmaniasis Co-infection: Influence on Disease Outcomes and Immune Response

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Malaria and Cutaneous Leishmaniasis (CL) are co-endemic throughout large regions in tropical countries and co-infection may impact the evolution of host-parasite interactions. In the present study, we evaluate Malaria/Leishmaniasis disease outcome, Th1/Th2 cytokine levels and the CD4 and CD8 T-cell profiles in a co-infection murine model (BALB/c) of *Plasmodium yoelii* 17XNL (Py) and *Leishmania amazonensis* (La) or *L. braziliensis* (Lb). Malaria parasitaemia was assessed through blood strains stained with Giemsa. *Leishmania* lesions were monitored with a digital caliper and parasite loads determined by limiting-dilution assay. Serum levels of IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10, and IL-17 were determined using multiplexed bead assay and expression of CD3, CD4, and CD8 T-cells markers were determined by Flow Cytometry in the thymus, spleens and lymph nodes. Parasitaemia in Lb+Py co-infected group was lower than in Py single-infected group, suggesting a protective effect of Lb co-infection in Malaria progression. In contrast, La+Py co-infection increased parasitaemia, patent infection and induced mortality in non-lethal Malaria infection. Regarding Leishmaniasis, Lb+Py co-infected group presented smaller lesions and less ulceration than Lb single-infected animals. In contrast, La+Py co-infected group presented only a transitory delay on the development of lesions when compared to La single-infected mice. Decreased levels of IFN-γ, TNF, IL-6, and IL-10 were observed in the serum of co-infected groups, demonstrating a modulation of Malaria immune response by *Leishmania* co-infections. We observed an intense thymic atrophy in Py single-infected and co-infected groups, which recovered earlier in co-infected animals. The CD4 and CD8 T cell profiles in thymus, spleens and lymph nodes did not differ between Py single and co-infected groups, except for a decrease in $CD4^+CD8^+$ T cells which also increased faster in co-infected mice. Our results suggest that Py and *Leishmania* co-infection may change disease outcome. Interestingly Malaria outcome can be altered according to the *Leishmania* specie involved. Alternatively Malaria infection reduced the severity or delayed the onset of leishmanial lesions. These alterations in Malaria and CL development seem to be closely related with changes in the immune response as demonstrated by alteration in serum cytokine levels and thymus/spleens T cell phenotypes dynamics during infection.

Keywords: co-infection, Malaria, Leishmaniasis, immunology, cytokine, *Plasmodium yoelii*, *Leishmania braziliensis*, *Leishmania amazonensis*

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INTRODUCTION

Malaria and Cutaneous Leishmaniasis (CL) are two of the world's most important vector-borne parasitic diseases (World Health Organization, 2010, 2015; Alvar et al., 2012). Malaria, an infectious disease caused by *Plasmodium* genus parasites, is an important cause of global mortality and morbidity. Half of the world population is at risk of contracting Malaria, with approximately 214 million cases and 438 000 deaths in 2015, amongst the 3.2 billion people living at risk of infection (World Health Organization, 2015). Humans can be infected by five *Plasmodium* species: *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi*. Although *P. falciparum* accounts for the great majority of morbidity and mortality, *P. vivax* has a wider geographic distribution and causes considerable symptomatic disease (Battle et al., 2014). Malaria infection has a variable clinical phenotype, ranging from a mild febrile illness to severe disease and death, but infection can also occur in the absence of clinical symptoms. These variations in disease pattern are attributable to many factors, including the genetic background of the host and pathogen, the complex relationship between the parasite and host immune response, the dynamics of parasite transmission and/or the biological interactions of the parasites within the host (Good and Doolan, 2010; van den Bogaart et al., 2012). Leishmaniasis is a complex disease caused by different species of intracellular protozoan parasites from the genus *Leishmania*, which also induces significant morbidity and mortality throughout the world. According to the World Health Organization (2016) 350 million people in 98 countries are at risk of infection. There are an estimated annual 1.3 million new cases worldwide, of which 300,000 cases are of Visceral Leishmaniasis (VL) and another 1 million of CL. Seventy (70) to 75% of the CL cases occur in Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Peru, Sudan, and Syria (World Health Organization, 2016). CL is caused by *L. major*, *L. tropica*, and *L. aethiopica* in the Old World, whereas in the New World it is most frequently caused by *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, and *L. guyanensis* (World Health Organization, 2010; Hartley et al., 2014). Symptoms range from the more prevalent single self-healing cutaneous lesions to uncontrolled parasite replication, producing non-healing cutaneous, mucosal or even visceral disease as well as chronic metastatic dissemination throughout the skin. This spectrum of manifestations is multifactorial and depends on complex interactions among parasite, host, and environmental factors, including the *Leishmania* specie, genetic background and immunological status of the host (Hartley et al., 2014).

The overlapping geographic distribution of Malaria and Leishmaniasis, especially in the tropical and subtropical countries demonstrate clearly that the potential for interaction among these parasites may occur and play a role in determining disease outcome (Hotez et al., 2006; van den Bogaart et al., 2012, 2014). Despite this natural coexistence, data from concomitant infections are so far not available in the literature (Ab Rahman and Abdullah, 2011; van den Bogaart et al., 2012, 2014). Therefore, the impact of the dual infections on the human population health remains unassessed particularly in what concerns CL.

In the eighties, two studies in the murine model evaluated the effect of non-lethal *P. yoelii* and *L. amazonensis* concomitant infections in the course of each disease (Coleman et al., 1988a,b). These studies demonstrated that severity and susceptibility to both diseases were enhanced during co-infection. Since these parasites do not compete for the same host cells, neither anatomical sites nor resources, the interaction among them is most likely indirect and related mostly to the host's immune responses induced by each pathogen. However, in both studies (Coleman et al., 1988a,b) the immunological mechanisms involved were not investigated. Alternatively, several studies have shown that outcome of Malaria and CL is determined, in part, by the balance of pro- and anti-inflammatory immune responses (Louis et al., 1998; Li et al., 2001; Silveira et al., 2009; Taylor-Robinson, 2010; Medina et al., 2011; Freitas do Rosario and Langhorne, 2012; Kedzierski and Evans, 2014).

The immune response against the blood stages of Malaria parasites operate in concert and sequentially to control and clear the parasitaemia by an early and strong pro inflammatory, type 1 response (Th1), limiting parasite growth, followed by a shift to anti-inflammatory, type 2 immune (Th2) response. The balance between cytokines produced by pro inflammatory and anti-inflammatory responses during different phases of the blood stage infection determines the outcome of the disease (Li et al., 2001). In contrast to the strong type 1 immune response at the beginning of the Malaria infection, CL caused by *L. amazonensis* generally results in type 2 cellular immune response polarization (Silveira et al., 2009; Gollob et al., 2014). On the other hand, in CL caused by *L. braziliensis*, there is evidence that increased production of inflammatory cytokines (IFN-γ and TNF-α) and absence of IL-10 is associated with tissue destruction and the development of mucosal lesions (Dutra et al., 2011; Gollob et al., 2014; Oliveira et al., 2014). In this context, the current study was designed to evaluate the outcome of Malaria-CL infections, the CD4 and CD8 T-cell profiles as well as the Th1/Th2 cytokine levels in a co-infection murine model (BALB/c) of *P. yoelii* 17XNL (non-lethal) and *L. amazonensis* or *L. braziliensis*. Furthermore, as far as we know, this is the first report utilizing *L. braziliensis* and *P. yoelii* 17XNL non-lethal strain as a co-infection model.

MATERIALS AND METHODS

Animals, Parasites, and Infections

Female BALB/c mice (5–6 weeks old) were obtained from the Center for Laboratory Animal Breeding of the Oswaldo Cruz Foundation (FIOCRUZ) (Rio de Janeiro, RJ, Brazil). Mice were maintained under specific pathogen-free conditions in Experimental Animal Center of Leônidas Deane building (FIOCRUZ). This research protocol was approved by the Ethical Committee for Animal Use of FIOCRUZ/MS (license LW-17/11).

P. yoelii 17XNL (non-lethal strain) was provided by Dr Fábio T.M. Costa at Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, UNICAMP, Campinas, SP, Brazil. Parasite

stabilates were stored at −196°C. To obtain the experimental inoculum of *P. yoelii* 17XNL, parasitized red blood cells (pRBCs) were defrosted and passed through three homologous donor mice.

Promastigotes of *L. braziliensis* (MCAN/BR/98/R69) and *L. amazonensis* (IFLA/BR/67/PH8), provided by Laboratório de Pesquisa em Leishmaniose, Instituto Oswaldo Cruz, FIOCRUZ, RJ, Brazil, were cultured in Schneider's medium supplemented with antibiotics (200 IU penicillin and 200 μg streptomycin/ml) and 10% inactivated fetal calf serum (all from Sigma–Aldrich, St Louis, MO, USA). For *L braziliensis* 2% of sterile human urine was also added to the cultures (Howard et al., 1991).

Eight to nine-week-old mice (∼22g each) were divided randomly into six groups, **Figure 1**. Group C: comprised uninfected mice; Group Py: mice were injected intraperitoneally (i.p.) with 10^6 *P. yoelii* 17XNL pRBCs (0.2 mL); Groups Lb and La: mice were inoculated intradermally in both ears with either 10^5 or 10^4 stationary phase promastigotes of *L. braziliensis* or *L. amazonensis,* respectively, as previously described (Belkaid et al., 2000); Groups Lb+Py and La+Py: mice were co-infected with both *P. yoelii* 17XNL and either *L. braziliensis* or *L. amazonensis*. Each group had 25 to 35 mice and experiments were repeated at least three times. First, mice were infected with *Leishmania* sp. and 3 days later with *P. yoelii* 17XNL, as shown in **Figure 1**. At 5, 10, 17, and 25 days post-*P. yoelii* 17XNL infection, six animals from each group were bled, for serum cytokine assay, and euthanized, for thymus, spleen, ears and lymph nodes removal. Mortality of mice was monitored daily, post-*P. yoelii* 17XNL infection.

Determination of Parasite Load, Leishmania Lesion Size, and Ulceration

Malaria parasitaemia was monitored every 2 days starting at day 3 post-infection. Blood smears were prepared from tail vein, methanol-fixed, stained with Giemsa and microscopically examined to determine parasitaemia in 1,000 erythrocytes. The percentage of infected erythrocytes was calculated as follows: Parasitaemia (%) = (number of infected erythrocytes x 100)/total number of erythrocytes counted (1,000).

The diameter of dermal *Leishmania* sp. lesions were measured weekly with a digital calipter. We also monitored the time elapsed until ulcer formation in nodules. Parasite numbers in the ears and draining lymph nodes were determined as previously described (Belkaid et al., 2000) and scored as the highest dilution containing viable parasites after incubation for 6 days at 26°C.

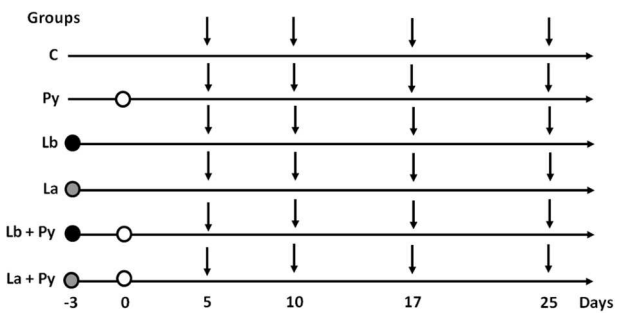

FIGURE 1 | Experimental design. Mice were infected with *Leishmania braziliensis*. (black circles) or *L. amazonensis* (gray circles) 3 days before *Plasmodium yoelii* infection (white circles). On days 5, 10, 17, and 25 post *P. yoelii* infection (vertical arrows), six mice from Py, Lb, La, Lb+Py, and La+Py groups and four mice from control (C) group were euthanized to evaluate parasite loads in the ears and draining lymph nodes (Lb/La and Lb+Py/La+Py groups), cytokine serum levels (all groups), thymus relative weight (all groups), absolute numbers of thymocytes (all groups) and phenotyping of T cells in the thymus, spleen and lymph nodes (all groups).

Cytokines Quantitation in the Serum

The serum levels of cytokines (interferon – IFN-γ, tumor necrose factor – TNF, interleukin (IL)-2, IL-4, IL-6, IL-10, and IL-17) were determined using the BD Cytometric Bead Array Kit Mouse Th1/Th2/Th17 Cytokines (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, 25 μL of mouse serum was incubated for 2 h at room temperature with 25 μL of cytokine capture bead and 25 μL of phycoerythrin (PE) detection reagent. Then, samples were washed with 1 mL of buffer by centrifugation (200 × *g*, 5 min). The supernatants were carefully aspirated and discarded from each assay tube. Finally, beads were resuspended in 300 μL of buffer for analysis on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The serum cytokine concentrations (pg/mL) were determined using a standard curve from recombinant cytokines provided by the kit.

Determination of Thymus Relative Weight

For determination of thymus relative weight, body and thymus of each mouse were weighted. Thymic index (TI) was calculated according to the following formula: TI = thymus weight (mg) / body weight (g).

T Cell Phenotyping by Flow Cytometry

Thymus, spleens, mesenteric nodes, subcutaneous nodes (cervical, axillary, brachial, and inguinal) and submandibular nodes (draining ears lesions) were dissected and mechanically disaggregated. Single-cell suspensions from control, infected and co-infected mice were obtained in RPMI-1640 supplemented with 10% fetal calf serum (Gibco-Invitrogen, USA). Cell numbers and viability were determined by Trypan blue exclusion using Neubauer chamber.

For T lymphocytes phenotypic characterization, 10^6 cells were stained with FITC labeled anti-mouse CD4, APC labeled anti- mouse CD8 and PE labeled anti-mouse CD3 monoclonal antibodies. After incubation for 30 min, followed by 2 washes with staining buffer (PBS, 0,1% BSA, 0,01% sodium azide, all from Sigma–Aldrich), cells were fixed with 1% paraformaldehyde solution (Sigma–Aldrich), washed and resuspended in staining buffer until acquisition. A minimum of 30,000 events per sample were acquired inside the lymphocytes gate, based on size and granularity properties using a FACSCantoII™ flow cytometer (BD Bioscience, USA) and analyzed using FlowJo 7.5.5 software (Tree Star Inc. Ashland, OR, USA).

Statistical Analysis

Sample size was determined *a priori* using the software G*Power version 3.1.9.2, based on the Mann–Whitney test, a significance level of 0.05, and a minimum power of 80%. Results were evaluated by a non-parametric test, the Mann–Whitney test, using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA). Data are expressed as means ± SEM and considered to be statistically significant when $p < 0.05$. Survival curves were analyzed by Mantel Cox and Gehan–Breslow–Wilcoxon tests and *Leishmania* lesions ulcerations were analyzed by Chi-square test using the same software.

RESULTS

L. braziliensis and *L. amazonensis* co-Infection exerts opposite effects on *P. yoelii* 17XNL Infection

Blood stage *P. yoelii* 17XNL parasites induced Malaria infection in all BALB/c mice from single-infected (Py) and *L. braziliensis* or *L. amazonensis* co-infected groups (Lb+Py and La+Py). Parasitaemia peak occurred between 10 and 17 days after infection and no parasite was detected after 23 days post-infection in blood smears of Py single-infected mice. The overall mean parasitaemia observed in Lb+Py co-infected group was lower than that observed in *P. yoelii* single-infected group (Py). Statistically significant differences observed in some days are suggestive for a protective effect of *L. braziliensis* co-infection in Malaria progression (**Figure 2A**). In contrast, co-infection with *L. amazonensis* appears to have a negative influence in acute Malaria (**Figure 2C**), and mortality in La+Py co-infected group enhanced in comparison to Py single infected group (**Figure 2D**). La+Py group exhibited higher parasitaemia on day 5 and a longer course of *P. yoelii* Malaria patency with mice remaining parasitemic 8 days longer than mice in Py single-infected group (**Figure 2C**). The higher rate of parasitemia in La+Py coinfeted mice seems to be associated with decreased survival. Four weeks after *P. yoelii* 17XNL infection, 30% of the co-infected La+Py mice were dead, compared to 100% of survival in the group infected only with *P. yoelii* 17XNL. Py single-infected groups showed no mortality during the whole experiment (**Figures 2B,D**).

Co-Infection is Able to Reduce Severity of *L. braziliensis* Lesions

BALB/c mice were intradermally infected with 10^5 stationary phase promastigotes of *L. braziliensis*. Lb+Py co-infected group showed an overall tendency to present smaller lesion sizes than Lb single infected group (**Figure 3A**). In both groups, lesions reached maximum size 5 weeks post-infection and started to heal afterward. To evaluate disease severity we followed the onset of open ulcers in *Leishmania*-infected and co-infected groups (**Figure 3B**). Ulcers started to appear 21 days post-infection in Lb group and after 28 days in Lb+Py. At week 11 after infection (77 days) Lb single-infected group presented a higher number of ulcerated nodules (83.3%) than Lb+Py (40.9%). At week 17 and 18 post infection, there was a complete resolution of lesions in Lb+Py and Lb groups, respectively (data not shown). Limiting-dilution assays performed in the ears and in draining lymph nodes showed an earlier detection of parasites in the ears of Lb single-infected animals than in co-infected group (14 and 21 days post-infection, respectively) with no difference in parasite loads between groups. On the other hand, in the draining lymph nodes parasites were detected 28 days after infection in both groups, and parasite loads were lower in Lb+Py co-infected group ($p = 0.006$, **Figures 3C,D**).

Co-Infection Reduce Severity of *L. amazonensis* Lesions Only during Acute Phase Malaria

Infection of BALB/c mice with 10^4 stationary phase promastigotes of *L. amazonensis* induced chronic lesions that did not heal over time in La and La+Py groups (**Figure 4A**). During murine Malaria (from day 0 to day 25) lesions in La+Py co-infected group were significantly smaller than in La single infected group. Although lesions of La group presented greater sizes than La+Py until 28 days of infection, after that time and until the end of the experiment both groups present similar lesion sizes. Ulcerated nodules were detected first in La group (21 days post-infection) while in La+Py group visual detection occurred 1 week later (28 days post-infection) (**Figure 4B**). However, after 63 days of infection all animals from both groups presented ulcerated nodules. Limiting dilution analysis performed at the site of infection and in draining lymph nodes showed higher parasite loads in La+Py group than La single-infected group (**Figures 4C,D**).

Co-Infection Modulate Serum Cytokine Levels Induced by *P. yoelii* 17XNL

Cytokine levels were measured in serum samples obtained from 4 to 6 animals of each experimental group. As expected, increased levels of the proinflammatory cytokine IFN-γ, were observed in all groups infected with *P. yoelii* 17XNL (Py, Lb+Py, and La+Py) in the first week of infection (day 5). The levels of TNF, IL-6 and IL-10 also increased in the serum of the same three groups during blood stage Malaria. Cytokine levels in co-infected groups (Lb+Py and La+Py) were clearly lower than those observed in Malaria single infected group (Py) suggesting a modulatory effect of co-infection (**Figure 5**). We were not able to detect any of the analyzed cytokines in serum samples from control and Lb or La single infected animals, except for IL-10 in La infected group, only at day 25th post infection. Although we could observe a tendency for an increased production of TNF, IL-6 and IL-10 in the first 10 days of Malaria infection for Lb+Py co-infected group when compared to La+Py co-infected animals, statistic difference was observed only for IL-6 at day 10 post Py infection ($P = 0.045$). We were not able to detect the cytokines IL-2, IL-4, and IL-17 in serum

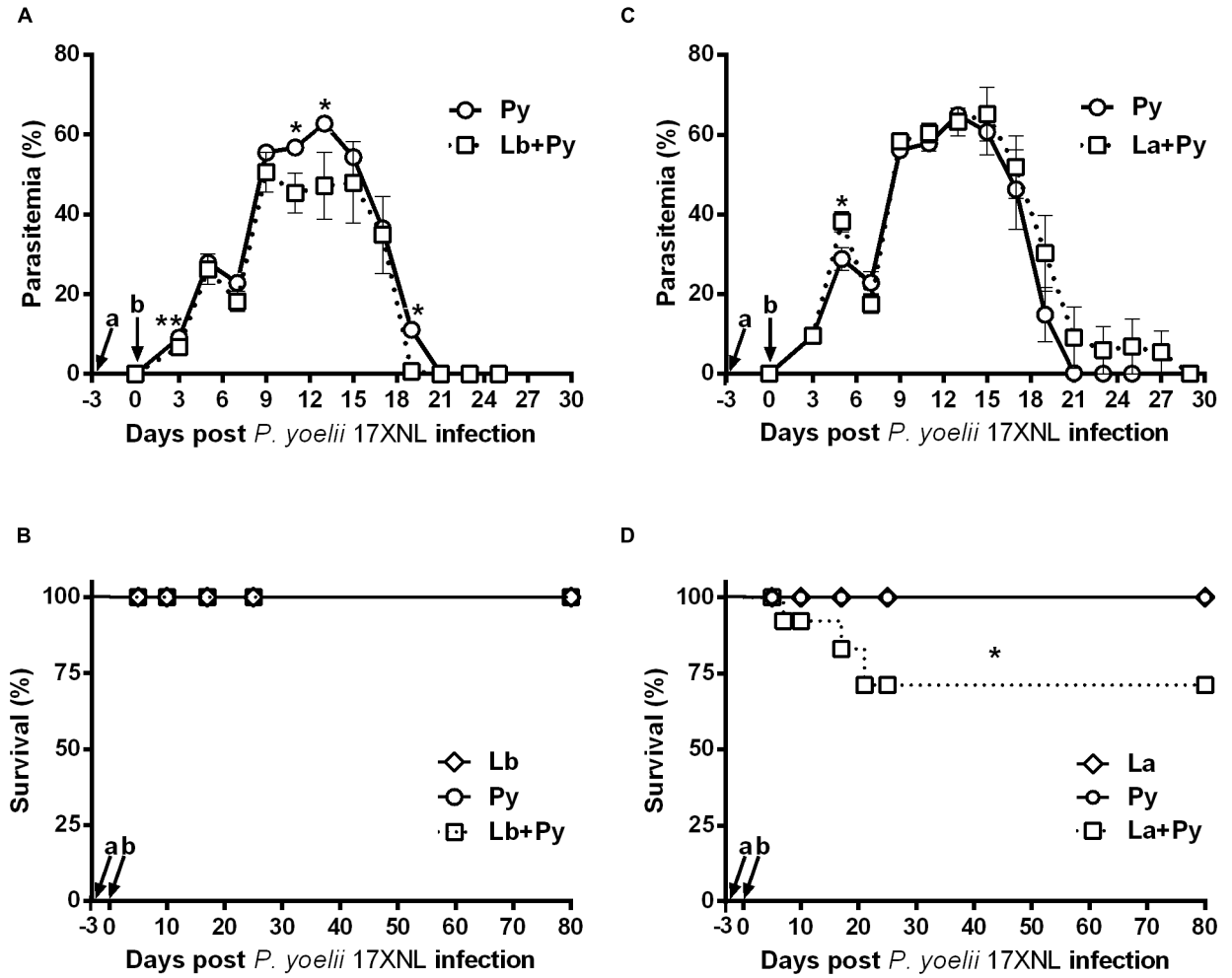

FIGURE 2 | Parasitaemia and survival rate of BALB/c mice infected with blood-stage *P. yoelli* 17XNL non-lethal strain and co-infected with *Leishmania* sp. and *P. yoelii* 17XNL. (A,C) Malaria parasitaemia in *P. yoelii* single-infected mice (Py) and in mice co-infected with *L. braziliensis/P. yoelii* (Lb+Py) (A) or with *L. amazonensis/P. yoelii* (La+Py) **(C)**. **(B,D)** Comparison of the survival rate in Py single-infected and Lb+Py co-infected **(B)** or La+Py co-infected mice **(D)**. Parasitaemia results are expressed by the mean ± SEM of at least 10 mice per group. The Mann–Whitney test was used to compare Py single-infected and co-infected groups. Survival rates were compared by Mantel-Cox test. $*p < 0.05$ and $**p < 0.01$, respectively. Arrows indicate the time of *Leishmania* (a) and *P. yoelii* (b) infection.

samples from all experimental groups, at any time of the study.

Recovery of Thymic Atrophy Occurs Earlier in Co-Infected Animals

In the course of Malaria infection, we observed a significant reduction of the thymus relative weight in comparison to the uninfected control group. Thymic atrophy was first observed 10 days post *P. yoelii* infection (dpi) and persisted until day 25th of infection in Py single-infected group. In co-infected groups (Lb+Py and La+Py) thymus relative weight came back to normal values at 25 day post-infection and no significant difference could be observed in comparison to the control group. During the entire experiment, *Leishmania* single-infected groups showed no differences in thymus relative weight when compared do the uninfected control group (**Figure 6**).

Flow cytometry analysis showed that the thymus of Py and co-infected groups underwent a decrease in $CD4^+CD8^+$ double-positive (DP) T cell subset and an increase in $CD4^+$ single-positive (SP), $CD8^+$SP and $CD4^-CD8^-$ double-negative (DN) T cell populations (**Figure 7**). A significant increase in $CD4^+$SP thymocytes occurred at 17 dpi and persisted up to 25 dpi in Py single-infected animals. In Lb+Py group, $CD4^+$SP thymocytes increased 10 days post infection, and returned to control values by day 25th post infection. In La+Py this transitory increase was observed latter at 17 dpi. The dynamics of $CD8^+$SP thymocytes in single infected and co-infected groups exhibited the same profile of $CD4^+$SP. When we evaluate this results together, it is possible to notice that in Lb+Py co-infection, the increase in $CD4^+$SP and $CD8^+$SP occurred sooner than in La+Py group (10

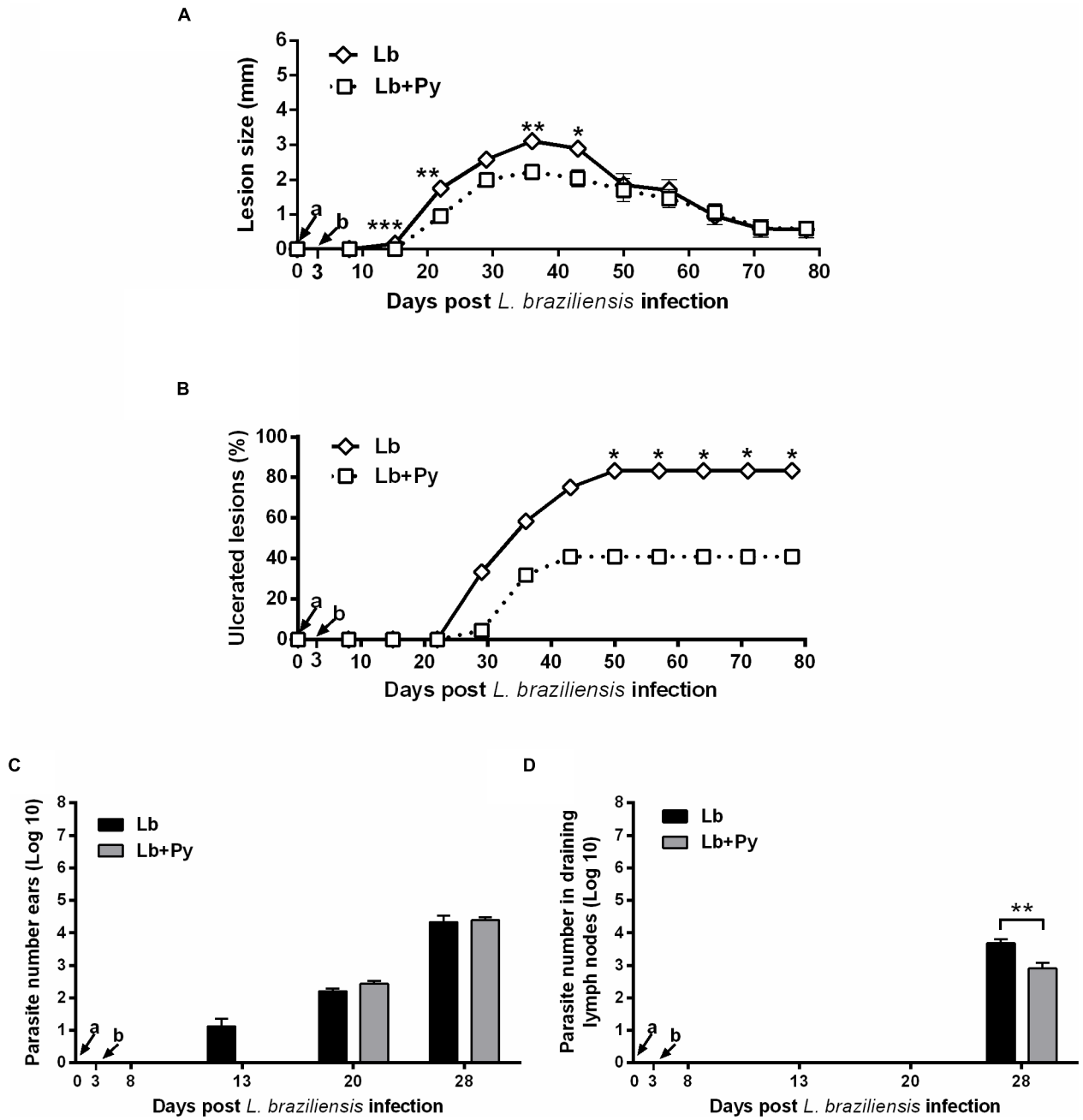

FIGURE 3 | Evaluation of *L. braziliensis* infection. (A) Lesion development, **(B)** onset of open ulcers, and **(C)** parasite quantitation in infected ears and **(D)** draining lymph nodes of BALB/c mice after intradermal infection in the ears with *L. braziliensis* promastigotes (Lb) or co-infected with *L. braziliensis* and *P. yoelii* (Lb+Py). Parasite loads were individually determined by a limited dilution assay. Results are expressed by the mean ± SEM of twelve ears and lymph nodes. The Mann–Whitney test was used to compare lesion size **(A)** and parasite loads in ears **(C)** and draining lymph nodes **(D)**. Chi-square test was used to evaluate ulcerations **(B)**. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively. Arrows indicate the time of *L. braziliensis* (a) and *P. yoelii* (b) infection.

and 17 dpi, respectively). Double-negative thymocytes increased in Py single-infection at 17 and 25 dpi. The same increase occurred in co-infected groups only at day 17 post infection. In parallel, $CD4^{+}CD8^{+}$ DP cells significantly decreased in the thymus of Py single infected animals at 17 dpi and remained lower at 25 dpi. This decrease was observed earlier is Lb+Py group (10 dpi) but, at day 25th post infection, DP cells increased in this group and were higher than in control and single infected groups. Interestingly, in La+Py co-infected group, the increase in DP thymocytes occurred earlier (10 dpi) and was followed by a rapid decrease at day 17th post infection, coming back to control values 25 dpi. Overall, thymus recovery seems to

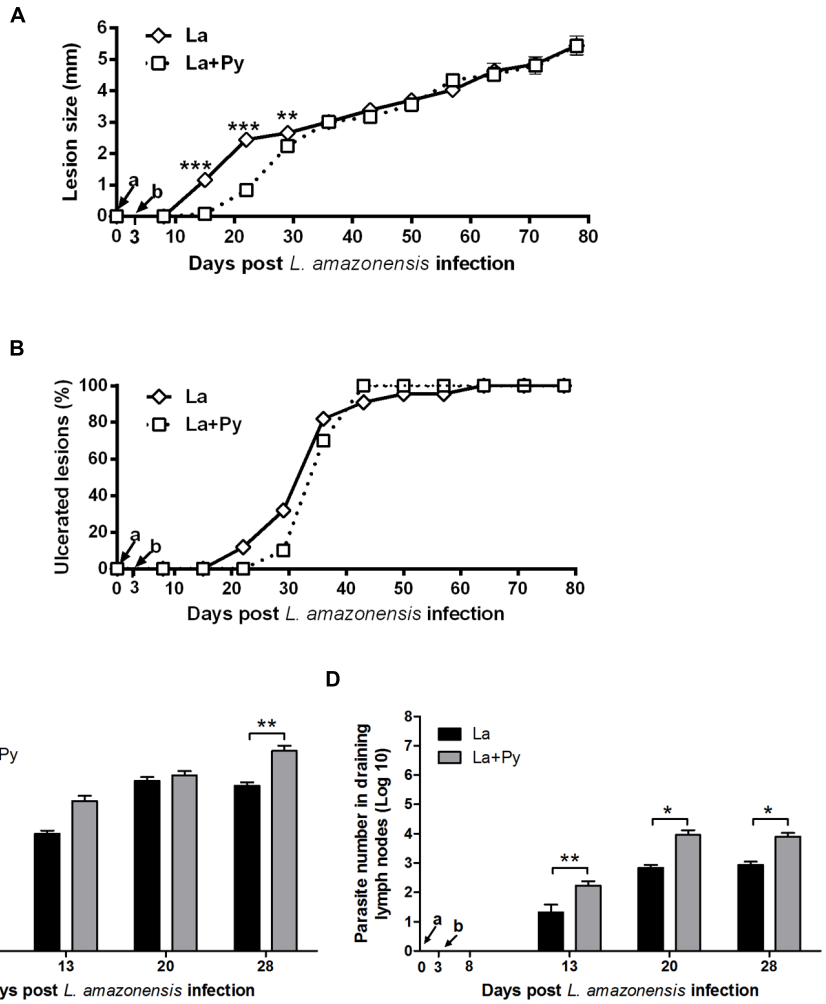

FIGURE 4 | Evaluation of *L. amazonensis* infection. (A) Lesion development, **(B)** onset of open ulcers, and **(C)** parasite quantitation in infected ears and **(D)** draining lymph nodes of BALB/c mice after intradermal infection in the ears with *L. amazonensis* promastigotes (La) or co-infected with *L. amazonensis* and *P. yoelii* (La+Py). Parasite loads were individually determined by a limited dilution assay. Results are expressed by the mean ± SEM of twelve ears and lymph nodes. The Mann–Whitney test was used to compare parasite loads in ears and draining lymph nodes. $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively. Arrows indicate the time of *L. braziliensis* (a) and *P. yoelii* (b) infection.

occur faster in Malaria/*Leishmania* co-infection than in Py single infection.

Leishmania Co-Infection Delay the Enhancement of Double Negative Cells Induced by *P. yoelii* 17XNL Infection in the Spleen

We also evaluated the T cell phenotypes present in the spleens, mesenteric, subcutaneous and submandibular nodes (the last ones as the draining nodes from ears lesions) in single infected and co-infected animals. No alterations were observed in the T cell phenotypes present in subcutaneous and submandibular nodes among the studied groups, while in mesenteric nodes and spleens we were able to observe similar patterns of alterations that were more evident in the spleens (**Figure 8**). After a transitory increase at 10 dpi in Py single infected and in co-infected groups, $CD4^+$ T cells dropped in Py single infected group at 17 dpi and remained bellow controls at 25 dpi. In Lb+Py co-infected group a significant decrease was observed only at 25 dpi. Py single infected animals also presented a significant drop in the percentages of $CD8^+$T cells. In co-infected groups, the percentages of $CD8^+$T cells were bellow controls at 10, 17, and 25 dpi. Low percentages of DP splenocytes were observed in Py single infected and co-infected groups, which were different form controls only when animals started to recover from malarial infection (17 and 25 dpi). On the other hand, *P. yoelii* infection increased the percentage of DN splenocytes during the entire experiment, and *Leishmania* co-infection was able to delay this enhancement. The percentages of CD4, CD8, DP, and DN splenocytes in *Leishmania* single infected groups (both Lb and La) remained similar to uninfected controls at all time points.

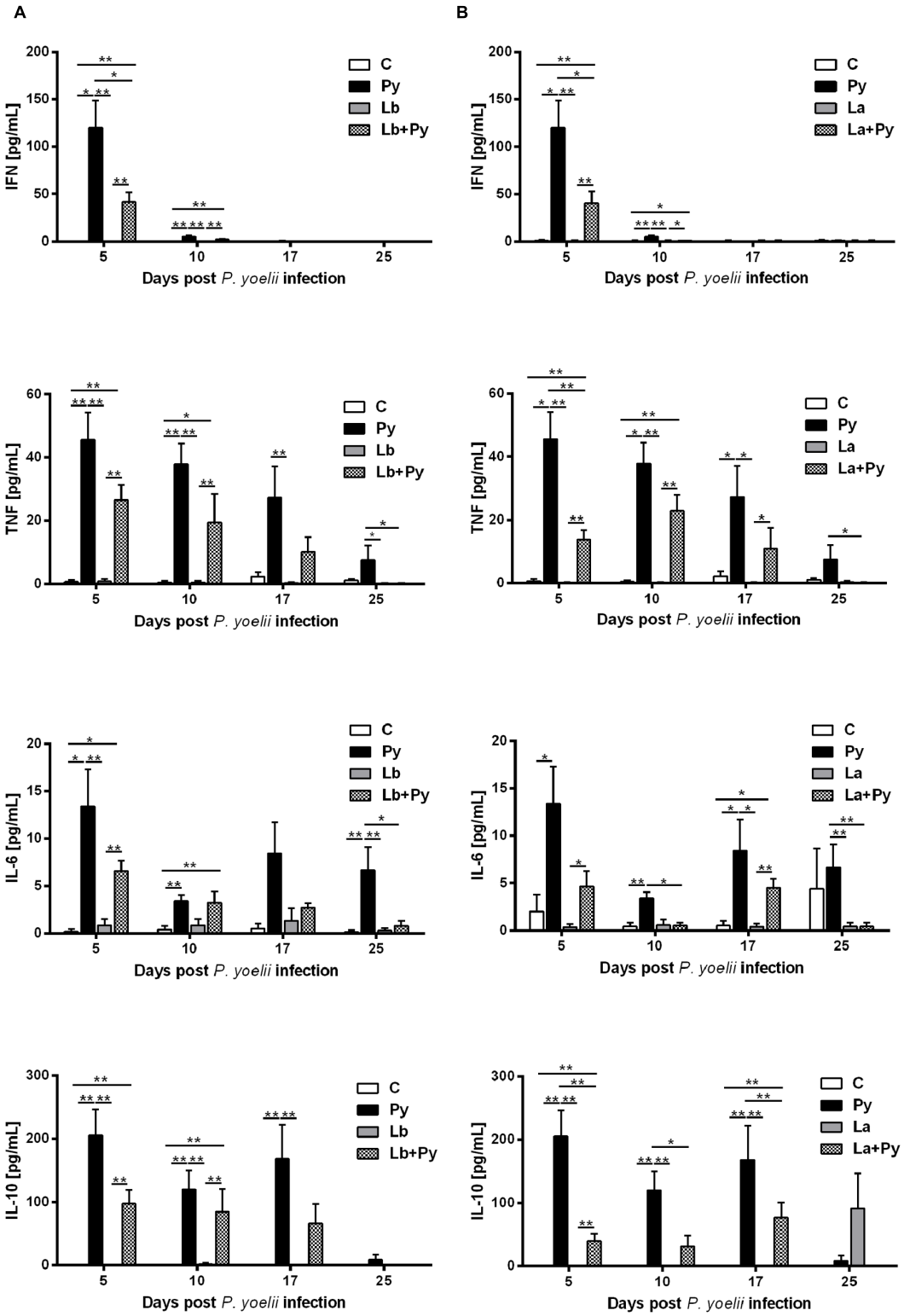

FIGURE 5 | Kinetics of cytokine levels in the serum of mice infected with *P. yoelii* (Py), *L. braziliensis* (Lb), *L. amazonensis* (La) or co-infection with *Leishmania* sp. and *P. yoelii*. Serum levels of IFN, TNF, IL-6, and IL-10 were determined by Cytokine Bead Array. Cytokine levels obtained in the serum of non-infected controls (open bars) were compared to those obtained in Py single-infected mice (black bars) and in animals co-infected (checkered bars) or single infected (gray bars) with *L. braziliensis* **(A)** or *L. amazonensis* promastigotes **(B)**. Results are expressed by the mean ± SEM of 4–6 mice per group. The Mann–Whitney test was used to compare serum cytokine levels between groups. $*p < 0.05$ and $**p < 0.01$, respectively.

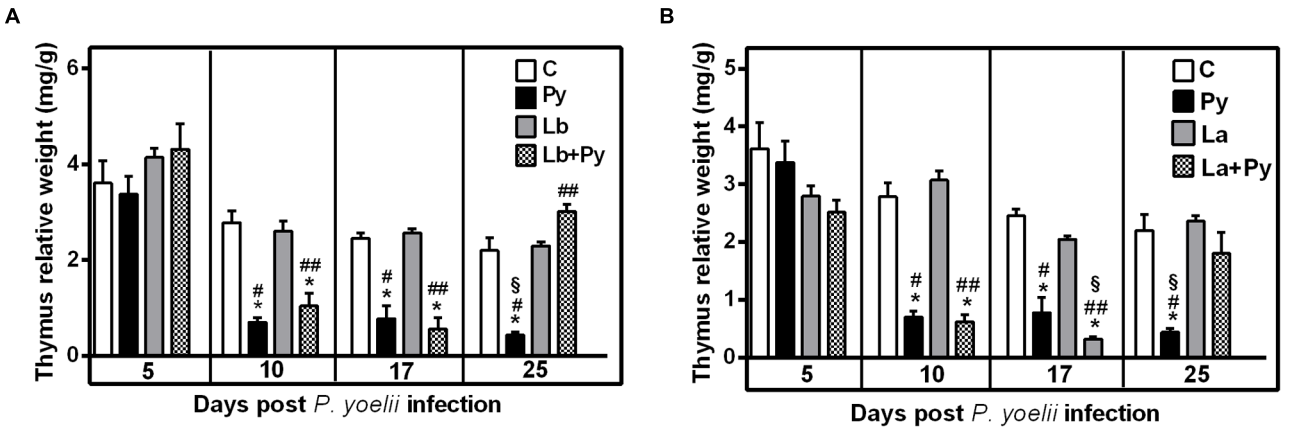

FIGURE 6 | Alterations in the thymus relative weight after infection with *P. yoelii* (Py), *L. braziliensis* (Lb), *L. amazonensis* (La), or co-infection with *Leishmania* sp. and *P. yoelii*. Thymus relative weight in uninfected controls (open bars) were compared to those obtained in Py single-infected mice (black bars) and in animals co-infected (checkered bars) or single infected (gray bars) with *L. braziliensis* **(A)** or *L. amazonensis* promastigotes **(B)**. Results are expressed by the mean ± SEM of 4–6 mice per group. The Mann–Whitney test was used to compare thymus relative weight between groups. (*) statistically different from control, (#) statistically different from *Leishmania* single infection and (§) statistically different from *Leishmania/P. yoelii* co-infected group.

DISCUSSION

Malaria and CL are neglected diseases spread over many tropical and subtropical regions of the World showing extensive overlapping geographic distribution. In addition, the increase in travel, migration and war refugees from endemic regions are factors that also contribute to the risk of simultaneous infections by *Plasmodium* spp. and *Leishmania* spp. parasites. Some cases of human Malaria and VL co-infection were described in the past and recently in different parts of the world (Yoeli, 1948; Ab Rahman and Abdullah, 2011; van den Bogaart et al., 2012, 2014; Bin Mohanna, 2015), demonstrating that co-infection may occur more frequently than we would expect. An increasing amount of evidence shows that co-infections may affect the natural outcome and progression of diseases due to the modulation of immune response (La Flamme et al., 2002; Kolbaum et al., 2012; Qi et al., 2013; van den Bogaart et al., 2014). In the present study, we established a co-infection model of American CL and a non-lethal murine Malaria and demonstrated that co-infection is able to affect the pathogenesis and outcome of both diseases.

Pilot studies were carried out in our Laboratory in which mice were co-infected at different times and orders. It was observed that the outcome of malaria or *Leishmania* infections were the same in any time and order of infections tested. For operational reasons we selected day 3 for Py infection. In addition, our results regarding the outcome of both diseases are similar to those published by Coleman et al. (1988a,b) in *P. yoelii* and *L. amazonensis* coinfection experiments.

Rodent Malaria parasites provide great models to investigate pathogenesis and immune mechanisms during infection (Li et al., 2001; Zuzarte-Luis et al., 2014). The non-lethal strain 17XNL of *P. yoelii* generally causes a transient disease with moderate parasitaemia, weight loss, splenomegaly, hypothermia, and anemia (Coleman et al., 1988b; Kobayashi et al., 1996; Niikura et al., 2008; Kolbaum et al., 2012; Karadjian et al., 2014). In our study, BALB/c mice infected with blood-stage *P. yoelii* 17XNL develop a self-limiting infection with mild parasitaemia that resolve in 4 weeks. Prior infection with *L. braziliensis* reduced parasitaemia, while animals previously infected with *L. amazonensis* showed a tendency to have higher parasitaemia than Py single infected group. Mortality during acute Malaria increased with *L. amazonensis* co-infection, demonstrating that the existence of intrinsic differences between this two *Leishmania* species during co-infection, may affect animal survival.

The course of experimental CL is dependent on a combination of factors: *Leishmania* and host species, parasite strain, site, and size of the inoculum among other aspects (Pereira and Alves, 2008; Pereira et al., 2009; Ribeiro-Gomes et al., 2014). The murine model of subcutaneous infection with *L. major* generated a great amount of data concerning the immune response in CL. It was in this model that could be demonstrated the importance of a type 1 (proinflammatory) specific immune response for healing the infection in genetically resistant strains of mice (such as C57BL/6), as well as the dominance of a type 2 immune response (with a strong production of IL-4 and IL-10) in susceptible BALC/c mice. On the other hand, all strains of mice that have been tested so far using the subcutaneous route of infection are susceptible to *L. amazonensis,* and non-permissive hosts for *L. braziliensis*.

The intradermal rout of infection with low doses of parasites in the ears, first developed for *L. major* infection (Belkaid et al., 2000), proven also to be an important experimental model for *L. braziliensis* studies. After intradermal infection in the ears with *L. braziliensis* promastigotes BALB/c are able to develop ulcerated lesions that heal spontaneously, similar to the most common clinical manifestation of American Tegumentary Leishmaniasis caused by *L. braziliensis*: the localized cutaneous form (de Moura et al., 2005; Falcão et al., 2012; Carregaro et al., 2013). In the present work, mice infected intradermally with *L. braziliensis*

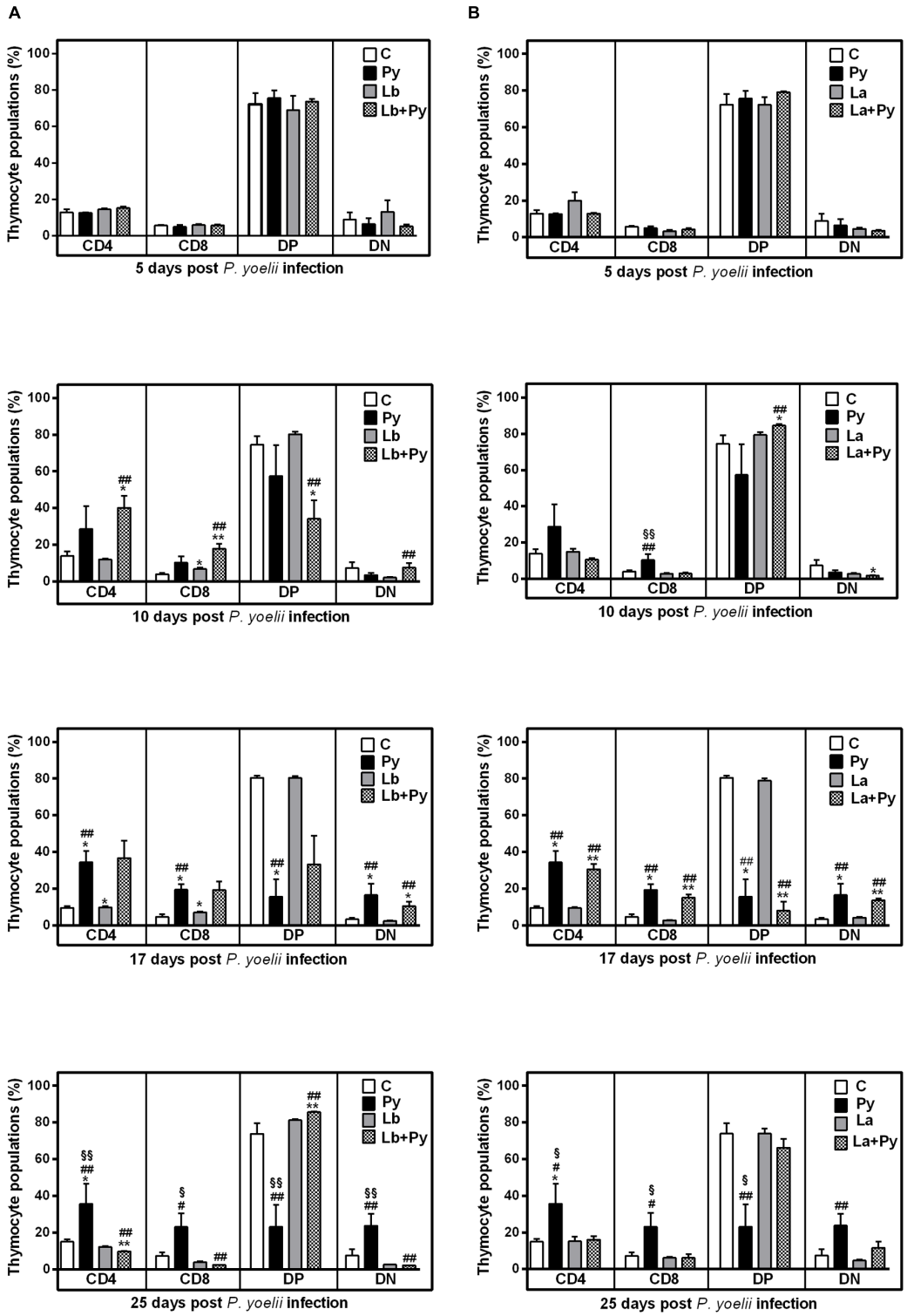

FIGURE 7 | Alterations in thymic T-cell subsets after infection with *P. yoelii* (Py), *L. braziliensis* (Lb), *L. amazonensis* (La), or co-infection with *Leishmania* sp. and *P. yoelii*. The phenotype of T subsets in the thymus were determined by flow cytometry at days 5, 10, 17, and 25 post *P. yoelii* infection. The percentages of $CD4^+$, $CD8^+$, $CD4^-CD8^-$ double negatives (DN), and $CD4^+CD8^+$ double positive cells (DP) obtained in uninfected controls (open bars) were compared to those obtained in Py single-infected mice (black bars) and in animals co-infected (checkered bars) or single infected (gray bars) with *L. braziliensis.* **(A)** or *L. amazonensis* promastigotes **(B)**. Results are expressed by the mean ± SEM of 4–6 mice per group. The Mann–Whitney test was used to compare groups. (*) statistically different from control, (#) statistically different from *Leishmania* sp. and (§) statistically different from *Leishmania* sp./*P. yoelii* co-infected group.

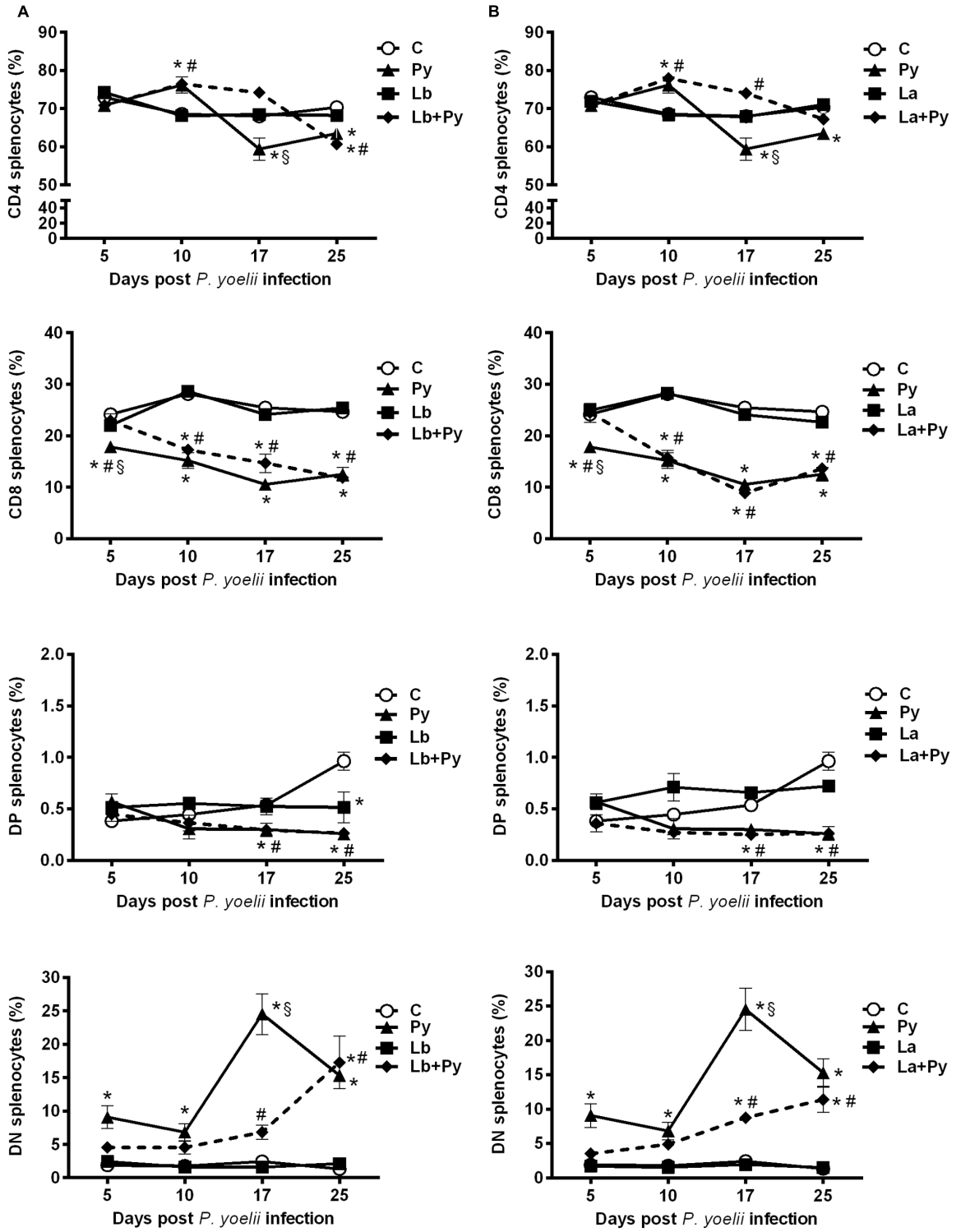

FIGURE 8 | Alterations in spleen T-cell subsets after infection with *P. yoelii* (Py), *L. braziliensis* (Lb), *L. amazonensis* (La), or co-infection with *Leishmania* sp. and *P. yoelii*. The phenotype of T subsets in the spleen were determined by flow cytometry at days 5, 10, 17, and 25 post *P. yoelii* infection. The percentages of $CD4^+$, $CD8^+$, $CD4^-CD8^-$ double negatives (DN), and $CD4^+CD8^+$ double positive cells (DP) obtained in uninfected controls (open circles) were compared to those obtained in Py single-infected mice (black triangles) and in animals co-infected (black diamonds with dashed line) or single infected (black squares) with *L. braziliensis*. **(A)** or *L. amazonensis* promastigotes **(B)**. Results are expressed by the mean ± SEM of 4–6 mice per group. The Mann–Whitney test was used to compare groups. (*) statistically different from control, (#) statistically different from *Leishmania* sp. and (§) statistically different from *Leishmania* sp./*P. yoelii* co-infected group.

developed a localized lesion that increased in size, became ulcerated and self-resolved by week 16 post infection, as expected (data not shown). Co-infected mice exhibited similar pattern in lesions development, but those were smaller and presented less ulcerations. *L. amazonensis* infection, on the other hand induced progressive non-healing lesions, and at the end of the study (11 weeks after *L. amazonensis* infection) 100% of the animals presented ulcerated lesions. Interestingly, during acute Py infection (in the 1st 4 weeks post *L. amazonensis* infection), co-infected group presented a transitory delay on the development of lesions as well as smaller lesions when compared to La single-infected mice. As mentioned before, the differences in susceptibility to *Leishmania* species in murine models, as well as in humans, have multifactorial causes, one of them being the type of immune response generated after infection. For this reason, the differences observed in the disease outcome of *L. braziliensis* and *L. amazonensis* infection during Malaria co-infection must be evaluated in the context of the intense systemic immune response elicited by *P. yoelii* 17XNL in the 1st weeks of *Leishmania* infection.

It is well described that Malaria erythrocytic stage triggers a strong IFN-γ response in both rodent and human Malaria (Shear et al., 1989; Chen et al., 2010; Perez-Mazliah and Langhorne, 2014). The first wave of proinflammatory cytokines is sustained by the innate immune system, when parasitaemia is still low, and is characterized by the release of IFN-γ, TNF-α, IL-2, and IL-12. The inflammatory environment sustains $CD4^+$ T cell polarization to a Th1 phenotype that provides more IFN-γ for macrophages activation and control of parasitaemia (Artavanis-Tsakonas and Riley, 2002; Artavanis-Tsakonas et al., 2003). In our experiments, we were able to detect an early and pronounced increase of IFN-γ levels in the serum of *P. yoelii* 17XNL single infected and co-infected groups, mainly in the beginning of the infection, as extensively described in literature (Shear et al., 1989; Kobayashi et al., 1996; Shan et al., 2012; Karadjian et al., 2014). While the enhanced levels of IFN-γ in the serum was only transitory (5 and 10 days after *P. yoelii* 17XNL) the levels of TNF and IL-6 also increased in animals infected with *P. yoelii* 17XNL, but remained elevated for a longer period of time (during patent parasitaemia) in the serum. Co-infected groups presented smaller lesions than *Leishmania* single infected groups and this effect occurred simultaneously with the increased cytokines levels in the serum, during Malaria erythrocytic phase. The strong proinflammatory environment caused by *P. yoelii* 17XNL infection could explain the delay on lesions development in co-infected groups, given the fact that IFN-γ and TNF play an essential role in controlling *Leishmania* replication inside macrophages (Ashok and Acha-Orbea, 2014). Obviously a clearly scenario can be achieve if we evaluate the *Leishmania*-specific immune response developed in *Leishmania* single infected and *Leishmania* /*P. yoelii* 17XNL co-infected groups. This matter is currently under investigation.

In CL as well as in Malaria, excessive inflammatory response can lead to pathology or even death. Therefore, the balance between pro and anti-inflammatory cytokines is important to control pathology and parasitaemia (Artavanis-Tsakonas et al., 2003; Antonelli et al., 2005; Oliveira et al., 2014). Overall we could observe a tendency for reduced levels of IFN-γ, TNF, IL-6, and IL-10 in the serum of co-infected groups, when compared to *P. yoelii* 17XNL single infected group, suggesting a modulation of *P. yoelii* 17XNL induced immune response by *Leishmania* co-infection. On the other hand, *L. braziliensis* and *L. amazonensis* co-infection exerts opposite effects on *P. yoelii* 17XNL infection (**Figures 2** and **3**). IL-10 is a key regulatory cytokine to protect mice against pathology during acute Malaria (Kobayashi et al., 1996; Freitas do Rosario and Langhorne, 2012). Interestingly the reduction in the serum levels of IL-10 reached statistically significance only *in L. amazonensis* co-infected group at days 5 and 10 post *P. yoelii* 17XNL infection. This significant reduction in serum IL-10 levels could be linked to the persistent parasitaemia and deaths observed in *L. amazonensis* co-infected group (**Figure 2**).

Similar to several other parasitic diseases, experimental Malaria infections can cause severe thymic alterations (Savino, 2006; De Meis et al., 2012). During infection the organ undergoes a strong atrophy with disruption of its architecture that may influence T cell maturation. It has been already demonstrated that mice infected with *P. chabaudi* or *P. berghei*, two other rodent Malaria species, exhibit an important thymic atrophy with cellular depletion and histological disorganization (Seixas and Ostler, 2005; Andrade et al., 2008; Francelin et al., 2011; Lima et al., 2012). Thymic atrophy caused by infectious diseases may occur in combination with one or more of the following events: impairment of thymocyte proliferation, increase in thymocyte death and increase in thymocyte migration to peripheral lymphoid tissue (De Meis et al., 2012). Increase in apoptosis and thymocyte migration has been described in a variety of diseases (Savino, 2006), including Malaria (Francelin et al., 2011).

Accordingly, in *P. yoelii* 17XNL infection we observed a profound thymic atrophy with a reduction in the percentage of double positive cells ($CD4^+CD8^+$) and an increase in the percentage of single positive ($CD4^+$ and $CD8^+$) and double negative cells ($CD4^-CD8^-$). We also observed a high degree of apoptosis and necrosis in the thymocytes of *P. yoelii* single infected and co-infected groups, with no difference between groups (data not shown). Besides the intense atrophy during acute Malaria, thymus was capable to recover its normal size and cellularity after *P. yoelii* elimination, in accordance with observations with *P. chaubaudi* non-lethal infection (Seixas and Ostler, 2005). Infections with both *Leishmania* species were not able to cause thymus involution in mice, probably due to the localized nature of the cutaneous lesions they cause, with no or little impact in the systemic immune environment. Remarkably, thymus recovery occurred faster in co-infected animals, what we believe is a reflection of the minor inflammatory environment observed in co-infected animals.

The reduction in double positive thymocytes has also been described as common feature in infectious diseases, and can be explained by a premature escape of immature cells to blood and peripheral lymphoid organs (De Meis et al., 2012). *P. yoelii* 17XNL single infected and co-infected groups showed decreased percentages of double positive $CD4^+CD8^+$ splenocytes when compared to *Leishmania* single infected animals and uninfected controls, suggesting that the reduced percentages of double

positive cells in the thymus of those groups were not due to increased migration to the peripheral lymphoid organs.

Among the mechanisms described to clear blood-stage Malaria parasites are mature isotypes antibodies and antibody-independent T cell mechanisms (Marsh and Kinyanjui, 2006; Beeson et al., 2008), but all of them require the activation of $CD4^+$ T cells. In our model, $CD4^+$ T cells presented a transitory increase at 10dpi but presented a significant drop on their percentages after 17 dpi when compared to controls. In *L. amazonensis* co-infected group they equalized controls only at 25 dpi, while in *L. braziliensis* co-infection they were lower them controls and *Leishmania* single infected group. The nature of this cells (e.g., if they are regulatory the cells or effector T cells), as well as the cytokines they produce after stimulation with *Leishmania* antigens or *P. yoelii* infected erythrocytes are under investigation.

Interestingly the percentages of double negatives CD4 and CD8 splenocytes increased in *P. yoelii* 17XNL infected groups, and *Leishmania* co-infection were able to delay this enhancement. We are also performing multiparametric flow cytometry experiments to better understand the phenotype of this $CD3^+CD4^-CD8^-$cells, since they can be NKT cells or γδT cells. Both cell types are components of the innate immune system and have been proposed to play significant roles in the clearance of blood-stage malarial parasites (Stevenson and Riley, 2004; Urban et al., 2005).

NKT cells are innate-like lymphocytes that account for approximately 5% of T lymphocytes in the spleen. They posses both T cell and NK cells surface markers since they can express CD4 or CD8 co receptors on their surface, or neither one of them (double-negative phenotype) as well as the NK1.1 surface molecule (Balato et al., 2009). Primary *P. yoelii* infection with non-lethal strain 265BY is able to induce an organ-specific and heterogeneous NKT cells response (Soulard et al., 2007). Hepatic NKT cells consisted mainly of CD1d-dependent $CD4^+$ and double negative NKT cells, whereas splenic NKT cells presented a CD1d-independent TCRhigh CD4high phenotype during infection (Soulard et al., 2007). It was also demonstrated that $CD49b^+$ $CD3^+$ natural killer T (NKT) cells increased in the liver after a primary infection with *P. yoelii* non-lethal strain (17XNL), and that CD1d-restricted NKT cells, which secrete IFNγ, are critical to reduce liver-stage burden in a secondary infection (Miller et al., 2014). Lack of type 1 IFN-alpha receptor signaling compromise the enhancement of NKT cells in the liver, showing a link between type I IFN signaling, cell recruitment, and subsequent parasite elimination.

Gamma delta T cells are the first to be generated in the ontogeny. They are able to respond quickly through the production of cytokines and can be divided into two types: interferon-producing cells (CD27hi) and IL-17-producing cells (CD27lo) (Zarin et al., 2015). In Malaria they are activated during pre-erythrocytic (liver) and erythrocyte stages and can produce INF-γ *in vitro* after stimulation with *P. falciparum* infected erythrocytes (Elloso et al., 1994; Farouk et al., 2004; Scholzen and Sauerwein, 2016). In BALB/c mice, the number of γδT cells increases considerably in the spleen during infection with *P. yoelii* non-lethal strain (17XNL) (Kopacz and Kumar, 1999), while this increase did not occur to the same extent in mice infected with the lethal strain of *P. yoelii* (17XL) suggesting that γδT cells play a protective role in Malaria (Kopacz and Kumar, 1999). In another work with *P. yoelii* non-lethal strain was observed an increase in the percentage of γδT cells in the spleen, liver and peripheral blood, peaking at day 21 of infection (Li et al., 2012). When the mice were depleted from γδT cells with monoclonal antibodies parasite elimination was delayed (Li et al., 2012).

Our results demonstrating an increase in $CD3^+CD4^-CD8^-$ cells during erythrocyte stage in *P. yoelii* 17XNL non-lethal strain infected animals both in the thymus (**Figure 7**) and in the spleen (**Figure 8**) does not discard the possibility that those can be either NK T or γδT cells, and the delay of this enhancement to occur in *Leishmania* co-infected groups when compared to *P. yoelii* single infected animals could have a correlation with the differences in cytokines production observed between *P. yoelii* 17XNL single infected and co-infected groups.

Taken together, our results suggest that coexisting infection with *P. yoelii* 17XNL and *L. braziliensis* or *L. amazonensis* may change diseases outcomes, and that Malaria outcome can be altered according to the *Leishmania* co-infection specie evaluated. On the other hand Malaria infection produced a transient delay on leishmanial lesions development. These alterations on Malaria and CL progress seem to be closely related to changes in the immune response as verified by alteration in serum cytokine levels and thymus dynamics during infection.

In areas where Malaria and CL are co-endemic, human populations are continuously exposed to Malaria and Leishmaniasis vectors bites and consequently are at risk of suffering repeated infections. These recurrent infections in endemic areas might cause permanent immunomodulatory effects, which can probably interfere in the outcome of both diseases. Moreover, the increase in Malaria parasitaemia, patency and mortality, observed in *L. amazonensis/P. yoelii* coinfection, might contribute to the severity of disease as well as to the maintenance of the malaria transmission in co-endemic areas. Considering the importance and the geographical distribution of Malaria and CL in tropical regions of the world, further investigation on immunomodulatory mechanisms during co-infection are necessary to understand how co-infection can affect not only the natural history and progression of diseases but also the treatment and prevention of both.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DB, PL, JF, and RP. Performed the experiments: RP, PL, DS, DV, DP, and DB. Analyzed the data: RP, PL, DV, DS, and DB. Contributed reagents/materials/analysis tools: DB, PL, and DV. Wrote the paper: RP, PL, and DB.

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REFERENCES

Ab Rahman, A. K., and Abdullah, F. H. (2011). Visceral Leishmaniasis (kala-azar) and Malaria co-infection in an immigrant in the state of Terengganu, Malaysia: a case report. *J. Microbiol. Immunol. Infect.* 44, 72–76. doi: 10.1016/j.jmii.2011.01.014

Alvar, J., Velez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE* 7:e35671. doi: 10.1371/journal.pone.0035671

Andrade, C. F., Gameiro, J., Nagib, P. R., Carvalho, B. O., Talaisys, R. L., Costa, F. T., et al. (2008). Thymic alterations in *Plasmodium berghei*-infected mice. *Cell Immunol.* 253, 1–4. doi: 10.1016/j.cellimm.2008.06.001

Antonelli, L. R. V., Dutra, W. O., Almeida, R. P., Bacellar, O., Carvalho, E. M., and Gollob, K. J. (2005). Activated inflammatory T cells correlate with lesion size in human Cutaneous Leishmaniasis. *Immunol. Lett.* 101, 226–230. doi: 10.1016/j.imlet.2005.06.004

Artavanis-Tsakonas, K., and Riley, E. M. (2002). Innate immune response to Malaria: rapid induction of IFN-gamma from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 169, 2956–2963. doi: 10.4049/jimmunol.169.6.2956

Artavanis-Tsakonas, K., Tongren, J. E., and Riley, E. M. (2003). The war between the Malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clin. Exp. Immunol.* 133, 145–152. doi: 10.1046/j.1365-2249.2003.02174.x

Ashok, D., and Acha-Orbea, H. (2014). Timing is everything: dendritic cell subsets in murine *Leishmania* infection. *Trends Parasitol.* 30, 499–507. doi: 10.1016/j.pt.2014.08.001

Balato, A., Unutmaz, D., and Gaspari, A. A. (2009). Natural killer T cells: an unconventional T-cell subset with diverse effector and regulatory functions. *J. Invest. Dermatol.* 129, 1628–1642. doi: 10.1038/jid.2009.30

Battle, K. E., Karhunen, M. S., Bhatt, S., Gething, P. W., Howes, R. E., Golding, N., et al. (2014). Geographical variation in *Plasmodium vivax* relapse. *Malar. J.* 13:144. doi: 10.1186/1475-2875-13-144

Beeson, J. G., Osier, F. H., and Engwerda, C. R. (2008). Recent insights into humoral and cellular immune responses against Malaria. *Trends Parasitol.* 24, 578–584. doi: 10.1016/j.pt.2008.08.008

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., and Sacks, D. (2000). A natural model of *Leishmania* major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J. Immunol.* 165, 969–977. doi: 10.4049/jimmunol.165.2.969

Bin Mohanna, M. A. (2015). Leishmaniasis, Malaria, and schistosomiasis concurrently in an 8-year-old boy. *Saudi Med. J.* 36, 494–496. doi: 10.15537/smj.2015.4.10757

Carregaro, V., Costa, D. L., Brodskyn, C., Barral, A. M., Barral-Netto, M., Cunha, F. Q., et al. (2013). Dual effect of *Lutzomyia longipalpis* saliva on *Leishmania braziliensis* infection is mediated by distinct saliva-induced cellular recruitment into BALB/c mice ear. *BMC Microbiol.* 13:102. doi: 10.1186/1471-2180-13-102

Chen, G., Feng, H., Liu, J., Qi, Z. M., Wu, Y., Guo, S. Y., et al. (2010). Characterization of immune responses to single or mixed infections with *P. yoelii* 17XL and *P. chabaudi* AS in different strains of mice. *Parasitol. Int.* 59, 400–406. doi: 10.1016/j.parint.2010.05.005

Coleman, R. E., Edman, J. D., and Semprevivo, L. H. (1988a). Interactions between *Plasmodium yoelii* and *Leishmania mexicana* amazonensis in *Leishmania* resistant C57B1/6 mice. *Am. J. Trop. Med. Hyg.* 39, 540–544.

Coleman, R. E., Edman, J. D., and Semprevivo, L. H. (1988b). *Leishmania mexicana*: effect of concomitant Malaria on Cutaneous Leishmaniasis. Development of lesions in a Leishmania-susceptible (BALB/c) strain of mouse. *Exp. Parasitol.* 65, 269–276. doi: 10.1016/0014-4894(88)90131-2

De Meis, J., Aurelio Farias-De-Oliveira, D., Nunes Panzenhagen, P. H., Maran, N., Villa-Verde, D. M., Morrot, A., et al. (2012). Thymus atrophy and double-positive escape are common features in infectious diseases. *J. Parasitol. Res.* 2012:574020. doi: 10.1155/2012/574020

de Moura, T. R., Novais, F. O., Oliveira, F., Clarencio, J., Noronha, A., Barral, A., et al. (2005). Toward a novel experimental model of infection to study American Cutaneous Leishmaniasis caused by *Leishmania braziliensis*. *Infect. Immun.* 73, 5827–5834. doi: 10.1128/IAI.73.9.5827-5834.2005

Dutra, W. O., De Faria, D. R., Lima Machado, P. R., Guimaraes, L. H., Schriefer, A., Carvalho, E., et al. (2011). Immunoregulatory and effector activities in human cutaneous and mucosal leishmaniasis: understanding mechanisms of pathology. *Drug Dev. Res.* 72, 430–436. doi: 10.1002/ddr.20449

Elloso, M. M., Van Der Heyde, H. C., Vande Waa, J. A., Manning, D. D., and Weidanz, W. P. (1994). Inhibition of *Plasmodium falciparum* in vitro by human gamma delta T cells. *J. Immunol.* 153, 1187–1194.

Falcão, S. C., De Moura, T. R., Clarencio, J., Brodskyn, C., Barral, A., and De Oliveira, C. I. (2012). The presence of Tregs does not preclude immunity to reinfection with *Leishmania braziliensis*. *Int. J. Parasitol.* 42, 771–780. doi: 10.1016/j.ijpara.2012.05.006

Farouk, S. E., Mincheva-Nilsson, L., Krensky, A. M., Dieli, F., and Troye-Blomberg, M. (2004). Gamma delta T cells inhibit in vitro growth of the asexual blood stages of *Plasmodium falciparum* by a granule exocytosis-dependent cytotoxic pathway that requires granulysin. *Eur. J. Immunol.* 34, 2248–2256. doi: 10.1002/eji.200424861

Francelin, C., Paulino, L. C., Gameiro, J., and Verinaud, L. (2011). Effects of Plasmodium berghei on thymus: high levels of apoptosis and premature egress of CD4(+)CD8(+) thymocytes in experimentally infected mice. *Immunobiology* 216, 1148–1154. doi: 10.1016/j.imbio.2011.03.009

Freitas do Rosario, A. P., and Langhorne, J. (2012). T cell-derived IL-10 and its impact on the regulation of host responses during Malaria. *Int. J. Parasitol.* 42, 549–555. doi: 10.1016/j.ijpara.2012.03.010

Gollob, K. J., Viana, A. G., and Dutra, W. O. (2014). Immunoregulation in human American Leishmaniasis: balancing pathology and protection. *Parasite Immunol.* 36, 367–376. doi: 10.1111/pim.12100

Good, M. F., and Doolan, D. L. (2010). Malaria vaccine design: immunological considerations. *Immunity* 33, 555–566. doi: 10.1016/j.immuni.2010.10.005

Hartley, M. A., Drexler, S., Ronet, C., Beverley, S. M., and Fasel, N. (2014). The immunological, environmental, and phylogenetic perpetrators of metastatic Leishmaniasis. *Trends Parasitol.* 30, 412–422. doi: 10.1016/j.pt.2014.05.006

Hotez, P. J., Molyneux, D. H., Fenwick, A., Ottesen, E., Ehrlich Sachs, S., and Sachs, J. D. (2006). Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and Malaria. *PLoS Med.* 3:e102. doi: 10.1371/journal.pmed.0030102

Howard, M. K., Pharoah, M. M., Ashall, F., and Miles, M. A. (1991). Human urine stimulates growth of *Leishmania in vitro*. *Trans. R. Soc. Trop. Med. Hyg.* 85, 477–479. doi: 10.1016/0035-9203(91)90226-O

Karadjian, G., Berrebi, D., Dogna, N., Vallarino-Lhermitte, N., Bain, O., Landau, I., et al. (2014). Co-infection restrains *Litomosoides sigmodontis* filarial load and plasmodial *P. yoelii* but not *P. chabaudi* parasitaemia in mice. *Parasite* 21:16. doi: 10.1051/parasite/2014017

Kedzierski, L., and Evans, K. J. (2014). Immune responses during cutaneous and Visceral Leishmaniasis. *Parasitology* 141, 1544–1562. doi: 10.1017/S003118201400095x

Kobayashi, F., Morii, T., Matsui, T., Fujino, T., Watanabe, Y., Weidanz, W. P., et al. (1996). Production of interleukin 10 during Malaria caused by lethal and nonlethal variants of *Plasmodium yoelii* yoelii. *Parasitol. Res.* 82, 385–391. doi: 10.1007/s004360050133

Kolbaum, J., Eschbach, M. L., Steeg, C., Jacobs, T., Fleischer, B., and Breloer, M. (2012). Efficient control of *Plasmodium yoelii* infection in BALB/c and C57BL/6 mice with pre-existing *Strongyloides ratti* infection. *Parasite Immunol.* 34, 388–393. doi: 10.1111/j.1365-3024.2012.01369.x

Kopacz, J., and Kumar, N. (1999). gamma delta T-cells may interfere with a productive immune response in *Plasmodium yoelii* infections. *Int. J. Parasitol.* 29, 737–742. doi: 10.1016/S0020-7519(99)00026-0

La Flamme, A. C., Scott, P., and Pearce, E. J. (2002). Schistosomiasis delays lesion resolution during *Leishmania* major infection by impairing parasite killing by macrophages. *Parasite Immunol.* 24, 339–345. doi: 10.1046/j.1365-3024.2002.00473.x

Li, C., Mannoor, K., Inafuku, M., Taniguchi, T., Inamine, Y., Miyazaki, T., et al. (2012). Protective function of an unconventional gammadelta T cell subset against Malaria infection in apoptosis inhibitor deficient mice. *Cell Immunol.* 279, 151–159. doi: 10.1016/j.cellimm.2012.09.012

Li, C., Seixas, E., and Langhorne, J. (2001). Rodent Malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. *Med. Microbiol. Immunol.* 189, 115–126. doi: 10.1007/s430-001-8017-8

Lima, A. C., Francelin, C., Ferrucci, D. L., Stach-Machado, D. R., and Verinaud, L. (2012). Thymic alterations induced by *Plasmodium berghei*: expression of matrix metalloproteinases and their tissue inhibitors. *Cell Immunol.* 279, 53–59. doi: 10.1016/j.cellimm.2012.09.006

Louis, J. A., Conceicao-Silva, F., Himmelrich, H., Tacchini-Cottier, F., and Launois, P. (1998). Anti-leishmania effector functions of CD4+ Th1 cells and early events instructing Th2 cell development and susceptibility to *Leishmania* major in BALB/c mice. *Adv. Exp. Med. Biol.* 452, 53–60. doi: 10.1007/978-1-4615-5355-7_7

Marsh, K., and Kinyanjui, S. (2006). Immune effector mechanisms in Malaria. *Parasite Immunol.* 28, 51–60. doi: 10.1111/j.1365-3024.2006.00808.x

Medina, T. S., Costa, S. P., Oliveira, M. D., Ventura, A. M., Souza, J. M., Gomes, T. F., et al. (2011). Increased interleukin-10 and interferon-gamma levels in *Plasmodium vivax* Malaria suggest a reciprocal regulation which is not altered by IL-10 gene promoter polymorphism. *Malar. J.* 10, 264. doi: 10.1186/1475-2875-10-264

Miller, J. L., Sack, B. K., Baldwin, M., Vaughan, A. M., and Kappe, S. H. (2014). Interferon-mediated innate immune responses against Malaria parasite liver stages. *Cell Rep.* 7, 436–447. doi: 10.1016/j.celrep.2014.03.018

Niikura, M., Kamiya, S., Kita, K., and Kobayashi, F. (2008). Co-infection with nonlethal murine Malaria parasites suppresses pathogenesis caused by *Plasmodium berghei* NK65. *J. Immunol.* 180, 6877–6884. doi: 10.4049/jimmunol.180.10.6877

Oliveira, W. N., Ribeiro, L. E., Schrieffer, A., Machado, P., Carvalho, E. M., and Bacellar, O. (2014). The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of human tegumentary Leishmaniasis. *Cytokine* 66, 127–132. doi: 10.1016/j.cyto.2013.12.016

Pereira, B. A., and Alves, C. R. (2008). Immunological characteristics of experimental murine infection with *Leishmania* (Leishmania) amazonensis. *Vet. Parasitol.* 158, 239–255. doi: 10.1016/j.vetpar.2008.09.015

Pereira, C. G., Silva, A. L., De Castilhos, P., Mastrantonio, E. C., Souza, R. A., Romao, R. P., et al. (2009). Different isolates from *Leishmania braziliensis* complex induce distinct histopathological features in a murine model of infection. *Vet. Parasitol.* 165, 231–240. doi: 10.1016/j.vetpar.2009.07.019

Perez-Mazliah, D., and Langhorne, J. (2014). CD4 T-cell subsets in Malaria: TH1/TH2 revisited. *Front. Immunol.* 5:671. doi: 10.3389/fimmu.2014.00671

Qi, Z., Han, X., Zhang, Y., Wang, J., and Cao, Y. M. (2013). Listeria monocytogenes inoculation protects mice against blood-stage *Plasmodium yoelii* infection. *Tohoku J. Exp. Med.* 229, 87–96. doi: 10.1620/tjem.229.87

Ribeiro-Gomes, F. L., Roma, E. H., Carneiro, M. B., Doria, N. A., Sacks, D. L., and Peters, N. C. (2014). Site-dependent recruitment of inflammatory cells determines the effective dose of *Leishmania* major. *Infect. Immun.* 82, 2713–2727. doi: 10.1128/IAI.01600-13

Savino, W. (2006). The thymus is a common target organ in infectious diseases. *PLoS Pathog.* 2:e62. doi: 10.1371/journal.ppat.0020062

Scholzen, A., and Sauerwein, R. W. (2016). Immune activation and induction of memory: lessons learned from controlled human Malaria infection with *Plasmodium falciparum*. *Parasitology* 143, 224–235. doi: 10.1017/S0031182015000761

Seixas, E., and Ostler, D. (2005). *Plasmodium chabaudi* chabaudi (AS): differential cellular responses to infection in resistant and susceptible mice. *Exp. Parasitol.* 110, 394–405. doi: 10.1016/j.exppara.2005.03.024

Shan, Y., Liu, J., Jiang, Y. J., Shang, H., Jiang, D., and Cao, Y. M. (2012). Age-related susceptibility and resistance to nonlethal *Plasmodium yoelii* infection in C57BL/6 mice. *Folia Parasitol. (Praha)* 59, 153–161. doi: 10.14411/fp.2012.021

Shear, H. L., Srinivasan, R., Nolan, T., and Ng, C. (1989). Role of IFN-gamma in lethal and nonlethal Malaria in susceptible and resistant murine hosts. *J. Immunol.* 143, 2038–2044.

Silveira, F. T., Lainson, R., De Castro Gomes, C. M., Laurenti, M. D., and Corbett, C. E. (2009). Immunopathogenic competences of *Leishmania* (V.) braziliensis and L. (L.) amazonensis in American Cutaneous Leishmaniasis. *Parasite Immunol.* 31, 423–431. doi: 10.1111/j.1365-3024.2009.01116.x

Soulard, V., Roland, J., Sellier, C., Gruner, A. C., Leite-De-Moraes, M., Franetich, J. F., et al. (2007). Primary infection of C57BL/6 mice with *Plasmodium yoelii* induces a heterogeneous response of NKT cells. *Infect. Immun.* 75, 2511–2522. doi: 10.1128/IAI.01818-06

Stevenson, M. M., and Riley, E. M. (2004). Innate immunity to Malaria. *Nat. Rev. Immunol.* 4, 169–180. doi: 10.1038/nri1311

Taylor-Robinson, A. W. (2010). Regulation of immunity to *Plasmodium*: implications from mouse models for blood stage Malaria vaccine design. *Exp. Parasitol.* 126, 406–414. doi: 10.1016/j.exppara.2010.01.028

Urban, B. C., Ing, R., and Stevenson, M. M. (2005). Early interactions between blood-stage plasmodium parasites and the immune system. *Curr. Top. Microbiol. Immunol.* 297, 25–70.

van den Bogaart, E., Berkhout, M. M., Adams, E. R., Mens, P. F., Sentongo, E., Mbulamberi, D. B., et al. (2012). Prevalence, features and risk factors for Malaria co-infections amongst Visceral Leishmaniasis patients from Amudat Hospital, Uganda. *PLoS Negl. Trop. Dis.* 6:e1617. doi: 10.1371/journal.pntd.0001617

van den Bogaart, E., Talha, A. B., Straetemans, M., Mens, P. F., Adams, E. R., Grobusch, M. P., et al. (2014). Cytokine profiles amongst Sudanese patients with Visceral Leishmaniasis and Malaria co-infections. *BMC Immunol.* 15:16. doi: 10.1186/1471-2172-15-16

World Health Organization (2010). Control of the leishmaniases. *World Health Organ. Tech. Rep. Ser.* 949, xii-xiii, 1–186.

World Health Organization (2015). *World Malaria Report*. Geneva: World Health Organization.

World Health Organization (2016). *Global Health Observatory (GHO) Data [Online]*. Available: http://www.who.int/gho/neglected_diseases/leishmaniasis/en/ [accessed March 15, 2016].

Yoeli, M. (1948). Non-pigmented Malaria parasites in the bone marrow from a mixed infection of *Leishmania* and *Plasmodium vivax*. *Trans. R. Soc. Trop. Med. Hyg.* 42:99. doi: 10.1016/0035-9203(48)90355-1

Zarin, P., Chen, E. L., In, T. S., Anderson, M. K., and Zuniga-Pflucker, J. C. (2015). Gamma delta T-cell differentiation and effector function programming, TCR signal strength, when and how much? *Cell Immunol.* 296, 70–75. doi: 10.1016/j.cellimm.2015.03.007

Zuzarte-Luis, V., Mota, M. M., and Vigario, A. M. (2014). Malaria infections: what and how can mice teach us. *J. Immunol. Methods* 410, 113–122. doi: 10.1016/j.jim.2014.05.001

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REVIEW
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Is the Antitumor Property of *Trypanosoma cruzi* Infection Mediated by Its Calreticulin?

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Eight to 10 million people in 21 endemic countries are infected with *Trypanosoma cruzi*. However, only 30% of those infected develop symptoms of Chagas' disease, a chronic, neglected tropical disease worldwide. Similar to other pathogens, *T. cruzi* has evolved to resist the host immune response. Studies, performed 80 years ago in the Soviet Union, proposed that *T. cruzi* infects tumor cells with similar capacity to that displayed for target tissues such as cardiac, aortic, or digestive. An antagonistic relationship between *T. cruzi* infection and cancer development was also proposed, but the molecular mechanisms involved have remained largely unknown. Probably, a variety of *T. cruzi* molecules is involved. This review focuses on how *T. cruzi* calreticulin (TcCRT), exteriorized from the endoplasmic reticulum, targets the first classical complement component C1 and negatively regulates the classical complement activation cascade, promoting parasite infectivity. We propose that this C1-dependent TcCRT-mediated virulence is critical to explain, at least an important part, of the parasite capacity to inhibit tumor development. We will discuss how TcCRT, by directly interacting with venous and arterial endothelial cells, inhibits angiogenesis and tumor growth. Thus, these TcCRT functions not only illustrate *T. cruzi* interactions with the host immune defensive strategies, but also illustrate a possible co-evolutionary adaptation to privilege a prolonged interaction with its host.

Keywords: calreticulin, *Trypanosoma cruzi*, trypomastigotes, complement system, C1q, cC1qR, tumor growth, immune response

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INTRODUCTION

Trypanosoma cruzi (the protozoan agent of Chagas' disease) cell infection is preceded by a variety of molecular interactions (1). Of relevance is the generation of a synapsis involving parasite endoplasmic reticulum (ER)-resident *T. cruzi* calreticulin (TcCRT) that, after translocation, interacts with complement component C1. C1 is then inactivated and recognized by cC1qR (a membrane form of mammalian CRT). The complement system, an important arm of innate and adaptive immune responses, is thus inhibited and parasite infectivity increased.

A significant decrease in experimental tumor growth is observed in experimental animals treated with recombinant TcCRT (rTcCRT) or infected with *T. cruzi*. A unifying molecular basis for these apparently unrelated phenomena is proposed herein. These molecular interactions do provide benefits for both the host and the parasite.

Through evolution, microbial agents have developed different mechanisms to resist the host immune response. In apparently unrelated strategies, some infectious agents elicit antitumor

immune responses, leading to inhibition of cancer progression (2). Although these antitumor effects have been reported for several decades now, for a variety of infections, information on pathogen molecules involved is scarce (3).

Eight to 10 million people in 21 endemic countries are infected with *T. cruzi*. In about 30% of those infected, manifests, Chagas' disease, a worldwide neglected tropical chronic illness (4, 5). The disease, originally endemic in Latin America, is now global, mainly because of migrations to USA, Canada, Europe, Oceania, and Asia (6), where transmission is mainly through blood transfusions, organ transplants, or congenital (7).

Eighty years ago, it was proposed that *T. cruzi* possesses an anticancer activity. Several *T. cruzi* strains displayed growth inhibitory effects over multiple transplanted or spontaneous tumors, in animal experimental models and humans (8, 9). This property was attributed to a "toxic substance" secreted by the parasite (10, 11). This "toxin" reduced pain, tumor growth, bleeding, and local inflammation in humans affected by a variety of tumors (12).

Chronically infected rats are more resistant to a carcinoma induced by 1,2-dimethylhidrazyne (9), and *T. cruzi* has a tropism for tumor cells, suggesting an antagonistic relationship between Chagas' disease and cancer development (8). Elemental Darwinian reasoning allows us to propose that, if host survival is favored, chances for improved parasite persistence are evident.

Some authors have proposed that tumor and parasites compete for nutrients with consequent inhibition of tumor growth (13). However, this hypothesis is not entirely satisfactory since tumor growth is a multistep and complex process involving development of new blood vessels (angiogenesis) that provide the tumor with the necessary nutrients, oxygen, and means for waste removal (14). Other investigators have demonstrated, using a recombinant non-pathogenic *T. cruzi* clone as vector of a testis tumor antigen, the activation of T cell-mediated immunity. This specific cell immunity could delay tumor development in infected mice (15). In this work, it would have been important to define whether the non-pathogenic *T. cruzi* clone used translocates-externalizes its CRT. Non-infective epimastigotes are strongly impaired in their capacity to translocate this chaperone (16). Moreover, hemiallelic *TcCRT* KO, wild type, and transgenic parasites, respectively carrying one, two, and three *TcCRT* gene copies, express increased levels of the protein, *in vitro* resistance to human complement, and higher infectivity (16, 17).

Most likely, multiple parasite molecules and mechanisms are involved in the tumor resistance mediated by *T. cruzi* infection. Understanding these mechanisms may contribute to identify new therapeutic targets against cancer and Chagas' disease.

Our laboratory has been working for more than 20 years now with TcCRT, a multifunctional ER-resident protein that the parasite translocates to the external milieu (as depicted in **Figures 1A,B**). TcCRT is involved in a multiplicity of

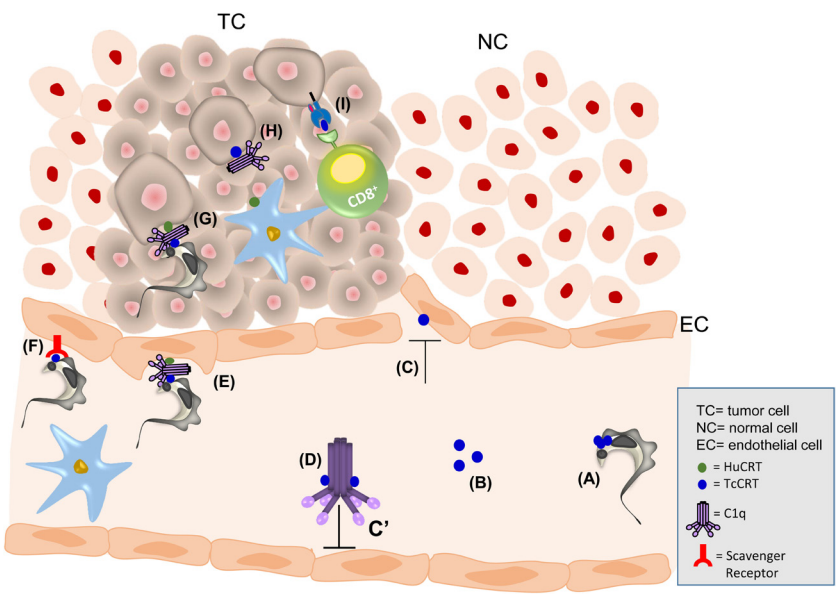

FIGURE 1 | The antitumor effect of *T. cruzi* infection may be explained by TcCRT. TcCRT is exposed on the parasite surface **(A)** and secreted **(B)**. TcCRT inhibits angiogenesis **(C)** and the activation of the classical pathway of the complement system through C1 inactivation **(D)**. TcCRT, present on the parasite surface, recruits C1. On the EC membrane, a trimolecular synapse is formed by HuCRT/C1q/TcCRT. This interaction increases the infectivity process **(E)**. TcCRT is also recognized by SRs on ECs, promoting infectivity **(F)**. The HuCRT/C1q/TcCRT interaction can also promote *T. cruzi* infectivity in TCs **(G)**. Moreover, TcCRT could mediate induction of an anamnestic antitumor immune response. Parasite could translocate TcCRT bound to the tumor cell with subsequent capture of host C1 **(H)**. This C1 will be recognized by HuCRT present on an antigen-presenting cell (APC), followed by internalization of this complex. Among many other possibilities, APCs will cross-process TcCRT, and specific peptides from this parasite protein will be loaded onto MHC I molecules. APCs will enter the regional lymph node and present these nTcCRT-specific peptides to cytotoxic T lymphocytes, thus leading to their activation. These $CD8^+$ cytotoxic T lymphocytes will leave the lymph node and kill tumor cells that also present TcCRT-derived peptides **(I)**.

host–pathogen interactions. Thus, TcCRT is a potent virulence factor that inhibits the angiogenesis and a likely responsible, for at least in important part, of the antitumor effects of *T. cruzi* infection.

IN EUKARYOTES, CALRETICULIN, AN ER-RESIDENT CHAPERONE PROTEIN, MEDIATES ANTITUMOR PROPERTIES

Calreticulin (CRT) is a 45 kDa protein, mainly residing in the ER (18). CRT participates in a variety of physiological and pathological processes in different cellular types (19). Thus, CRT contributes in multiple physiological processes such as control of glycoprotein folding quality system and binding to monoglucosylated high mannose glycans (20). Moreover, CRT is involved in quality control process during protein synthesis, including integrins, surface receptors, and transporters (21), and it is considered as an intracellular Ca^{2+} regulator (22).

Calreticulin is also found in the cytosol, nucleus, secretory granules, on the plasma membrane, and free in the extracellular milieu (18), accelerating cutaneous wound healing (23–25) and regulating cell adhesion by interacting with the cytosolic tail of the integrin alpha subunit (18); nuclear export of some steroid hormone receptors (26–28) and the stability or translation of a variety of RNAs (29–33). CRT is an mRNA binding protein that regulates mRNA stability (19).

Calreticulin also participates in the immune response against apoptotic cancer cells (34–38), and surface exposure of CRT participates as an "eat me" signal required for phagocytosis on dying tumor cells (39). Tumor tissues express significant higher levels of CRT compared to normal tissues (40). Indeed, its expression is related to the clinical stage and lymph node metastasis in several types of cancer (41, 42).

Over 40 functions have been described for human CRT (HuCRT) (43). These functions reside in three different domains: globular N-terminal (N), proline-rich (P), and acidic C-terminus (18). HuCRT and its N-terminal fragment bind laminin (44) with antiangiogenic properties *in vitro* and *in vivo* (45, 46) and inhibit the growth in several tumor models (47–49).

Vasostatin, a CRT 180 amino acid N-terminal fragment, is an endogenous inhibitor of angiogenesis and suppressor of tumor growth. It inhibits vascular endothelial growth factor (VEGF)-induced endothelial cell (EC) proliferation and tube formation in Matrigel and induces cell apoptosis under oxygen deprivation (50).

Calreticulin is present in humans (51), insects (52, 53), nematodes (54–57), protozoans (58–61), and plants (62). A high identity is shared among CRTs from different species. Thus, *Onchocerca volvulus*, *Schistosoma mansoni*, and *Leishmania donovani* share 50% of the identity in amino acid sequence with HuCRT.

Examples of important evasive strategies performed by CRTs from different parasite species are *Amblyomma americanum* [secretes CRT during the feeding process (63)] and *Schistosoma cercariae* [uses CRT in the penetration of gland cells or skin and parasite migration (54)].

HOW DOES *T. CRUZI* CALRETICULIN PARTICIPATE IN THE HOST–PARASITE INTERPLAY?

Given the important pleiotropic HuCRT behavior, the CRT model opens interesting research opportunities on how this protein, alone or interacting with others, intervenes in the host–parasite interactions.

For 25 years now, our laboratory has worked with TcCRT. This protein is coded by only one gene with a variable number of copies whose involvement in TcCRT expression will depend on the *T. cruzi* clone and strain studied (unpublished data). A TcCRT gene was cloned, sequenced, and expressed in our laboratory in 1991 (58). We identified variable low plasma levels of anti-native TcCRT antibodies in *T. cruzi*-infected humans (64), thus revealing the immunogenic capacity of the native protein.

Trypanosoma cruzi calreticulin also binds monoglucosylated glycans (60) and participates in the maturation of cruzipain, a lysosomal protease (65) present in *T. cruzi*. Although TcCRT locates mainly in the ER, it is also found in the Golgi complex, reservosomes, flagellar pocket, cell surface, cytosol, nucleus, and kinetoplast (66, 67). However, the mechanisms involved in these diverse TcCRT localizations are unknown. Thus, TcCRT, in spite of its KEDL-ER retention sequence [KDEL in mammal CRTs (18)], translocates from the ER to the extracellular environment (**Figures 1A,B**) where, besides inhibiting complement (66) and acting as a virulence factor (68), it mediates antitumor effects.

In spite of the long evolutionary distance, TcCRT still shares 50% of overall sequence homology with HuCRT, reaching up to 80% in critical functional domains. Moreover, the general globular N-domain, responsible of antiangiogenic properties and the structural features of the extended arm P-domain also share structure homologies, thus announcing the possibility of functional similarities (69).

Two important TcCRT functions may explain the relationship between *T. cruzi* infection and cancer. First, TcCRT is an important complement inhibitor (**Figure 1D**) and virulence factor (**Figure 1E**). Second, TcCRT inhibits angiogenesis (**Figure 1C**). Both functions are central to inhibit tumor growth.

TcCRT IS AN IMPORTANT VIRULENCE FACTOR IN *T. CRUZI*

Similar to HuCRT (70, 71), TcCRT inhibits the complement system by interacting with C1 (**Figure 1D**), the first component of its classical pathway (66, 72–74). TcCRT is translocated from the ER to the area of flagellum emergence (**Figure 1A**) (66), where C1 is recruited by parasite-bound TcCRT and inhibited at the earliest complement activation step (C4b generation) (**Figure 1D**). TcCRT also affects the ability of C1s to activate C4, in a calcium-independent manner (74). Inhibition of C1 is a significant complement evasion strategy, with consequences in the host–parasite relationships. Although HuCRT and TcCRT prevent binding of the serine proteases to C1q, they do not displace the serine proteases from the preformed stabilized C1 (C1q, r_2, and s_2) complex (74). TcCRT also binds to MBL and

Ficolins (75). C1, MBL, and Ficolins are three complement "danger signal" recognition macromolecular modules present in plasma. These molecular complexes are genetically, structurally, and functionally related, but they differ in the nature of the recognized danger signals (76). More recently, we have proposed that L-Ficolin binds TcCRT, inhibiting the lectin pathway. This inhibition may represent other *T. cruzi* strategy to inhibit the host immune response (75). In agreement with these findings, TcCRT is present on the parasite surface co-localizing with C1q (66).

Human CRT is also a membrane receptor for C1q [cC1qR (77)], and it may bridge TcCRT on the parasite surface with HuCRT present on the host cell (**Figure 1E**) (78). The TcCRT/C1q/HuCRT synapsis represents the culmination of an important molecular mimicry strategy. Apoptotic cells to be phagocytized use a similar mechanism (34, 36, 37). The CRT/C1q complex is recognized as an "eat me" signal by cC1qR on phagocytes. This signal is also used by *T. cruzi* as an "apoptotic mimicry" strategy (i.e., by capturing C1 in the area of flagellum emergence), thus facilitating the invasion/infectivity of host cells (79). This TcCRT-C1q-mediated parasite infectivity correlates with significant increases in TcCRT mRNA levels during early (cell contact and penetration) infection stages (36, 66, 68, 69, 72, 79). The TcCRT–C1q interaction can be prevented with anti-TcCRT $F(ab')_2$ fragments (devoid of the C1-binding Fc domains) (80). Indeed, passive immunization of mice with these fragments decreases infectivity (68). Congenital transmission is an important *T. cruzi* transmission pathway. Human pregnancy is a condition of elevated circulating CRT (81, 82). Moreover, human placenta expresses high CRT levels (83). We have recently proposed that the TcCRT/C1q/HuCRT interaction is very important in an *ex vivo* model of infection of human placenta (84), indicating a possible mechanism to explain the congenital transmission.

TcCRT PARTICIPATES IN THE INHIBITION OF TUMOR GROWTH

Cancer is omnipresent in human history, and it also affects most of the living animal species, as a natural phenomenon of sporadic cellular dysfunction. Mammary, prostate, lung, cervix/uterine are just a few examples of cancer that, taken together, have epidemic proportions.

Interestingly, in patients infected with *T. cruzi*, cancer is rare (10, 12). About 80 years ago, Roskin, Ekzempliarskaia, and Klyuyeva, researchers from the former Soviet Union, postulated an experimental anticancer toxic activity derived from this infection. When they inoculated *T. cruzi* extracts, directly in a peritumoral area, in different tumors, both in experimental animals and in humans, similar results related to reduction of tumor size were obtained (10–13, 85, 86). More recently, the parasite capacity to infect preferentially tumor cells, as compared to normal host cells, was described (8). Although, in general, these data suggest an antagonism between *T. cruzi* infection and tumor growth (8), and research progress in these areas was seriously hampered by the intense international political problems of those years (i.e., the Cold War) (11). Although several publications on these issues have appeared during the last decades, the molecular basis of this phenomenon has remained elusive.

We propose that TcCRT is an important mediator of the antitumor effects of *T. cruzi* infection. Similar to HuCRT, TcCRT is antiangiogenic in *in vitro*, *ex vivo*, and *in vivo* models (**Figure 1C**) (3, 87, 88). Moreover, TcCRT inhibits the growth of a mammary adenocarcinoma and a melanoma in different experimental animal models (3, 87–89). The inhibition of tumor angiogenesis was proposed as a cancer therapy almost 40 years ago (90). For this reason, molecules or drugs with capacity to inhibit angiogenesis are currently applicable to a wide variety of tumors, often as a complement to other therapies (91).

Trypanosoma cruzi calreticulin and its N-terminal domain (N-TcCRT) were studied in different experimental set ups in mammals, *Homo sapiens* included (3). Thus, rTcCRT and its N-TcCRT inhibit capillary growth *ex vivo* in *Rattus rattus* aortic rings, morphogenesis, proliferation, and chemotaxis in human umbilical cord endothelial cells (HUVECs) (3) and *in vivo* angiogenesis in the *Gallus gallus* chorioallantoid membrane (CAM) assay (87). TcCRT was overall more effective, in molar terms, than HuCRT (3). Interestingly, in the CAM assay, the antiangiogenic TcCRT effect was fully reverted by polyclonal antibodies against rTcCRT (88).

In agreement with the previously described facts, the *in vivo* antitumor capacity of *T. cruzi* infection is paralleled by the inoculation of rTcCRT, with inhibits by 60–70% the time-course development of a murine mammary methotrexate multiresistant adenocarcinoma (TA3-MTX-R) (7).

T. CRUZI INFECTS NEOPLASTIC CELLS AND PROMOTES AN IMMUNE RESPONSE

Native TcCRT (nTcCRT) on the parasite contacts ECs, mediating internalization of *T. cruzi* and inhibition of tumor growth. This nTcCRT/EC contact may be indirect, mediated by C1q (**Figure 1E**) or by direct binding to scavenger receptors (SRs) (**Figure 1F**). TcCRT has affinity for collagenous structures, a possible explanation for its binding to human C1 and to SRs (66, 68). Fluid-phase Fucoidan, bearing extensive collagen-like sequences, inhibits the binding of CRT to SR-A present on both phagocytic cells (92) and the internalization of TcCRT by ECs (3).

IS NATIVE TcCRT RESPONSIBLE FOR THE ANTITUMOR EFFECT OF *T. CRUZI* INFECTION?

Recombinant TcCRT has important *in vivo* antiangiogenic and antitumor activities (3, 88). The antitumor effect of *T. cruzi* extract has been recently reproduced in a rat model. Experimental animals showed a strong cytotoxic response against tumor, with activation of $CD4^+$ and $CD8^+$ T cells and splenocytes. Moreover, a humoral adaptive immune response is generated. These anti-*T. cruzi* antibodies cross-reacted with tumor cells, inducing an antibody-dependent cellular toxicity *in vitro* (93). In a mouse model, we have reverted the antitumor effect of a *T. cruzi* epimastigote extract with specific antibodies against rTcCRT. Moreover,

anti-rTcCRT $F(ab')_2$ antibodies (devoid of their capacity to interact with C1) neutralize the antitumor activity of *T. cruzi* infection, thus identifying nTcCRT as a mediator of this effect (unpublished data).

HOW DOES TcCRT INHIBIT TUMOR GROWTH IN INDIVIDUALS INFECTED WITH *T. CRUZI*?

We propose that, during *T. cruzi* infection, nTcCRT mediates key alterations in the tumor cell microenvironment leading to an adaptive immune response, with significant antitumor effects. Once in the circulation, *T. cruzi* must swiftly invade ECs (**Figures 1E,F**). Translocated-exteriorized TcCRT (**Figures 1A,B**) (92) will recruit and inactivate plasma complement C1 (**Figure 1D**) and inhibits angiogenesis (**Figure 1C**). This will allow the parasite to contact ECs *via* cC1qR (**Figure 1E**) (77, 94). Otherwise, the chaperone protein could interact directly with SR-A1 on ECs (**Figure 1F**) (95–97). Both pathways may lead to antiangiogenesis and generate a stressful environment where tumor cells will externalize their CRT, as previously shown with other stressing agents, such as Antracyclins (37). C1 recruitment and increased tumor cell phagocytosis by dendritic cells will follow (**Figure 1H**).

On the other hand, an adaptive immune response may be invoked by inoculated TcCRT or by its native counterpart timely externalized by infecting trypomastigotes (66) or present in epimastigote extracts (75). The chaperone protein should reach the surface of tumor cells (or ECs), thus generating a site for C1 binding (**Figure 1G**), followed by phagocytosis of these complexes by dendritic cells (**Figure 1H**). Targeting these activities on tumor cells should be favored by the parasite tropism for these tissues. The relevant novelty of parasite TcCRT is its difference in amino acidic sequence with the mammal (murine, in this case) counterpart. This difference may reach 50%, while mammal CRTs differ among them by no more than 10% (73). Upon arrival to the regional lymph nodes, these dendritic cells will present antigenic peptides derived from TcCRT, thus activating cytotoxic T lymphocytes, among other possibilities. Whether tumor cells can cross-present peptides derived from endocytosed TcCRT to cytotoxic T cells (**Figure 1I**) is a matter of current research in our laboratory. Activated cytotoxic T cells should then return to the tumor site and act against neoplastic tumor cells. Activation of $CD4^+$ T cells *via* MHC II presentation, with stimulation of B cells and resulting ADCC against tumor cells, is a possibility that should also be entertained.

In our murine models, these antitumor effects are better performed by TcCRT, as compared to HuCRT. Among mammals, CRTs are at least 95% homologous in amino acidic differences. CRT immunogenicity across mammal species is thus restricted. On the other hand, because of extensive evolutionary distances, TcCRT amino acidic sequence differs by 50% with its mammal counterparts. Thus, TcCRT is more capable of generating immunogenic epitopes on the surface of mammal tumors. Recently, the expression of CRT has been correlated with a favorable prognosis of cancer. The high expression of CRT on tumor cells has been associated with a high density of infiltrating mature dendritic cells and effector memory T-cell subsets, suggesting that CRT triggers the activation of an adaptive immune response in the tumor microenvironment (98). Thus, TcCRT expressed and secreted by the parasite may be also important in this regard.

CONCLUDING REMARKS

Infection with *T. cruzi* correlates with increased resistant to tumors. Since, during infection, nTcCRT is translocated to the parasite exterior and experimental parenteral administration of rTcCRT mimics the antitumor effects of the infection, nTcCRT is the most likely responsible molecule for these effects. Moreover, the antitumor effects of parasite infection can be specifically reverted by anti-rTcCRT antibodies. Since, in a large set of experimental animals treated with rTcCRT, no clinical deleterious effects have been detected by standard clinical veterinary criteria, we can now propose that rTcCRT or derived domains are interesting immunological tools to be considered in more advanced preclinical trials (e.g., rTcCRT capacity to bind to human mammary tumor cell lines *in vitro*, to subsequently incorporate C1, with increased capacity to induce phagocytosis).

AUTHOR CONTRIBUTIONS

GR-T, PA, and AF designed experiments. GR-T and PA performed experiments. GR-T, PA, and AF interpreted the data. GR-T, PA, and AF generated key reagents. GR-T, PA, and AF wrote, revised, and edited the manuscript. GR-T, PA, and AF approved the manuscript.

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REFERENCES

1. Yoshida N. Molecular basis of mammalian cell invasion by *Trypanosoma cruzi*. *An Acad Bras Cienc* (2006) 78:87–111. doi:10.1590/S0001-37652006000100010
2. Oikonomopoulou K, Brinc D, Hadjisavvas A, Christofi G, Kyriacou K, Diamandis EP. The bifacial role of helminths in cancer: involvement of immune and non-immune mechanisms. *Crit Rev Clin Lab Sci* (2014) 51:138–48. doi:10.3109/10408363.2014.886180
3. Lopez NC, Valck C, Ramirez G, Rodriguez M, Ribeiro C, Orellana J, et al. Antiangiogenic and antitumor effects of *Trypanosoma cruzi* Calreticulin. *PLoS Negl Trop Dis* (2010) 4:e730. doi:10.1371/journal.pntd.0000730
4. Coura JR, Vinas PA. Chagas disease: a new worldwide challenge. *Nature* (2010) 465:S6–7. doi:10.1038/nature09221
5. Perez-Molina JA, Perez AM, Norman FF, Monge-Maillo B, Lopez-Velez R. Old and new challenges in Chagas disease. *Lancet Infect Dis* (2015) 15:1347–56. doi:10.1016/S1473-3099(15)00243-1
6. Tanowitz HB, Weiss LM, Montgomery SP. Chagas disease has now gone global. *PLoS Negl Trop Dis* (2011) 5:e1136. doi:10.1371/journal.pntd.0001136
7. Ramirez-Toloza G, Aguilar-Guzman L, Valck C, Abello P, Ferreira A. Is it all that bad when living with an intracellular protozoan? The role of *Trypanosoma cruzi* calreticulin in angiogenesis and tumor growth. *Front Oncol* (2014) 4:382. doi: 10.3389/fonc.2014.00382

8. Kallinikova VD, Matekin PV, Ogloblina TA, Leikina MI, Kononenko AF, Sokolova NM, et al. [Anticancer properties of flagellate protozoan *Trypanosoma cruzi* Chagas, 1909]. *Izv Akad Nauk Ser Biol* (2001) 3:299–311.
9. Oliveira EC, Leite MS, Miranda JA, Andrade AL, Garcia SB, Luquetti AO, et al. Chronic *Trypanosoma cruzi* infection associated with low incidence of 1,2-dimethylhydrazine-induced colon cancer in rats. *Carcinogenesis* (2001) 22:737–40. doi:10.1093/carcin/22.5.737
10. Klyuyeva NG, Roskin GI. *Biotherapy of Malignant Tumours*. New York: Pergamon Press (1963). 315 p.
11. Krementsov NL. *The Cure: A Story of Cancer and Politics from the Annals of the Cold War*. Chicago: University of Chicago Press (2002). 261 p.
12. Roskin G. Toxin therapy of experimental cancer; the influence of protozoan infections upon transplanted cancer. *Cancer Res* (1946) 6:363–5.
13. Hauschka TS, Goodwin MB. *Trypanosoma cruzi* Endotoxin (KR) in the treatment of malignant mouse tumors. *Science* (1948) 107:600–2. doi:10.1126/science.107.2788.600
14. Folkman J. Fundamental concepts of the angiogenic process. *Curr Mol Med* (2003) 3:643–51. doi:10.2174/1566524033479465
15. Junqueira C, Santos LI, Galvao-Filho B, Teixeira SM, Rodrigues FG, DaRocha WD, et al. *Trypanosoma cruzi* as an effective cancer antigen delivery vector. *Proc Natl Acad Sci U S A* (2011) 108:19695–700. doi:10.1073/pnas.1110030108
16. Sanchez Valdez FJ, Perez Brandan C, Zago MP, Labriola C, Ferreira A, Basombrio MA. *Trypanosoma cruzi* carrying a monoallelic deletion of the calreticulin (TcCRT) gene are susceptible to complement mediated killing and defective in their metacyclogenesis. *Mol Immunol* (2013) 53:198–205. doi:10.1016/j.molimm.2012.08.001
17. Sanchez-Valdez FJ, Perez Brandan C, Ramirez G, Uncos AD, Zago MP, Cimino RO, et al. A monoallelic deletion of the TcCRT gene increases the attenuation of a cultured *Trypanosoma cruzi* strain, protecting against an in vivo virulent challenge. *PLoS Negl Trop Dis* (2014) 8:e2696. doi:10.1371/journal.pntd.0002696
18. Michalak M, Groenendyk J, Szabo E, Gold LI, Opas M. Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem J* (2009) 417:651–66. doi:10.1042/BJ20081847
19. Lu YC, Weng WC, Lee H. Functional roles of calreticulin in cancer biology. *Biomed Res Int* (2015) 2015:526524. doi:10.1155/2015/526524
20. Michalak M, Milner RE, Burns K, Opas M. Calreticulin. *Biochem J* (1992) 285(Pt 3):681–92. doi:10.1042/bj2850681
21. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M. Calreticulin: one protein, one gene, many functions. *Biochem J* (1999) 344(Pt 2):281–92. doi:10.1042/0264-6021:3440281
22. Baksh S, Michalak M. Expression of calreticulin in *Escherichia coli* and identification of its Ca2+ binding domains. *J Biol Chem* (1991) 266:21458–65.
23. Arias I, Sepulveda C, Bravo P, Hamilton-West C, Maldonado I, Ferreira A. Comparative effect of human and *Trypanosoma cruzi* calreticulin in wound healing. *J Tissue Eng Regen Med* (2012) 9(1):41–54. doi:10.1002/term.1613
24. Gold LI, Rahman M, Blechman KM, Greives MR, Churgin S, Michaels J, et al. Overview of the role for calreticulin in the enhancement of wound healing through multiple biological effects. *J Investig Dermatol Symp Proc* (2006) 11:57–65. doi:10.1038/sj.jidsymp.5650011
25. Nanney LB, Woodrell CD, Greives MR, Cardwell NL, Pollins AC, Bancroft TA, et al. Calreticulin enhances porcine wound repair by diverse biological effects. *Am J Pathol* (2008) 173:610–30. doi:10.2353/ajpath.2008.071027
26. Burns K, Duggan B, Atkinson EA, Famulski KS, Nemer M, Bleackley RC, et al. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* (1994) 367:476–80. doi:10.1038/367476a0
27. Dedhar S, Rennie PS, Shago M, Hagesteijn CY, Yang H, Filmus J, et al. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* (1994) 367:480–3. doi:10.1038/367480a0
28. Holaska JM, Black BE, Love DC, Hanover JA, Leszyk J, Paschal BM. Calreticulin is a receptor for nuclear export. *J Cell Biol* (2001) 152:127–40. doi:10.1083/jcb.152.1.127
29. Iakova P, Wang GL, Timchenko L, Michalak M, Pereira-Smith OM, Smith JR, et al. Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. *EMBO J* (2004) 23:406–17. doi:10.1038/sj.emboj.7600052
30. Nickenig G, Michaelsen F, Muller C, Berger A, Vogel T, Sachinidis A, et al. Destabilization of AT(1) receptor mRNA by calreticulin. *Circ Res* (2002) 90:53–8. doi:10.1161/hh0102.102503
31. Singh NK, Atreya CD, Nakhasi HL. Identification of calreticulin as a rubella virus RNA binding protein. *Proc Natl Acad Sci U S A* (1994) 91:12770–4. doi:10.1073/pnas.91.26.12770
32. Timchenko LT, Iakova P, Welm AL, Cai ZJ, Timchenko NA. Calreticulin interacts with C/EBPalpha and C/EBPbeta mRNAs and represses translation of C/EBP proteins. *Mol Cell Biol* (2002) 22:7242–57. doi:10.1128/MCB.22.20.7242-7257.2002
33. Totary-Jain H, Naveh-Many T, Riahi Y, Kaiser N, Eckel J, Sasson S. Calreticulin destabilizes glucose transporter-1 mRNA in vascular endothelial and smooth muscle cells under high-glucose conditions. *Circ Res* (2005) 97:1001–8. doi:10.1161/01.RES.0000189260.46084.e5
34. Obeid M, Panaretakis T, Joza N, Tufi R, Tesniere A, van Endert P, et al. Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis. *Cell Death Differ* (2007) 14:1848–50. doi:10.1038/sj.cdd.4402201
35. Obeid M, Panaretakis T, Tesniere A, Joza N, Tufi R, Apetoh L, et al. Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from "silent" to immunogenic. *Cancer Res* (2007) 67:7941–4. doi:10.1158/0008-5472.CAN-07-1622
36. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* (2007) 13:54–61. doi:10.1038/nm1523
37. Obeid M, Tesniere A, Panaretakis T, Tufi R, Joza N, van Endert P, et al. Ecto-calreticulin in immunogenic chemotherapy. *Immunol Rev* (2007) 220:22–34. doi:10.1111/j.1600-065X.2007.00567.x
38. Tesniere A, Apetoh L, Ghiringhelli F, Joza N, Panaretakis T, Kepp O, et al. Immunogenic cancer cell death: a key-lock paradigm. *Curr Opin Immunol* (2008) 20:504–11. doi:10.1016/j.coi.2008.05.007
39. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* (2005) 123:321–34. doi:10.1016/j.cell.2005.08.032
40. Zamanian M, Veerakumarasivam A, Abdullah S, Rosli R. Calreticulin and cancer. *Pathol Oncol Res* (2013) 19:149–54. doi:10.1007/s12253-012-9600-2
41. Chen CN, Chang CC, Su TE, Hsu WM, Jeng YM, Ho MC, et al. Identification of calreticulin as a prognosis marker and angiogenic regulator in human gastric cancer. *Ann Surg Oncol* (2009) 16:524–33. doi:10.1245/s10434-008-0243-1
42. Lwin ZM, Guo C, Salim A, Yip GW, Chew FT, Nan J, et al. Clinicopathological significance of calreticulin in breast invasive ductal carcinoma. *Mod Pathol* (2010) 23:1559–66. doi:10.1038/modpathol.2010.173
43. Eggleton P, Bremer E, Dudek E, Michalak M. Calreticulin, a therapeutic target? *Expert Opin Ther Targets* (2016) 25:1–11. doi:10.1517/14728222.2016.1164695
44. Yao L, Pike SE, Tosato G. Laminin binding to the calreticulin fragment vasostatin regulates endothelial cell function. *J Leukoc Biol* (2002) 71:47–53.
45. Pike SE, Yao L, Jones KD, Cherney B, Appella E, Sakaguchi K, et al. Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. *J Exp Med* (1998) 188:2349–56. doi:10.1084/jem.188.12.2349
46. Pike SE, Yao L, Setsuda J, Jones KD, Cherney B, Appella E, et al. Calreticulin and calreticulin fragments are endothelial cell inhibitors that suppress tumor growth. *Blood* (1999) 94:2461–8.
47. Cai KX, Tse LY, Leung C, Tam PK, Xu R, Sham MH. Suppression of lung tumor growth and metastasis in mice by adeno-associated virus-mediated expression of vasostatin. *Clin Cancer Res* (2008) 14:939–49. doi:10.1158/1078-0432.CCR-07-1930
48. Jazowiecka-Rakus J, Jarosz M, Kozlowska D, Sochanik A, Szala S. Combination of vasostatin and cyclophosphamide in the therapy of murine melanoma tumors. *Acta Biochim Pol* (2007) 54:125–33.
49. Yao L, Pike SE, Pittaluga S, Cherney B, Gupta G, Jaffe ES, et al. Anti-tumor activities of the angiogenesis inhibitors interferon-inducible protein-10 and the calreticulin fragment vasostatin. *Cancer Immunol Immunother* (2002) 51:358–66. doi:10.1007/s00262-002-0294-2
50. Shu Q, Li W, Li H, Sun G. Vasostatin inhibits VEGF-induced endothelial cell proliferation, tube formation and induces cell apoptosis under oxygen deprivation. *Int J Mol Sci* (2014) 15:6019–30. doi:10.3390/ijms15046019
51. McCauliffe DP, Zappi E, Lieu TS, Michalak M, Sontheimer RD, Capra JD. A human Ro/SS-A autoantigen is the homologue of calreticulin and is highly homologous with onchocercal RAL-1 antigen and an aplysia "memory molecule". *J Clin Invest* (1990) 86:332–5. doi:10.1172/JCI114704

52. Ferreira CA, Da Silva Vaz I, da Silva SS, Haag KL, Valenzuela JG, Masuda A. Cloning and partial characterization of a Boophilus microplus (Acari: Ixodidae) calreticulin. *Exp Parasitol* (2002) 101:25–34. doi:10.1016/S0014-4894(02)00032-2
53. Smith MJ. Nucleotide sequence of a *drosophila melanogaster* gene encoding a calreticulin homologue. *DNA Seq* (1992) 3:247–50. doi:10.3109/10425179209034025
54. Kasper G, Brown A, Eberl M, Vallar L, Kieffer N, Berry C, et al. A calreticulin-like molecule from the human hookworm *Necator americanus* interacts with C1q and the cytoplasmic signalling domains of some integrins. *Parasite Immunol* (2001) 23:141–52. doi:10.1046/j.1365-3024.2001.00366.x
55. Khalife J, Pierce RJ, Godin C, Capron A. Cloning and sequencing of the gene encoding *Schistosoma mansoni* calreticulin. *Mol Biochem Parasitol* (1993) 62:313–5. doi:10.1016/0166-6851(93)90120-M
56. Rokeach LA, Zimmerman PA, Unnasch TR. Epitopes of the *Onchocerca volvulus* RAL1 antigen, a member of the calreticulin family of proteins, recognized by sera from patients with onchocerciasis. *Infect Immun* (1994) 62:3696–704.
57. Smith MJ. A C. elegans gene encodes a protein homologous to mammalian calreticulin. *DNA Seq* (1992) 2:235–40. doi:10.3109/10425179209020808
58. Aguillon JC, Ferreira L, Perez C, Colombo A, Molina MC, Wallace A, et al. Tc45, a dimorphic *Trypanosoma cruzi* immunogen with variable chromosomal localization, is calreticulin. *Am J Trop Med Hyg* (2000) 63:306–12.
59. Joshi M, Pogue GP, Duncan RC, Lee NS, Singh NK, Atreya CD, et al. Isolation and characterization of *Leishmania donovani* calreticulin gene and its conservation of the RNA binding activity. *Mol Biochem Parasitol* (1996) 81:53–64. doi:10.1016/0166-6851(96)02676-X
60. Labriola C, Cazzulo JJ, Parodi AJ. *Trypanosoma cruzi* calreticulin is a lectin that binds monoglucosylated oligosaccharides but not protein moieties of glycoproteins. *Mol Biol Cell* (1999) 10:1381–94. doi:10.1091/mbc.10.5.1381
61. Oladiran A, Belosevic M. *Trypanosoma carassii* calreticulin binds host complement component C1q and inhibits classical complement pathway-mediated lysis. *Dev Comp Immunol* (2010) 34:396–405. doi:10.1016/j.dci.2009.11.005
62. Denecke J, Carlsson LE, Vidal S, Hoglund AS, Ek B, van Zeijl MJ, et al. The tobacco homolog of mammalian calreticulin is present in protein complexes in vivo. *Plant Cell* (1995) 7:391–406. doi:10.1105/tpc.7.4.391
63. Jaworski DC, Higgins JA, Radulovic S, Vaughan JA, Azad AF. Presence of calreticulin in vector fleas (Siphonaptera). *J Med Entomol* (1996) 33:482–9. doi:10.1093/jmedent/33.3.482
64. Marcelain K, Colombo A, Molina MC, Ferreira L, Lorca M, Aguillon JC, et al. Development of an immunoenzymatic assay for the detection of human antibodies against *Trypanosoma cruzi* calreticulin, an immunodominant antigen. *Acta Trop* (2000) 75:291–300. doi:10.1016/S0001-706X(00)00062-0
65. Conte I, Labriola C, Cazzulo JJ, Docampo R, Parodi AJ. The interplay between folding-facilitating mechanisms in *Trypanosoma cruzi* endoplasmic reticulum. *Mol Biol Cell* (2003) 14:3529–40. doi:10.1091/mbc.E03-04-0228
66. Ferreira V, Valck C, Sanchez G, Gingras A, Tzima S, Molina MC, et al. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*. *J Immunol* (2004) 172:3042–50. doi:10.4049/jimmunol.172.5.3042
67. Souto-Padron T, Labriola CA, de Souza W. Immunocytochemical localisation of calreticulin in *Trypanosoma cruzi*. *Histochem Cell Biol* (2004) 122:563–9. doi:10.1007/s00418-004-0724-7
68. Ramirez G, Valck C, Molina MC, Ribeiro CH, Lopez N, Sanchez G, et al. *Trypanosoma cruzi* calreticulin: a novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity. *Immunobiology* (2011) 216:265–73. doi:10.1016/j.imbio.2010.04.001
69. Ramirez G, Valck C, Ferreira VP, Lopez N, Ferreira A. Extracellular *Trypanosoma cruzi* calreticulin in the host-parasite interplay. *Trends Parasitol* (2011) 27:115–22. doi:10.1016/j.pt.2010.12.007
70. Stuart GR, Lynch NJ, Day AJ, Schwaeble WJ, Sim RB. The C1q and collectin binding site within C1q receptor (cell surface calreticulin). *Immunopharmacology* (1997) 38:73–80. doi:10.1016/S0162-3109(97)00076-3
71. Stuart GR, Lynch NJ, Lu J, Geick A, Moffatt BE, Sim RB, et al. Localisation of the C1q binding site within C1q receptor/calreticulin. *FEBS Lett* (1996) 397:245–9. doi:10.1016/S0014-5793(96)01156-8
72. Ferreira V, Molina MC, Schwaeble W, Lemus D, Ferreira A. Does *Trypanosoma cruzi* calreticulin modulate the complement system and angiogenesis? *Trends Parasitol* (2005) 21:169–74. doi:10.1016/j.pt.2005.02.005
73. Ferreira V, Molina MC, Valck C, Rojas A, Aguilar L, Ramirez G, et al. Role of calreticulin from parasites in its interaction with vertebrate hosts. *Mol Immunol* (2004) 40:1279–91. doi:10.1016/j.molimm.2003.11.018
74. Valck C, Ramirez G, Lopez N, Ribeiro CH, Maldonado I, Sanchez G, et al. Molecular mechanisms involved in the inactivation of the first component of human complement by *Trypanosoma cruzi* calreticulin. *Mol Immunol* (2010) 47:1516–21. doi:10.1016/j.molimm.2010.01.019
75. Sosoniuk E, Vallejos G, Kenawy H, Gaboriaud C, Thielens N, Fujita T, et al. *Trypanosoma cruzi* calreticulin inhibits the complement lectin pathway activation by direct interaction with L-Ficolin. *Mol Immunol* (2014) 60:80–5. doi:10.1016/j.molimm.2014.03.014
76. Ramirez G, Valck C, Aguilar L, Kemmerling U, Lopez-Munoz R, Cabrera G, et al. Roles of *Trypanosoma cruzi* calreticulin in parasite-host interactions and in tumor growth. *Mol Immunol* (2012) 52:133–40. doi:10.1016/j.molimm.2012.05.006
77. Eggleton P, Tenner AJ, Reid KB. C1q receptors. *Clin Exp Immunol* (2000) 120:406–12. doi:10.1046/j.1365-2249.2000.01218.x
78. Malhotra R. Collectin receptor (C1q receptor): structure and function. *Behring Inst Mitt* (1993) (93):254–61.
79. Rimoldi MT, Tenner AJ, Bobak DA, Joiner KA. Complement component C1q enhances invasion of human mononuclear phagocytes and fibroblasts by *Trypanosoma cruzi* trypomastigotes. *J Clin Invest* (1989) 84:1982–9. doi:10.1172/JCI114388
80. Aguilar L, Ramirez G, Valck C, Molina MC, Rojas A, Schwaeble W, et al. F(ab')2 antibody fragments against *Trypanosoma cruzi* calreticulin inhibit its interaction with the first component of human complement. *Biol Res* (2005) 38:187–95. doi:10.4067/S0716-97602005000200008
81. Crawford KE, Kalionis B, Stevenson JL, Brennecke SP, Gude NM. Calreticulin has opposing effects on the migration of human trophoblast and myometrial endothelial cells. *Placenta* (2012) 33:416–23. doi:10.1016/j.placenta.2012.02.003
82. Gu VY, Wong MH, Stevenson JL, Crawford KE, Brennecke SP, Gude NM. Calreticulin in human pregnancy and pre-eclampsia. *Mol Hum Reprod* (2008) 14:309–15. doi:10.1093/molehr/gan017
83. Houen G, Koch C. Human placental calreticulin: purification, characterization and association with other proteins. *Acta Chem Scand* (1994) 48:905–11. doi:10.3891/acta.chem.scand.48-0905
84. Castillo C, Ramirez G, Valck C, Aguilar L, Maldonado I, Rosas C, et al. The interaction of classical complement component C1 with parasite and host calreticulin mediates *Trypanosoma cruzi* infection of human placenta. *PLoS Negl Trop Dis* (2013) 7:e2376. doi:10.1371/journal.pntd.0002376
85. Hauschka T, Sake L, Rlair M. *Trypanosoma cruzi* in treatment of mouse tumours. *J Natl Cancer Inst* (1947) 7:189.
86. Malisoff WM. The action of the endotoxin of *Trypanosoma cruzi* (KR) on malignant mouse tumors. *Science* (1947) 106(2763):591–4. doi:10.1126/science.106.2763.591-a
87. Molina MC, Ferreira V, Valck C, Aguilar L, Orellana J, Rojas A, et al. An in vivo role for *Trypanosoma cruzi* calreticulin in antiangiogenesis. *Mol Biochem Parasitol* (2005) 140:133–40. doi:10.1016/j.molbiopara.2004.12.014
88. Toledo V, Ramirez G, Valck C, Lopez N, Ribeiro CH, Maldonado I, et al. Comparative in vivo antiangiogenic effects of calreticulin from *Trypanosoma cruzi* and Homo sapiens sapiens. *Biol Res* (2010) 43:287–9. doi:10.4067/S0716-97602010000300004
89. Aguilar-Guzman L, Lobos-Gonzalez L, Rosas C, Vallejos G, Falcon C, Sosoniuk E, et al. Human survivin and *Trypanosoma cruzi* calreticulin act in synergy against a murine melanoma in vivo. *PLoS One* (2014) 9:e95457. doi:10.1371/journal.pone.0095457
90. Baillie CT, Winslet MC, Bradley NJ. Tumour vasculature – a potential therapeutic target. *Br J Cancer* (1995) 72:257–67. doi:10.1038/bjc.1995.323
91. Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev* (2000) 52:237–68.
92. Berwin B, Hart JP, Rice S, Gass C, Pizzo SV, Post SR, et al. Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. *EMBO J* (2003) 22:6127–36. doi:10.1093/emboj/cdg572
93. Ubillos L, Freire T, Berriel E, Chiribao ML, Chiale C, Festari MF, et al. *Trypanosoma cruzi* extracts elicit protective immune response against chemically induced colon and mammary cancers. *Int J Cancer* (2016) 138:1719–31. doi:10.1002/ijc.29910

94. van den Berg RH, Faber-Krol MC, Sim RB, Daha MR. The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. *J Immunol* (1998) 161:6924–30.
95. Adachi H, Tsujimoto M, Arai H, Inoue K. Expression cloning of a novel scavenger receptor from human endothelial cells. *J Biol Chem* (1997) 272:31217–20. doi:10.1074/jbc.272.50.31217
96. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol* (2013) 13:621–34. doi:10.1038/nri3515
97. Zani IA, Stephen SL, Mughal NA, Russell D, Homer-Vanniasinkam S, Wheatcroft SB, et al. Scavenger receptor structure and function in health and disease. *Cells* (2015) 4:178–201. doi:10.3390/cells4020178
98. Fucikova J, Becht E, Iribarren K, Goc J, Remark R, Damotte D, et al. Calreticulin expression in human non-small cell lung cancers correlates with increased accumulation of antitumor immune cells and favorable prognosis. *Cancer Res* (2016) 76:1746–56. doi: 10.1158/0008-5472.CAN-15-1142

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REVIEW
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Dendritic Cells: A Double-Edged Sword in Immune Responses during Chagas Disease

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Dendritic cells (DCs) are the most important member of the antigen presenting cells group due to their ability to recognize antigen at the infection site and their high specialized antigen internalization capacity. These cells have central role in connecting the innate and adaptive immune responses against *Trypanosoma cruzi*, the causative agent of Chagas disease. These first line defense cells modulate host immune response depending on type, maturation level, cytokine milieu and DC receptor involved in the interactions with *T. cruzi*, influencing the development of the disease clinic forms. Here, we present a review of DCs–*T. cruzi* interactions both in human and murine models, pointing out the parasite ability to manipulate DCs activity for the purpose of evading innate immune response and assuring its own survival and persistence.

Keywords: chagas disease, *Trypanosoma cruzi*, dendritic cell, immunoregulation, evasion strategy

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INTRODUCTION

More than a century has passed since Carlos Chagas discovered the pathogen *Trypanosoma cruzi* and the natural way of its transmission to humans and animals, and elucidated the corresponding human disease it causes (Kropf and Sá, 2009). Chagas disease is also transmitted through blood transfusions or organ transplants, vertically from mother to child through the placenta, and through contaminated food (Andrade et al., 2014). This neglected disease affects predominantly deprived people and induces social and economic impacts by decreasing patient's productivity and earning capacity (Hotez et al., 2007).

With 28,000 new cases per year and 8,000 newborns infected during gestation, Chagas disease affects about 8 million people, beyond 65 million live in areas of exposure and are at risk of contracting the disease (World Health Organization, 2015). Despite the efforts to reduce Chagas disease and the number of infections over the last two decades, it is still endemic in Latin America (Rassi et al., 2010; Strasen et al., 2013) and, as result of global migration, the number of people infected with *T. cruzi* is increasing in North America, Europe, Japan and Oceania (non-endemic areas) (Schmunis, 2007). Since Chagas disease crosses boundaries and spreads, it becomes not only a burden for the endemic countries but a worldwide health concern (Andrade et al., 2014).

Treatment of Chagas disease is one of the greatest therapeutic challenges in tropical medicine since the only drugs approved for human treatment — Nifurtimox and Benznidazole — date from the early 1970s and have carcinogenic properties conferred to nitrofuran and nitroimidazole, their active chemical groups, respectively (Bern, 2011; Wilkinson et al., 2011). Nevertheless, both

medicines share similar features: necessity of prolonged administration, effectiveness related to the acute phase and different susceptibility among *T. cruzi* strains (Bermudez et al., 2015). Aditionally, Nifurtimox prescription has been abolished in Brazil, Argentina, Uruguay, Chile and US due to its toxic effects over central nervous system, genotoxicity, and reduced efficacy (Wilkinson et al., 2011).

The natural disease progression consists of an acute phase that can be followed by an asymptomatic indeterminate phase, which represents most of the cases and, one-third of infected population progress to the chronic phase that may lead to death (Machado et al., 2013). Early clinical manifestations include headache, fever, and cough, which are non-specific signs; consequently most of infected individuals are neither notified nor treated. Symptomatic chronic stage of the disease usually occurs from 10 to 25 years after infection and typical manifestations are mild to severe cardiomyopathy and/or dilated digestive tract (megaoesophagus and megacolon) (Steverding, 2014). The mechanisms responsible for patient progression from the indeterminate to the symptomatic chronic phase are not completely understood, although the immunological events initiated during the acute phase undoubtedly drive the organism toward the development of a protective or deleterious immune response (Andrade et al., 2014).

It is unanimity that during the acute phase, dendritic cells (DC), along with macrophages, and natural killer (NK) cells, guarantee the host first line of defense against the parasite (Watanabe Costa et al., 2016). Guide by this context, our purpose is to present a review about DC–*T. cruzi* interaction pointing out the parasite ability to evade innate immune response to assure its own survival and persistence.

Trypanosoma cruzi

Trypanosoma cruzi is an obligate intracellular protozoan of the Kinetoplastea Class, characterized by the presence of one flagellum and a single mitochondrion comprising the kinetoplast, a specialized DNA-containing structure. The Order Trypanosomatida comprises other parasites responsible for severe diseases in humans and other species, for instance *Leishmania* sp. (leishmaniasis), *Trypanosoma brucei* (African trypanosomiasis), *Phytomonas* sp. (plant diseases), and *Crithidia* sp. (arthropods diseases) (Stevens, 2008), that show adaptability toward their hosts with numerous sophisticated immune evasion strategies (D'Avila-Levy et al., 2015).

Trypanosoma cruzi strains present diversity in morphology, infection capacity, cell surface predominant antigens and other biological and biochemical features (de Lana et al., 2010). In 2009, *the Second Satellite Meeting* held in Buzios, Brazil suggested six Discrete Typing Units (DTUs) for classifying the several *T. cruzi* strains, named TcI to TcVI (Zingales et al., 2009). A DTU is a population set that is more genetically related among themselves than to any other population, showing common genetic, immunological and molecular markers (Tibayrenc, 1998). These DTUs subsets have different distribution among the American continent due to parasite adaptations to different vectors and reservoirs (Zingales et al., 2012), and, although, some characteristics are shared within the same DTU, differences in many aspects can be found among strains. *T. cruzi* Colombian and G strains, belonging to TcI group, are a clear example of this intra-DTU diversity. Colombian strain, isolated from humans, is highly infective (Ramírez et al., 2010), but no human infections were reported from G strain, which was isolated from anal gland secretions of an opossum (Deane et al., 1984). *T.* cruzi G strain has gp35/50 as predominant surface glycoprotein that appears to be related to its poor internalization by humans cells (Yoshida, 2006).

T. cruzi life cycle alternates between reduviid bug vectors and mammal hosts. Domestic and wild animals like opossum, bats, armadillo, and monkey may also act as reservoir host (Coura et al., 2002). The parasite presents different forms during its life cycle – epimastigotes remains in the insect gut; non-divinding and infective metacyclic trypomastigotes (MT) find in the insect feces and/or urine; bloodstream trypomastigotes that can circulate in the mammalian blood and, finally, the intracellular, proliferative and rounded amastigote form. A triatomine insect picks up the parasite trypomastigote forms by feeding on the blood of an infected mammal (Teixeira et al., 2009) and, once inside the vector, those forms differentiate into epimastigotes and multiply in midgut (Manchola et al., 2015). After migration to the bug's hindgut, epimastigotes attach to the waxy gut cuticle by their flagella and differentiate into infectious MT, which will be deposited along with feces/urine on the skin of the victim (Muñoz-Saravia et al., 2012). The parasite penetrates the new host through lesions caused by its bite or a variety of mucosal membranes. MTs invade host cells entering a parasitophorous vacuole (Romano et al., 2012), which fuses to lysosomes. Once free in the cytoplasm, the parasite differentiates into amastigotes that undergo binary fission multiplication and transform back to trypomastigotes, which are released upon rupture of the host cell membrane and infect neighboring cells or enter the bloodstream (Stecconi-Silva et al., 2003; Tonelli et al., 2004). Bloodstream trypomastigotes may begin another infection cycle when they are taken in the blood feeding of the vector (Bern, 2015). Humans and animals can be infected orally through the ingestion of food and drink contaminated by crushed infected insect vectors or their feces (Nóbrega et al., 2009; Bastos et al., 2010).

Immune evasion strategies developed by *T. cruzi* are essential to the establishment of a long-life infection. Complement inactivation, escape from phagolysosome, antibodies with no *T-cruzi* specificity and delayed immune response are some examples of those strategies (Reviewed by Nardy et al., 2015; Cardoso et al., 2016). Another mechanism involved in parasite persistence is the manipulation of DC functions, which impairs an adequate host immune defense.

DENDRITIC CELLS

Dendritic cells are bone-marrow-derived cells that belong to the antigen presenting cells (APC) group, being considered the most important member due to their high capacity to recognize and

internalize antigens at the infection site (Haniffa et al., 2009; Collin et al., 2013). They link innate and adaptive immune responses by capturing, processing and expressing antigens in the cell surface membrane (Planelles et al., 2003; Steinman, 2007). Immature DCs have a wide range of innate receptors that enables the recognition of pathogens via pattern recognition receptors (PRRs), which activate DCs through signaling pathways eliciting their maturation (Pearce and Everts, 2015). Toll-like receptors (TLRs), abundant in APC, are one of the best-characterized PPRs and efficiently detect pathogen-associated molecular patterns (PAMPs) that are located on the cell surface or in the lumen of endosomes. The presence of TLRs on the cell surface or in the lumen of endosomes enables efficient pathogen recognition and the development of an adequate innate immune response; i.e., TLR2, located in DCs plasma membrane, senses various components of pathogens and its stimulation induces the production of various proinflammatory cytokines. TLR9, located in DCs endolysosome, is involved in virus, bacteria, protozoa nucleic acid recognition and its activation also leads to the production of proinflammatory cytokines (Takeuchi and Akira, 2010). After antigen recognition, DCs can travel along the body from peripheral tissues to lymphoid organs/tissues where they present the processed antigens through their major histocompatibility complexes (MHC) to T cells (Lipscomb and Masten, 2002). Maturation process comprises differentiation from antigen-capturing specialized cells to presenting and stimulating specialized cells. Mature DCs can be identified by morphological aspects like cytoplasmic extensions and abundant intracellular structures (lysosomes, endosomes, granules, etc.) related to antigen processing and by modulation of molecular markers such as up-regulation of CD83, of co-stimulatory molecules like CD80 (or B7-1) and CD86 (or B7-2) and MHC (O'Neill et al., 2004).

Dendritic cells present antigens to lymphocytes $CD8^+$ and $CD4^+$ T by MHC class I and MHC class II, respectively (Blum et al., 2013). For MHC class I molecules, these antigens originate from intracellular sources; for MHC class II, from exogenous sources. Some DCs have an atypical ability, called cross-presentation that allows to load peptides from exogenous antigens onto MHC class I molecules (Vyas et al., 2008; Neefjes et al., 2011; Segura and Amigorena, 2015). MHC class I molecules are expressed by all nucleated cells and their antigen presentation pathway consists in a series of reactions: (1) intracellular proteins are degraded by the proteasome; (2) the peptides are delivered to the endoplasmic reticulum by the transporter associated with antigen processing complex; (3) antigens are loaded onto MHC class I molecules; (4) peptide–MHC class I complexes are transported via the Golgi to cell surface for presentation to $CD8^+$ T cells (Vyas et al., 2008; Neefjes et al., 2011). Recently it was demonstrated that infection of HeLa cells with *T. cruzi* Y strain promotes a down-regulation of the immunoproteasome subunits biosynthesis as well as the MHC class I molecule expression, which could be considered a mechanism of parasite persistence inside the cell (Camargo et al., 2014). Unlike MHC class I expression, MHC class II are mainly expressed by APCs such as DCs, macrophages and B cells (Vyas et al., 2008; Neefjes et al., 2011). Extracellular antigens are taken up by APCs and placed into the phagosome. This compartment fuses with lysosomes to form phagolysosomes, where MHC class II molecules interact with the antigens. Peptide-loaded MHC class II molecules are then transported to the cell surface where they engage antigen-specific $CD4^+$ T cells (Vyas et al., 2008; Neefjes et al., 2011). Curiously, MHC II molecules are in constant recycle and degradation process in immature DCs, but mature DCs exhibits a stable and prolonged antigen presentation (den Haan et al., 2014). It is worth mentioning that DCs antigen presentation is not enough for T lymphocytes activation and proliferation. Co-stimulatory molecules expression and cytokine production are also required and they are efficiently provided by mature DCs (den Haan et al., 2014). Following activation via TLRs, DCs may produce acute innate cytokines involved in local and systemic responses such as IL-1β, IL-6, IL-8, IL-12, and TNF-α (Verhasselt et al., 1997; Holdsworth and Can, 2015), however, DCs, under specific conditions, are also able to produce IL-10 and TGF-β for directing a regulatory response (**Table 1**).

Different DC subsets and maturation levels can produce distinct kinds of cytokines or co-stimulatory molecules that can lead either to an inflammatory or a regulatory response. Among DC subsets, myeloid (mDC or classical DC), plasmocytoid (pDC), Langerhans cells (LCs), and derived from monocytes (monocyte DC) are some well-known examples. In murines, mDCs are composed of two main groups: resident and migratory, which are further divided into two subsets: Batf3-dependent and IRF4-dependent DCs (Reviewed by Segura, 2016). mDC Batf3-dependent/migratory expresses CD11c, Clec9A, XCR1, CD103, and CD207; the resident one expresses CD11c, Clec9A, XCR1, and CD8. On the other hand, mDC IRF4-dependent/resident expresses CD11c, CD11b, CD172a; the same markers are found in the migratory subsets along with CD206. The major markers for pDC are CD11c, Ly6c, B220 and SiglecH. For monocyte DC, they are CD11c, CD11b, CD64, FcεRI, CD206, CD14, CD172a, and Ly6c (Reviewed by Segura, 2016). Finally, LCs is a special DC population present in epidermis and other stratified squamous epithelia, such as oral and genital mucous membranes and bronchus. Despite of being associated with these tissues, LCs may differentiate into migratory cells for antigen presentation; their principal markers are CD11c, CD207, EpCAM, and E-cadherin. Human mDCs classical markers are $CD1c^+$, Dectin 1 (CLEC 7A), and Dectin 2 (CLEC6A). Regarding pDCs, CD303 (CLEC4C), CD304 (neuropilin), and CD123 (IL-3R) are human usual markers. Monocyte DCs may express CD14, CD209 (DC-SIGN), CD16, and CD1c (Reviewed by Collin et al., 2013). Lewis and Reizis (2012) have proposed the existence of two more kinds of DCs within these subsets: "receptors", cells more specialized in capturing antigens and producing cytokines and "presenters", cells that benefit from these cytokines and travel to lymph nodes for presenting the antigens. It represents another level of specialization among DCs, stating their diversity and their capacity to guide polarity, magnitude and specificity of immune responses (Lewis and Reizis, 2012).

Some studies have demonstrated that acute and chronic phases of Chagas disease require different polarizations of

TABLE 1 | Study conditions in murine models.

DC source	Markers	*T. cruzi* strain	Study conditions	Disease phase	Result	Reference
Spleen	CD11c	Tehuantepec	Tehuantepec *in vivo* infection	Acute	↓CD86; ↓ migration capacity	Chaussabel et al., 2003
Bone-marrow	CD11b; CD11c	RA	RA *in vitro* infection	Acute	↓Cytokine production; ↓endocytic capacity	Poncini et al., 2008
Spleen	CD11c	RA	RA *in vivo* infection	Acute	↓Cytokine production; ↓MHC II expression	Alba Soto et al., 2003
Spleen	CD11c	K98	K98 *in vivo* infection	Acute	↑Co-stimulatory molecules expression; ↑MHC II expresion	Alba Soto et al., 2003
Bone-marrow	CD11b; CD11c	AQ1.7, MUTUM (TcI); 1849, 2369 (TcII)	*In vitro* infections	Acute	↑Anti-inflammatory cytokine production; ↑ TLR2 expression	da Costa et al., 2014
Spleen	CD11c	Y	*In vivo* infection using different mouse lineages	Acute	↓Co-stimulatory molecules expression; ↓MHC II expression	Planelles et al., 2003
Bone-marrow	CD11c	Queretaro	*In vivo* infection using MIF-deficient mice	Acute	IL-12 role in protection	Terrazas et al., 2011
Bone-marrow	CD11b; CD11c	RA	IL-10-deficient DCs were injetec along RA strain in mice	From acute to chronic	↓Cytokine production; ↓MHC II expression	Alba Soto et al., 2010
Bone-marrow	CD11b; CD11c	RA	*In vitro* infections	Acute	↑Anti-inflammatory cytokine production; ↓T cell induction	Poncini et al., 2010
Bone-marrow DCs and NK cells	CD11c	RA	Study of NK cells and DCs interaction in mice	Acute	Unbalanced DC population	Batalla et al., 2013
Spleen	CD11b; CD11c	Colombian	300, 3000, and 30000 initial inocula in mice	From acute to chronic	Less favorable host response in medium inocula	Borges et al., 2012
Bone-marrow		CL Brener	*In vitro* assay using CpG islands	Acute	Recognition by TLR9; ↑IL-12 and IFN-γ production	Bartholomeu et al., 2008
Spleen	CD11c	Y	*In vivo* infection studying TLR9 expression	Acute	↑Recognition by TLR9; ↑inflammatory cytokine production	Gravina et al., 2013
Bone-marrow		Tulahuen	*In vivo* and *in vitro* infection using MyD88/TRIF-deficient mice	Acute	No IFN-β production and no parasite clearance	Koga et al., 2006
Spleen	CD11c	CL Brener	*In vivo* infection using UNC93B1deficient mice	Acute	No correct TLR activation; ↓IL-12p40 and IFN-γ	Caetano et al., 2011
Spleen	CD11c	Dm28c	*In vitro* infections using B2R-deficient DCs	Acute	No IL-12 production	Monteiro et al., 2007
Spleen	CD11c	Dm28c	*In vitro* infections using C5a antagonist	Acute	No IL-12p40/70 and IFN-γ production	Schmitz et al., 2014
Spleen	CD11c	RA	*In vitro* infections using Gal-1-deficient DCs	Acute	Regulatory T Cell induction	Poncini et al., 2015
Spleen	CD11c	Tehuantepec	*In vivo* infections studying Gal-3 expression	Acute	↑Gal-3 expression;↓Migration capacity	Vray et al., 2004
Bone-marrow	CD11c	Tulahuen	*In vitro* infections blocking Siglec-E	Acute	↓Inflammatory cytokine production; ↓T cell induction	Erdmann et al., 2009
Bone-marrow	CD11c	Tehuantepec	*In vitro* infections blocking Siglec-E	Acute	Parasite clearance	Erdmann et al., 2009
Spleen	CD11b; CD11c	Y	*In vitro* infectionn using Slamf1-deficient splenic DCs	From acute to chronic	↓IFN-γ production; no intracellular *T. cruzi* replication	Calderón et al., 2012

immune response: Th1 profile is protective in an acute state and regulatory responses are important in preventing the chronic phase (Andrade et al., 2014; Dutra et al., 2015). For this reason, DCs are a key group of cells in Chagas disease: they could modulate response depending on type, maturation level, cytokine milieu and DC receptor involved, having a fundamental role in the development of the disease clinic forms including the undetermined stage.

DENDRITIC CELL–*T. cruzi* INTERACTION: MURINE MODELS

Studies on acute Chagas disease in humans are limited due to lack of unique symptoms that characterize this disease state. Nonetheless, extending our knowledge about the acute phase is important because immunological events that take place in this stage have a great influence on the possible development of the chronic phase (Andrade et al., 2014). In this context, experimental murine infection may mimic the human disease, giving us a similar view on what happens at the beginning of *T. cruzi* infection.

Unlikely the habitual DCs response during an infection, the expression of important surface molecules like MHC, CD80 or CD86 can be reduced when DCs recognize *T. cruzi* antigens in a strain dependent manner, limiting DC maturation and antigen presenting capacity. During acute phase, splenic DCs infected by *T. cruzi* Tehuantepec strain shows low expression of CD86 molecules and are not able to migrate toward lymphoid organs/tissues (Chaussabel et al., 2003). *T. cruzi* high virulent RA strain (TcVI) induces bone-marrow DCs downregulation of cytokine production and of endocytic capacity added to a suppression of MHC class II, compared to non-infected cells (Poncini et al., 2008). These data are in concordance with the regulation studied by Alba Soto and coworkers, where they also detected a diminished MHC II expression in infected splenic DCs by the same *T. cruzi* strain. Additionally, they showed that the DC manipulated behavior induced by RA strain is not repeated for non-virulent K98 *T. cruzi* strain (TcI) (**Figure 1**; Alba Soto et al., 2003). Another comparative infection study, using mDCs, was performed using TcI (AQ1.7 and MUTUM) and TcII strains (1849 and 2369). The results demonstrated that both *T. cruzi* DTUs may modulate DCs to different extents and this modulation varied more between strains than between DTUs themselves. In general, both DTUs induced the production and expression of anti-inflammatory molecules, such as IL-10, production and PD-L1 and TLR2 expression. Regarding TLR2 expression, it seems that *T. cruzi* has molecules that bind this receptor promoting the production of anti-inflammatory cytokines such as IL-10. Oppositely, proinflammatory molecules did not present a pattern, varying depending on the strain. Finally, they also demonstrated that DC expression of differentiation and activation molecules was not polarized, which suggests that each strain of *T. cruzi* has possibly evolved specific evasion strategies (da Costa et al., 2014). Interestingly, when two mice lineages were infected by high virulent *T. cruzi* Y strain (TcII), the susceptible one showed splenic DCs with reduced capacity of antigen presentation and lower expression of CD40 and CD86 molecules compared with resistant lineages (Planelles et al., 2003). It is well known that the deficiency of co-stimulatory signals during cross-presentation may reduce T cell stimulation or lead to an anergic state (Boussiotis et al., 1996). Thus, it seems that impaired function by DC maybe helps parasite to evade the host immune system.

Cytokine production is another important aspect during DCs–*T. cruzi* interaction, particularly the production of IL-12, considered a protective molecule during the acute infection once it drives a polarized Th1 adaptive response that enables the host to adequately control parasite growth through IFN-γ (Andrade et al., 2014). In concordance, DCs from *T. cruzi*-resistant mouse lineages overexpressed IL-12 and TNF-α while the susceptible lineages produced the Th2 cytokine IL-4 (Planelles et al., 2003). IL-4 is known to mediate host susceptibility to *T. cruzi* but is also required for preventing immune hyperactivity and organ immunopathology (Abrahamsohn and Coffman, 1996; Laucella et al., 1996). Terrazas et al. (2011) demonstrated the protective role of IL-12 through *T. cruzi* Queretaro (TcI) strain infection of MIF (macrophage migration inhibitory factor)-deficient mice. MIF is a pleiotropic cytokine produced by multiple different cell types, including DCs that modulates the expression of several proinflammatory molecules (Cooke et al., 2009). It seems that it favors DC maturation through IL-12 secretion and activation of p38 MAPK protein, a kinase already known to be involved in DC maturation. On the other hand, MIF-deficient mice showed lower levels of IL-12 production and immature bone-marrow DCs, leading to a susceptibility of *T. cruzi* Queretaro (TcI) strain infection (Terrazas et al., 2011). The regulatory IL-10 cytokine is also associated with host susceptibility during acute stage of Chagas disease by limiting DCs induction of antimicrobial effector mechanism such as suppressing DC trafficking to draining lymph nodes (Corinti et al., 2001; Demangel et al., 2002; Alba Soto et al., 2010). A single intravenous injection of IL-10-deficient DCs that were pulsed with parasite antigens conferred an effective control against a lethal challenge with *T. cruzi* RA strain in mice. IL-10 deficient DCs were high Th1 cytokines producers and inducers of antigen-specific T lymphocytes after immunization (Alba Soto et al., 2010). On the other hand, Poncini et al. (2010) have showed that interaction of DCs with *T. cruzi* trypomastigotes was not able to activate the DCs, and these cells became TGF-β and IL-10 producers and were not efficient as lymphocyte stimulators, being classified as regulatory DCs. Furthermore, mature DCs have the capacity to induce NK cells activation and proliferation. NK cells play a significant role in innate immune response and surveillance as a result of their cytokine production and cytolysis of infected cells. Moreover, NK cells secrete IFN-γ, TNF-α, and GM-CSF, which promote DC maturation and activation of T-cell (Gerosa et al., 2002; Piccioli et al., 2002). Batalla et al. (2013) used RA and K98 *T. cruzi* strains to demonstrate the role of NK cells in regulating the maturation level of DCs. During both infections, NK cell was functionally activated and produced IFN-γ but also IL-10; NK cells from mice infected with *T. cruzi* RA strain (high virulence) exhibit reduced ability to lyse and fail to induce maturation

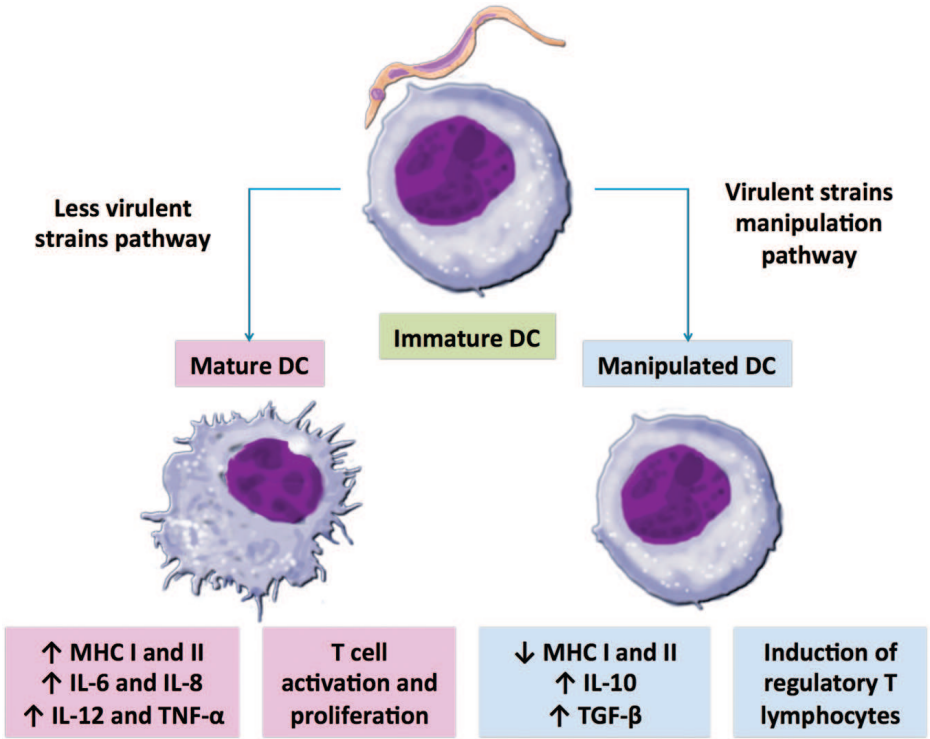

FIGURE 1 | Possible scenarios after dendritic cells (DCs)–*Trypanosoma cruzi* interaction. DCs may maturate, up-regulating major histocompatibility complexes (MHC) molecule expression and proinflammatory cytokine production, which may stimulate antigen-specific T lymphocyte activation and proliferation to control parasitemia (magenta); or DCs may be manipulated and the resultant low MHC expression together with high regulatory cytokine production may lead to a weak T cell activation, helping parasite establishment (blue). DC deviant behavior pathway could represent a potent immune evasion strategy of virulent *T. cruzi* strains to successfully set host infection. On the other hand, less virulent *T. cruzi* strains lack DC modulation capacity, which enables host immune response and parasite control.

of bone marrow-derived immature DCs. This unbalanced DC population could difficult T cell stimulation for an adequate response. Finally, that IL-10 production observed by NK cells after infection with *T. cruzi* RA strain might lead to parasite persistence but can also limit the induction of a vigorous tissue-damaging T-cell response (Batalla et al., 2013). Cytokine production may also be modulated by parasite initial inocula. Three different inocula of *T. cruzi* Colombian strain were used to infect mice, resulting in differential expression of IFN-γ, IL-17, TNF-α, IL-4, and IL-23 by immune cells in heart infiltrates. Curiously, the medium inoculum showed the less favorable host response, which may indicate the existence of an ideal initial inoculum to help parasite evade host immune response (Borges et al., 2012).

It also has been shown the important role of TLRs in *T. cruzi* recognition by first line of defense cells, including DCs. Campos et al. (2004) demonstrated that *T. cruzi* employs a myeloid differentiation factor 88 (MyD88)-, a key adaptor for most TLRs, dependent pathway to elicit cytokine production by the host cells. Intraperitoneal macrophages lacking MyD88 produced less IFN-γ, IL-12, TNF-α and reactive nitrogen intermediates, they also presented higher parasitemia and mortality (Campos et al., 2004). The role of TLRs in the establishment of critical effector mechanisms mediated by $CD8^+$ T cells during *T. cruzi* infection was also investigated (Oliveira et al., 2010). The analysis of induction of IFN-γ and cytotoxic activity *in vivo* in TLR2-, TLR4-, TLR9-, or MyD88-deficient mice during infection showed that neither the absence of TLR2, TLR4, or TLR9 individually, nor the ablation of all MyD88-mediated pathways affect the development of cytotoxic and IFN-γ-producing $CD8^+$ T cells. Nonetheless, TLR4 deficient macrophages presented a reduced production of TNF-α and nitric oxide (NO), pointing to an important role of the TLR4 pathway and NO production to the innate immune response against *T. cruzi* infection (Oliveira et al., 2010). With regard specifically to DCs, infection with *T. cruzi* parasites promotes recruitment of TLR9 to the DC endolysosome compartment, promoting their interaction during initial phagocytosis. Stimulatory motifs containing CpG islands of *T. cruzi* CL Brener, particularly those formed by genes coding for mucin like proteins, also led TLR9 into lysosomes of bone-marrow DCs and the induction of IL-12 as well as IFN-γ synthesis (Bartholomeu et al., 2008). Such potent proinflammatory activity and, consequently, control of parasite replication could lead to host resistance to infection or avoiding host lethality and maintenance of parasite life cycle long-term parasite persistence. The second hypothesis suggests another adaptation of *T. cruzi* to the host cell-mediated immunity (Bartholomeu et al., 2008). Gravina et al. (2013) stated that DC population constitutes

the main source of IL-12/IL-23p40 production in a TLR9-dependent manner in *T. cruzi* Y strain infection. Moreover, when DCs were unable to produce IL-12/IL-23p40, macrophages recovered their capacity to respond to TLR9 agonist, which may represent a compensatory response. Therefore, modulation of TLR9 is important to control the inflammatory response in the different cell populations but TLR9 acts fundamentally on DC inflammatory activity in *T. cruzi* infection (Gravina et al., 2013). Synergy among TLRs in parasite infected DC has also been studied. When MyD88/TRIF (Toll/IL-1R domain-containing adaptor-inducing IFN-β) deficient mice (i.e., with no functional activation of TLRs) were infected with *T. cruzi* Tulahuen strain (TcVI), parasite clearance was impaired mainly by absence of IFN-β production (Koga et al., 2006). In the same work, it was proposed that proinflammatory cytokine production is a MyD88-dependent induction and the expression of IFN-β is a TRIF-dependent. In any case, both TLR adaptors contribute to innate immune responses against *T. cruzi* infection (Koga et al., 2006). UNC93B1, a protein that interacts with TLR3, TLR7, and TLR9, seems to play an essential role in host protection against *T. cruzi* infection (Caetano et al., 2011). UNC93B1 mice deficient were more susceptible to *T. cruzi* infection and produced lower concentration of IL-12p40 and IFN-γ. Such susceptibility was also achieved during TLR3/TLR7/TLR9-deficient mice *T. cruzi* infection, showing that nucleic acid-sensing TLRs are critical determinants of host resistance to primary infection with *T. cruzi* (Caetano et al., 2011).

Other receptors also have been proposed to play an important role during the acute phase of Chagas disease. G-protein-coupled bradykinin (BK) B2 receptors (B_2R)-deficient mice were more susceptible to *T. cruzi* Dm28c strain (TcI) infection than WT animals (Monteiro et al., 2007). B_2Rs recognize *T. cruzi* released kinins, mediators related to bradykinin that activate immature DCs (Monteiro et al., 2006). Splenic DCs without B_2R receptor do not produce IL-12, appointing a critical role for the kinin signaling pathway in the development of type-1 effector T cells (Monteiro et al., 2007). In a recent study, the same group demonstrated that blockage of B_2R along with C5a receptor resulted in splenic DCs unable to produce IL-12p40/70 and IFN-γ (Schmitz et al., 2014). C5a is an anaphylatoxin derived from proteolysis of C5 complex of complement system, whose biological function is to activate cells from myeloid lineage (Klos et al., 2009). Yet, they showed that, as for kinins, C5a molecules seems to be produced through *T. cruzi* cruzipain activity during infection and can promote DC activation and a Th1 protective response (Schmitz et al., 2014). Galectins, a lectin receptor, can also act as pathogen recognition receptors and as modulators of innate and adaptive response (Vasta, 2009). It has been shown that those receptors are widely expressed in B cell, macrophages and DCs during *T. cruzi* infection (Vray et al., 2004; Zúñiga et al., 2001a,b). Concerning DCs, Galectin-1 seems to be a negative immune regulator that limits the host protective response by driving tolerogenic circuits in DCs. Those tolerogenic DCs induce regulatory T cells activation, which would favor parasite persistence in host tissues or limit collateral tissue damage through suppression of inflammatory responses (Poncini et al., 2015). Galectin-3 (Gal-3) and its specific ligands were over-expressed in splenic DCs after infection by *T. cruzi* Tehuantepec strain, which lead to DC increased adhesiveness and reduced migration (Vray et al., 2004). Therefore, *T. cruzi* modulates Gal-3 and its ligands functionality to improve infection, another immunomodulatory property of *T. cruzi* (Vray et al., 2004). Another lectin-like receptor expressed by immune system cells, Siglec-E (sialic acid-binding Ig-like lectin-E), has also been implicated in *T. cruzi* infection. It is well known that transference of sialic acid by *T. cruzi trans*-sialidase (TS) from host cell to parasite surface mucin-like molecules confers resistance to human complement, contributing to infection (Tomlinson et al., 1994). In this context, pathogenic *T. cruzi* Tulahuen strain (high TS activity) interacted more to Siglec-E than non-pathogenic *T. cruzi* Tehuantepec strain (reduced TS activity). This interaction led to an inhibitory effect on DCs modulation, suppressing the production of cytokine IL-12 and subsequent T-cell activation. In contrast, *T. cruzi* Tehuantepec strain could not install an important parasitemia (Erdmann et al., 2009). Together, those findings suggest that *T. cruzi* (or parasite products) may lead to immunosuppression through its interaction with DC lectin receptors (Terrazas et al., 2010). Slamf1 (self-ligand adhesion molecule - CD150) is a co-stimulatory molecule present in myeloid lineage and required at the interface of antigen presenting cells and T cells (van Driel et al., 2016). *In vitro* and *in vivo* experiments revealed that Slamf1-deficient myeloid cells showed altered production of cytokines and reduced parasite replication. For instance, much lower IFN-γ production was detected in the heart of Slamf1 deficient mice than in the heart of WT mice. Additionally, Slamf1 deficient mice presented reduced cardiac damage despite of the comparable number of infiltrating DCs, macrophages, CD4 and CD8 T lymphocytes to that of WT animals. Therefore, *T. cruzi* requires Slamf1 to replicate in DCs and its absence leads to less production of myeloid cell specific factors by DCs, which are key compounds to host immune response and infection outcomes (Calderón et al., 2012).

Currently, there is an enormous amount of data that states a direct association between mouse DC functional specializations (antigen presentation or pathogen sensing) and their subsets. However, after our meticulous review of literature in the field, it is not unrealistic to conclude that the role of DC subsets in innate immune response against *T. cruzi* needs to be more properly addressed by researches since most cited works emphasize only one surface phenotype as if all DCs are equally functional. The same statement is valid for *T. cruzi* strains, a missing pattern respect to DCs subsets and their level of activation and to *T. cruzi* strains, which hampers an association concerning published data. **Table 1** summarizes the limited actual data in the murine DC-*T. cruzi* system.

HUMAN DC–*T. cruzi* INTERACTION: *IN VITRO* STUDIES

The first experiment that confirmed the ability of *T. cruzi* to infect and reproduce inside a human DC was performed in 1999 (Van Overtvelt et al., 1999), a biological process that had already

been known for *Leishmania* (Moll et al., 1995). The authors also demonstrated that DCs, derived from monocytes and infected with *T. cruzi* Tehuantepec strain, significantly reduced HLA-DR and CD40 expression. In addition, these infected DCs were neither IL-12 nor TNF-α producers (Van Overtvelt et al., 1999). In a different study, using the same *T. cruzi* strain, Van Overtvelt and co-workers showed that *T. cruzi* soluble factor(s) released by the parasite itself into the DC culture medium inhibits LPS induced MHC class I up-regulation on the surface of human DC. Such inhibition may decrease the protective effect of specific $CD8^+$ T since infected DCs had a weaker capacity of cross-presentation. This reduction of DC function may influence the *in vivo* host's ability to competently combat *T. cruzi* infection (Van Overtvelt et al., 2002). It is well known that a small family of type 1 glycoinositolphospholipids (GIPLs) is abundant in *T. cruzi* cell surface and, therefore, such molecules seem to have immunoregulatory activities (Brodskyn et al., 2002; Medeiros et al., 2007). GIPLs isolated from *T. cruzi* G (TcI) and Y (TcII) strains were incubated along with LPS to stimulate DCs derived from monocytes. The results showed that *T. cruzi* GIPL antigens direct the down-regulation of both proinflammatory cytokines, such as TNF-α and IL-12 and anti-inflammatory, such IL-10, in DCs. The parasite GIPL also inhibited the expression of co-stimulatory molecules HLA-DR, CD83, CD86, CD80, and CD40 on DC surface. Similar results were achieved when the ceramide portion of GIPL molecule alone was used to stimulate DC, suggesting that fragments from the parasite glycoproteins could represent an evasion strategy of *T. cruzi*. Altogether, GIPL seems to contribute to parasite protection from the innate responses, allowing the beginning of infection and also acts in an inhibitory way on DC maturation, postponing an adequate immune response against *T. cruzi* (Brodskyn et al., 2002). Otherwise, a parasite released protein belonging to thiol-disulfide oxidoreductase family (Tc52) binds to and induces human and murine DC maturation by TLR2 activation. DCs derived from monocytes treated with Tc52 showed higher expression of CD83, CD86, CD54 and HLA-DR and an elevated production of IL-8, MCP-1, MIP-1α. These *in vitro* data suggest that Tc52 may provide local recruitment and activation of leukocyte and then DC migration to the lymph node, where they can trigger B and T cell immune responses (Ouaissi et al., 2002).

Yet, *T. cruzi* Tulahuen strain parasites enhance expression of CD40 and CD80 on cord blood mDCs in a higher level compared to mDCs from adult donors. $CD8^+$ T cells proliferation was also stimulated by those cord blood mDCs. In early life, immune responses are considered of partial effectiveness, owing to the relative immaturity of the human immune system therefore it is possible that maternally transmitted IgGs might contribute to overcome some deficiency of fetal/neonatal DCs and to protect the fetus/newborn against pathogens that have already come into contact with the mother (Rodriguez et al., 2012a). Another study, performed by the same group, showed that *T. cruzi* can induce maturation of this DC type without infecting them. Rodriguez and coworkers found that blood cord and adult mDCs that had contact with parasite but were not yet infected also expressed high levels of CD80 and CD83. In addition, they demonstrated that either infected mDCs or *T. cruzi* lysates co-incubated mDCs have a similar expression pattern of their surface molecules. The authors also showed that infection rate in mDCs is lower than in monocytes and granulocytes, maybe due to their enhanced capacity of phagocytosis when compared to mature DCs (Rodriguez et al., 2012b). Consistent with the results shown in murine model, modulation of DCs function varies according to *T. cruzi* strain. In a comparative study, Magalhães and colleagues demonstrated that *T. cruzi* Col cl1.7 (TcI) but not Y (TcII) strain induces higher CD80 and CD86 expression, while *T. cruzi* Y strain induces up-regulation of IL-10, TNF-α and granzyme A production. Also, $CD8^+$ T lymphocytes activated by Col cl1.7 strain produced higher level of IL-17. Then, TcI strain were capable of a higher monocyte activation, while the profile induced by TcII was more inflammatory (Magalhães et al., 2015). **Figure 2** summarizes DC receptors referred to in this review.

The present knowledge about the interaction between human DCs and *T. cruzi* is restricted to *in vitro* models where expression of some cytokines and surface molecules were analyzed, but the specific functions of human DC subsets are only beginning to be unraveled. Contradictory studies have been published concerning DC–*T. cruzi* interactions, both in human and mouse models. However it seems that *T. cruzi* virulent strains probably take advantage of susceptible DCs to overcome host immune response and successfully install the infection. Remarkably, those modulated DCs will conduct a weak adaptive response with low expression of MHC class I and II molecules and proinflammatory cytokines, which are fundamental for controlling parasite survival (Poncini et al., 2008, 2010; Alba Soto et al., 2010; da Costa et al., 2014).

FUTURE: NEEDS AND EXPECTATIONS

Dendritic cells are crucial decision-making cells of the immunological system as they direct tolerance, anergy, and initiation/regulation of the adaptive immune responses (Banchereau and Steinman, 1998). For these reasons, DC has been proposed as targets for immunotherapy in diseases related to autoimmunity and exacerbated immune responses, such as autoimmune encephalomyelitis (Menges et al., 2002), thyroiditis (Verginis et al., 2005), and arthritis (Jaen et al., 2009) or to improve unsatisfactory immune responses toward tumor or pathogens (Dubsky et al., 2005). Targeting these cells with recombinant antibodies conjugated to autoantigens or pathogen antigens could direct a less exacerbated response against certain disease (Lutz, 2012).

ASP-2, an amastigote protein from *T. cruzi* Y strain, was conjugated with αDEC205 antibody, DEC205 is a C-type lectin endocytic receptor expressed in some DC populations and is widely used for targeting DCs. Recombinant ASP-2/DEC205 antibody was injected in mice, resulting in higher IFN-γ production by splenocytes and high proliferation of antigen-specific $CD4^+$ T cells (Rampazo et al., 2015). Thus, targeted regulatory DCs could be used as an immunotherapy strategy during disease undetermined form to prevent evolution to

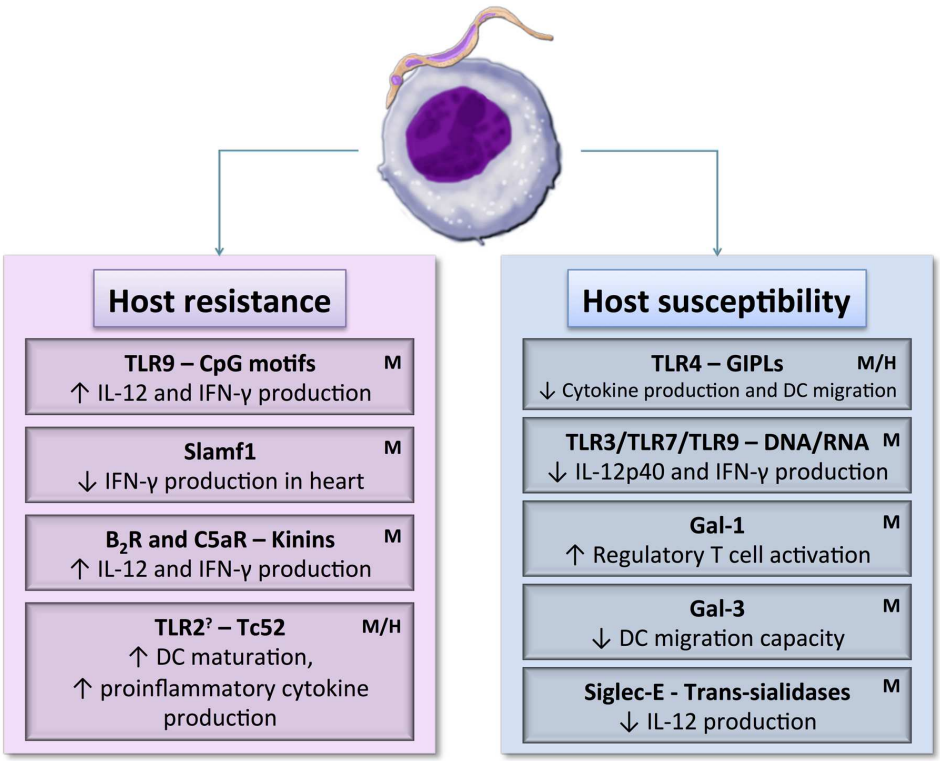

FIGURE 2 | Dendritic cell receptor–*T. cruzi* antigen interactions may induce host resistance or susceptibility to parasite by activating different DCs signaling pathways. DCs maturation, cytokine production, and migration capacity could be up or down-regulated depending on the signaling pathways triggered during the initial contact between DCs and the parasite. **H**: DC receptors already known to interact with parasite in human model. **M**: DC receptors already known to interact with parasite in murine model. **?**: May act as a maturation inducer or as regulatory cytokines activator.

the chronic phase. Nevertheless, host receptor and parasite antigen should be carefully elected because they could influence maturation signals received by DCs for orchestration of the immune response (Cohn and Delamarre, 2014). Furthermore, trypomastigote lysate -pulsed IL-10-deficient DC conferred protection against *T. cruzi* infection to recipient mice by secreting increased amounts of IFN-γ, enhancing antigen-specific production and inducing endogenous DC activation. This DC-based vaccination against *T. cruzi* also demonstrated that IL-10 produced by sensitizing DC has a key role in inhibiting the protection response (Alba Soto et al., 2010). DC-based vaccine has also been successfully tested in *Leishmania*. Freshly isolated pDC from mice pulsed with *Leishmania* antigen and reinjected into host resulted in a protective effect, presenting mixed Th1/Th2 response with secretion of IFN-γ, IL-4 and IL-10 (Remer et al., 2007).

Unfortunately, DC immunizations have an elevated cost, which turns up this approach less attractive added to relative efficiency of the current treatment for Chagas disease during acute phase (Rassi et al., 2010; Cohn and Delamarre, 2014). Nonetheless, this neglected disease remains with no vaccines or antiparasitic drugs proven efficient in chronically infected adults, when most patients are diagnosed. Thus, future DCs immunization researches could be directed toward treatment for the undetermined stage targeting tolerogenic DCs.

CONCLUSION

Although efforts have been devoted to deciphering DCs–*T. cruzi* interaction, there is still much to be investigated before the complete understanding of DC role in the induction of immunity against *T. cruzi*. Moreover, our knowledge about that interaction is mostly based on the regulation, differentiation and function of the DC lineage from mouse. A challenge that needs to be overcome is the difficulty in isolating subsets of DCs from human tissue; only then we might be able to improve the understanding of human DCs in a molecular level and perhaps develop vaccines for the prevention or treatment of Chagas disease. Our review reiterates that *T. cruzi* capacity to modulate host DCs is an indispensable strategy to escape from innate immune response with the purpose of its own survival. Nevertheless, DCs present an efficient machinery to capture, process, and present antigens to T cells and to activate B cells therefore their immunotherapeutic potential may not be disregarded.

AUTHOR CONTRIBUTIONS

NG-J: participated in design and manuscript writing. FM: participated in design and manuscript writing. CF: participated in design and manuscript writing. IB: participated in design,

coordination, and manuscript writing. JS: participated in design, coordination, and manuscript writing.

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REFERENCES

Abrahamsohn, I. A., and Coffman, R. L. (1996). *Trypanosoma cruzi*: IL-10, TNF, IFN-gamma, and IL-12 regulate innate and acquired immunity to infection. *Exp. Parasitol.* 84, 231–244. doi: 10.1006/expr.1996.0109

Alba Soto, C. D., Mirkin, G. A., Solana, M. E., and González Cappa, S. M. (2003). *Trypanosoma cruzi* infection modulates in vivo expression of major histocompatibility complex class II molecules on antigen-presenting cells and T-cell stimulatory activity of dendritic cells in a strain-dependent manner. *Infect. Immun.* 71, 1194–1199. doi: 10.1128/IAI.71.3.1194

Alba Soto, C. D., Solana, M. E., Poncini, C. V., Pino-Martinez, A. M., Tekiel, V., and González-Cappa, S. M. (2010). Dendritic cells devoid of IL-10 induce protective immunity against the protozoan parasite *Trypanosoma cruzi*. *Vaccine* 28, 7407–7413. doi: 10.1016/j.vaccine.2010.08.105

Andrade, D. V., Gollob, K. J., and Dutra, W. O. (2014). Acute chagas disease: new global challenges for an old neglected disease. *PLoS Negl. Trop. Dis.* 8:e3010. doi: 10.1371/journal.pntd.0003010

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* 392, 245–252. doi: 10.1038/32588

Bartholomeu, D. C., Ropert, C., Melo, M. B., Parroche, P., Junqueira, C. F., Teixeira, S. M. R., et al. (2008). Recruitment and endo-lysosomal activation of TLR9 in dendritic cells infected with *Trypanosoma cruzi*. *J. Immunol.* 181, 1333–1344. doi: 10.4049/jimmunol.181.2.1333

Bastos, C. J. C., Aras, R., Mota, G., Reis, F., Dias, J. P., de Jesus, R. S., et al. (2010). Clinical outcomes of thirteen patients with acute chagas disease acquired through oral transmission from two urban outbreaks in Northeastern Brazil. *PLoS Negl. Trop. Dis.* 4:e711. doi: 10.1371/journal.pntd.0000711

Batalla, E. I., Pino Martínez, A. M., Poncini, C. V., Duffy, T., Schijman, A. G., González Cappa, S. M., et al. (2013). Impairment in natural killer cells editing of immature dendritic cells by infection with a virulent *Trypanosoma cruzi* population. *J. Innate Immun.* 5, 494–504. doi: 10.1159/000350242

Bermudez, J., Davies, C., Simonazzi, A., Real, J. P., and Palma, S. (2015). Current drug therapy and pharmaceutical challenges for Chagas disease. *Acta Trop.* 156, 1–16. doi: 10.1016/j.actatropica.2015.12.017

Bern, C. (2011). Antitrypanosomal therapy for chronic Chagas' disease. *N. Engl. J. Med.* 364, 2527–2534. doi: 10.1056/NEJMct1014204

Bern, C. (2015). Chagas' Disease. *N. Engl. J. Med.* 373, 456–466. doi: 10.1056/NEJMra1410150

Blum, J. S., Wearsch, P. A., and Cresswell, P. (2013). Pathways of antigen processing. *Annu. Rev. Immunol.* 31, 443–473. doi: 10.1146/annurev-immunol-032712-095910

Borges, D. C., Araújo, N. M., Cardoso, C. R., and Lazo Chica, J. E. (2012). Different parasite inocula determine the modulation of the immune response and outcome of experimental *Trypanosoma cruzi* infection. *Immunology* 138, 145–156. doi: 10.1111/imm.12022

Boussiotis, V. A., Freeman, G. J., Gribben, J., and Nadler, L. (1996). The role of B7-1/B7-2:CD28/CTLA-4 pathways in the prevention of anergy, induction of productive immunity and down-regulation of the immune response. *Immunol. Rev.* 153, 5–26. doi: 10.1111/j.1600-065X.1996.tb00918.x

Brodskyn, C., Patricio, J., Oliveira, R., Lobo, L., Arnholdt, A., Mendonça-previato, L., et al. (2002). Glycoinositolphospholipids from *Trypanosoma cruzi* interfere with macrophages and dendritic cell responses. *Infect. Immun.* 70, 3736–3743. doi: 10.1128/IAI.70.7.3736-3743.2002

Caetano, B. C., Carmo, B. B., Melo, M. B., Cerny, A., dos Santos, S. L., Bartholomeu, D. C., et al. (2011). Requirement of UNC93B1 reveals a critical role for Toll-Like Receptor 7 in host resistance to primary infection with *Trypanosoma cruzi*. *J. Immunol.* 187, 1903–1911. doi: 10.4049/jimmunol.1003911

Calderón, J., Maganto-Garcia, E., Punzón, C., Carrión, J., Terhorst, C., and Fresno, M. (2012). The receptor Slamf1 on the surface of myeloid lineage cells controls susceptibility to infection by *Trypanosoma cruzi*. *PLoS Pathog.* 8:e1002799. doi: 10.1371/journal.ppat.1002799

Camargo, R., Faria, L. O., Kloss, A., Favali, C. B. F., Kuckelkorn, U., Kloetzel, P. M., et al. (2014). *Trypanosoma cruzi* infection down-modulates the immunoproteasome biosynthesis and the MHC class I cell surface expression in HeLa cells. *PLoS ONE* 9:e95977. doi: 10.1371/journal.pone.0095977

Campos, M. A., Closel, M., Valente, E. P., Cardoso, J. E., Akira, S., Alvarez-leite, J. I., et al. (2004). Impaired production os proinflammatory cytokines and host resistance to acute infection with *Trypanosoma cruzi* in mice lacking functional myeliod differentation factor 88. *J. Immunol.* 172, 1711–1718. doi: 10.4049/jimmunol.172.3.1711

Cardoso, M. S., Reis-cunha, J. L., Bartholomeu, D. C., Fernandez, M. M., and Bartholomeu, D. C. (2016). Evasion of the Immune Response by *Trypanosoma cruzi* during Acute Infection. *Front. Immunol.* 6:659. doi: 10.3389/fimmu.2015.00659

Chaussabel, D., Pajak, B., Vercruysse, V., Bisseyé, C., Garzé, V., Habib, M., et al. (2003). Alteration of migration and maturation of dendritic cells and T-Cell depletion in the course of experimental *Trypanosoma cruzi* infection. *Lab. Investig.* 83, 1373–1382. doi: 10.1097/01.LAB.0000087587.93781.6F

Cohn, L., and Delamarre, L. (2014). Dendritic cell-targeted vaccines. *Front. Immunol.* 5:255. doi: 10.3389/fimmu.2014.00255

Collin, M., Mcgovern, N., and Haniffa, M. (2013). Human dendritic cell subsets. *Immunology* 140, 22–30. doi: 10.1111/imm.12117

Cooke, G., Armstrong, M. E., and Donnelly, S. C. (2009). Macrophage migration inhibitory factor (MIF), enzymatic activity and the inflammatory response. *Biofactors* 35, 165–168. doi: 10.1002/biof.27

Corinti, S., Albanesi, C., la Sala, A., Pastore, S., and Girolomoni, G. (2001). Regulatory activity of autocrine IL-10 on dendritic cell functions. *J. Immunol.* 166, 4312–4318. doi: 10.4049/jimmunol.166.7.4312

Coura, J. R., Dias, J. C. P., Frasch, A. C. C., Guhl, F., Lazzari, J. O., Lorca, M., et al. (2002). Control of Chagas disease. *Adv. Parasitol.* 61, 129–165. doi: 10.1016/S0065-308X(05)61004-4.

da Costa, T. A., Silva, M. V., Mendes, M. T., Carvalho-Costa, T. M., Batista, L. R., Lages-Silva, E., et al. (2014). Immunomodulation by *Trypanosoma cruzi*: toward understanding the association of dendritic cells with infecting TcI and TcII populations. *J. Immunol. Res.* 2014, 1–12. doi: 10.1155/2014/962047

D'Avila-Levy, C. M., Boucinha, C., Kostygov, A., Santos, H. L. C., Morelli, K. A., Grybchuk-Ieremenko, A., et al. (2015). Exploring the environmental diversity of kinetoplastid flagellates in the high-throughput DNA sequencing era. *Mem. Inst. Oswaldo Cruz* 110, 956–965. doi: 10.1590/0074-02760150253

de Lana, M., Marques, E., and Machado, D. M. (2010). *Biology of "Trypanosoma cruzi" and Biological Diversity*, 1st Edn. Amsterdam: Elsevier Inc.

Deane, M. P., Lenzi, H. L., and Jansen, A. (1984). *Trypanosoma cruzi*: vertebrate and invertebrate cycles in the same mammal host, the opossum *Didelphis marsupialis*. *Mem. Inst. Oswaldo Cruz* 79, 513–515. doi: 10.1590/S0074-02761984000400021

Demangel, C., Bertolino, P., and Britton, W. J. (2002). Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur. J. Immunol.* 32, 994–1002. doi: 10.1002/1521-4141(200204)32:4<994::AID-IMMU994>3.3.CO;2-Y

den Haan, J. M. M., Arens, R., and van Zelm, M. C. (2014). The activation of the adaptive immune system: cross-talk between antigen-presenting cells, T cells and B cells. *Immunol. Lett.* 162, 103–112. doi: 10.1016/j.imlet.2014.10.011

Dubsky, P., Ueno, H., Piqueras, B., Connolly, J., Banchereau, J., and Palucka, A. K. (2005). Human dendritic cell subsets for vaccination. *J. Clin. Immunol.* 25, 551–572. doi: 10.1007/s10875-005-8216-7

Dutra, W. O., Menezes, C. A. S., Magalhães, L. M. D., and Gollob, K. J. (2015). Immunoregulatory networks in human Chagas disease. *Parasite Immunol.* 36, 377–387. doi: 10.1111/pim.12107

Erdmann, H., Steeg, C., Koch-nolte, F., Fleischer, B., and Jacobs, T. (2009). Sialylated ligands on pathogenic *Trypanosoma cruzi* interact with Siglec-E (sialic acid-binding Ig-like lectin-E). *Cell. Microbiol.* 11, 1600–1611. doi: 10.1111/j.1462-5822.2009.01350.x

Gerosa, F., Baldani-Guerra, B., Nisii, C., Marchesini, V., Carra, G., and Trinchieri, G. (2002). Reciprocal activating interaction between natural killer cells and dendritic cells. *J. Exp. Med.* 195, 327–333. doi: 10.1084/jem.20010938

Gravina, H. D., Antonelli, L., Gazzinelli, R. T., and Ropert, C. (2013). Differential Use of TLR2 and TLR9 in the regulation of immune responses during the infection with *Trypanosoma cruzi*. *PLoS ONE* 8:e63100. doi: 10.1371/journal.pone.0063100

Haniffa, M., Ginhoux, F., Wang, X.-N., Bigley, V., Abel, M., Dimmick, I., et al. (2009). Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation. *J. Exp. Med.* 206, 371–385. doi: 10.1084/jem.20081633

Holdsworth, S. R., and Can, P. Y. (2015). Cytokines: names and numbers you should care about. *Clin. J. Am. Soc. Nephrol.* 10, 2243–2254. doi: 10.2215/CJN.07590714

Hotez, P. J., Molyneux, D. H., Fenwick, A., Kumaresan, J., Sachs, S. E., Sachs, J. D., et al. (2007). Control of neglected tropical diseases. *N. Engl. J. Med.* 357, 1018–1027. doi: 10.1056/NEJMra064142

Jaen, O., Rullé, S., Bessis, N., Zago, A., Boissier, M. C., and Falgarone, G. (2009). Dendritic cells modulated by innate immunity improve collagen-induced arthritis and induce regulatory T cells in vivo. *Immunology* 126, 35–44. doi: 10.1111/j.1365-2567.2008.02875.x

Klos, A., Tenner, A. J., Johswich, K. O., Ager, R. R., Reis, E. S., and Köhl, J. (2009). The role of the anaphylatoxins in health and disease. *Mol. Immunol.* 46, 2753–2766. doi: 10.1016/j.molimm.2009.04.027

Koga, R., Hamano, S., Kuwata, H., Atarashi, K., Ogawa, M., Hisaeda, H., et al. (2006). TLR-dependent induction of IFN- β mediates host defense against *Trypanosoma cruzi*. *J. Immunol.* 177, 7059–7066. doi: 10.4049/jimmunol.177.10.7059

Kropf, S. P., and Sá, M. R. (2009). The discovery of *Trypanosoma cruzi* and Chagas disease (1908-1909): tropical medicine in Brazil. *Hist. Cienc. Saude Manguinhos.* 16(Suppl. 1), 13–34. doi: 10.1590/S0104-59702009000500002

Laucella, S. A., Rottenberg, M. E., and de Titto, E. H. (1996). Role of cytokines in resistance and pathology in *Trypanosoma cruzi* infection. *Rev. Argent Microbiol.* 28, 99–109.

Lewis, K. L., and Reizis, B. (2012). Dendritic cells: arbiters of immunity and immunological tolerance. *Cold Spring Harb. Perspect. Biol.* 4:a007401. doi: 10.1101/cshperspect.a007401

Lipscomb, M. F., and Masten, B. J. (2002). Dendritic cells: immune regulators in health and disease. *Physiol. Rev.* 82, 97–130. doi: 10.1152/physrev.00023.2001

Lutz, M. B. (2012). Therapeutic potential of semi-mature dendritic cells for tolerance induction. *Front. Immunol.* 3:123. doi: 10.3389/fimmu.2012.00123

Machado, F. S., Dutra, W. O., Esper, L., Gollob, K., Teixeira, M., Factor, S. M., et al. (2013). Current understanding of immunity to *Trypanosoma cruzi* infection and pathogenesis os Chagas disease. *Semin. Immunopathol.* 34, 753–770. doi: 10.1007/s00281-012-0351-7

Magalhães, L. M. D., Viana, A., Chiari, E., Galvão, L. M. C., Gollob, K. J., and Dutra, W. O. (2015). Differential activation of human monocytes and lymphocytes by distinct strains of *Trypanosoma cruzi*. *PLoS Negl. Trop. Dis.* 9:e0003816. doi: 10.1371/journal.pntd.0003816

Manchola, N. C., Rapado, L. N., Barison, M. J., and Silber, A. M. (2015). Biochemical characterization of branched chain amino acids uptake in *Trypanosoma cruzi*. *J. Eukaryot. Microbiol.* 63, 299–308. doi: 10.1111/jeu.12278

Medeiros, M. M., Peixoto, J. R., Oliveira, A.-C., Cardilo-Reis, L., Koatz, V. L. G., Van Kaer, L., et al. (2007). Toll-like receptor 4 (TLR4)-dependent proinflammatory and immunomodulatory properties of the glycoinositolphospholipid (GIPL) from *Trypanosoma cruzi*. *J. Leukoc. Biol.* 82, 488–496. doi: 10.1189/jlb.0706478

Menges, M., RöBner, S., Voigtländer, C., Schindler, H., Kukutsch, N. A., Bogdan, C., et al. (2002). Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* 195, 15–21. doi: 10.1084/jem.20011341

Moll, H., Flohé, S., and Röllinghoff, M. (1995). Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. *Eur. J. Immunol.* 25, 693–699. doi: 10.1002/eji.1830250310

Monteiro, A. C., Schmitz, V., Morrot, A., de Arruda, L. B., Nagajyothi, F., Granato, A., et al. (2007). Bradykinin B 2 receptors of dendritic cells, acting as sensors of kinins proteolytically released by *Trypanosoma cruzi*, are critical for the development of protective Type-1 responses. *PLOS Pathog.* 3:e185. doi: 10.1371/journal.ppat.0030185

Monteiro, A. C., Schmitz, V., Svensjo, E., Gazzinelli, R. T., Almeida, I. C., Todorov, A., et al. (2006). Cooperative activation of TLR2 and bradykinin B2 receptor is required for induction of type 1 immunity in a mouse model of subcutaneous infection by *Trypanosoma cruzi*. *J. Immunol.* 177, 6325–6335. doi: 10.4049/jimmunol.177.9.6325

Muñoz-Saravia, S. G., Haberland, A., Wallukat, G., and Schimke, I. (2012). Chronic Chagas' heart disease: a disease on its way to becoming a worldwide health problem: epidemiology, etiopathology, treatment, pathogenesis and laboratory medicine. *Heart Fail. Rev.* 17, 45–64. doi: 10.1007/s10741-010-9211-5

Nardy, A. F., Freire-de-Lima, C. G., and Morrot, A. (2015). Immune evasion strategies of *Trypanosoma cruzi*. *J. Immunol. Res.* 2015:7. doi: 10.1155/2015/178947

Neefjes, J., Jongsma, M. L., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11, 823–836. doi: 10.1038/nri3084

Nóbrega, A. A., Garcia, M. H., Tatto, E., Obara, M. T., Costa, E., Sobel, J., et al. (2009). Oral transmission of chagas disease by consumption of Açaí palm fruit, Brazil. *Emerg. Infect. Dis.* 15, 653–655. doi: 10.3201/eid1504.081450

Oliveira, A., Alencar, B. C., De Tzelepis, F., Klezewsky, W., Raquel, N., Neves, F. S., et al. (2010). Impaired innate immunity in Tlr4 2 / 2 mice but preserved CD8 + T cell responses against *Trypanosoma cruzi* in Tlr4-, Tlr2-, Tlr9- or myd88-deficient mice. *PLoS Pathog.* 6:e1000870. doi: 10.1371/journal.ppat.1000870

O'Neill, D. W., Adams, S., and Bhardwaj, N. (2004). Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood J.* 104, 2235–2246. doi: 10.1182/blood-2003-12-4392

Ouaissi, A., Guilvard, E., Delneste, Y., Caron, G., Magistrelli, G., Herbault, N., et al. (2002). The *Trypanosoma cruzi* Tc52-released protein induces human dendritic cell maturation, signals via toll-like receptor 2, and confers protection against lethal infection. *J. Immunol.* 168, 6366–6374. doi: 10.4049/jimmunol.168.12.6366

Pearce, E. J., and Everts, B. (2015). Dendritic cell metabolism. *Nat. Rev. Immunol.* 15, 18–29. doi: 10.1038/nri3771

Piccioli, D., Sbrana, S., Melandri, E., and Valiante, N. M. (2002). Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J. Exp. Med.* 195, 335–341. doi: 10.1084/jem.20010934

Planelles, L., Thomas, M. C., Marañón, C., Morell, M., and López, M. C. (2003). Differential CD86 and CD40 co-stimulatory molecules and cytokine expression pattern induced by *Trypanosoma cruzi* in APCs from resistant or susceptible mice. *Clin. Exp. Immunol.* 131, 41–47. doi: 10.1046/j.1365-2249.2003.02022.x

Poncini, C. V., Giménez, G., Pontillo, C. A., Alba-Soto, C. D., de Isola, E. L. D., Piazzón, I., et al. (2010). Central role of extracellular signal-regulated kinase and Toll-like receptor 4 in IL-10 production in regulatory dendritic cells induced by *Trypanosoma cruzi*. *Mol. Immunol.* 47, 1981–1988. doi: 10.1016/j.molimm.2010.04.016

Poncini, C. V., Ilarregui, J. M., Batalla, E. I., Engels, S., Cerliani, J. P., Cucher, M. A., et al. (2015). *Trypanosoma cruzi* infection imparts a regulatory program in dendritic cells and T cells via galectin-1–dependent mechanisms. *J. Immunol.* 195, 3311–3324. doi: 10.4049/jimmunol.1403019

Poncini, C. V., Soto, C. D. A., Batalla, E., Solana, M. E., and Gonzalez Cappa, S. M. (2008). *Trypanosoma cruzi* induces regulatory dendritic cells in vitro. *Infect. Immun.* 76, 2633–2641. doi: 10.1128/IAI.01298-07

Ramírez, J. D., Guhl, F., Rendón, L. M., Rosas, F., Marin-Neto, J. A., and Morillo, C. A. (2010). Chagas cardiomyopathy manifestations and *Trypanosoma cruzi* genotypes circulating in chronic chagasic patients. *PLoS Negl. Trop. Dis.* 4:e899. doi: 10.1371/journal.pntd.0000899

Rampazo, E. V., Amorim, K. N. S., and Yamamoto, M. M. (2015). Antigen targeting to dendritic cells allows the identification of a CD4 T-Cell epitope within an immunodominant *Trypanosoma cruzi* antigen. *PLoS ONE* 10:e0117778. doi: 10.1371/journal.pone.0117778

Rassi, A., Rassi, A., and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* 375, 1388–1402. doi: 10.1016/S0140-6736(10)60061-X

Remer, K. A., Apetrei, C., Schwarz, T., Linden, C., and Moll, H. (2007). Vaccination with plasmacytoid dendritic cells induces protection against infection with *Leishmania major* in mice. *Eur. J. Immunol.* 37, 2463–2473. doi: 10.1002/eji.200636780

Rodriguez, P., Carlier, Y., and Truyens, C. (2012a). Activation of cord blood myeloid dendritic cells by *Trypanosoma cruzi* and parasite-specific antibodies, proliferation of CD8 + T cells, and production of IFN-gamma. *Med. Microbiol. Immunol.* 201, 157–169. doi: 10.1007/s00430-011-0217-y

Rodriguez, P., Carlier, Y., and Truyens, C. (2012b). *Trypanosoma cruzi* activates cord blood myeloid dendritic cells independently of cell infection. *Med. Microbiol. Immunol.* 201, 287–296. doi: 10.1007/s00430-012-0230-9

Romano, P. S., Cueto, J. A., Casassa, A. F., Vanrell, M. C., Gottlieb, R. A., and Colombo, M. I. (2012). Molecular and cellular mechanisms involved in the *Trypanosoma cruzi*/host cell interplay. *IUBMB Life* 64, 387–396. doi: 10.1002/iub.1019

Schmitz, V., Almeida, L. N., Svensjö, E., Monteiro, A. C., Köhl, J., and Scharfstein, J. (2014). C5a and bradykinin receptor cross-talk regulates innate and adaptive immunity in *Trypanosoma cruzi* infection. *J. Immunol.* 193, 3613–3623. doi: 10.4049/jimmunol.1302417

Schmunis, G. A. (2007). Epidemiology of Chagas disease in non-endemic countries: the role of international migration. *Mem. Inst. Oswaldo Cruz* 102, 75–85. doi: 10.1590/S0074-02762007005000093

Segura, E. (2016). "Review of mouse and human dendritic cell subsets," in *Dendritic Cell Protocols*, eds E. Segura and N. Onai (New York, NY: Springer Science+Business Media), 3–15. doi: 10.1007/978-1-4939-3606-9_1

Segura, E., and Amigorena, S. (2015). *Cross-Presentation in Mouse and Human Dendritic Cells*, 1st Edn. Amsterdam: Elsevier Inc.

Stecconi-Silva, R. B., Andreoli, W. K., and Mortara, R. A. (2003). Parameters affecting cellular invasion and escape from the parasitophorous vacuole by different infective forms of *Trypanosoma cruzi*. *Mem. Inst. Oswaldo Cruz* 98, 953–958. doi: 10.1590/S0074-02762003000700016

Steinman, R. M. (2007). Dendritic cells: versatile controllers of the immune system. *Nat. Med.* 13, 1155–1159. doi: 10.1038/nm1643

Stevens, J. R. (2008). Kinetoplastid phylogenetics, with special reference to the evolution of parasitic trypanosomes. *Parasite* 15, 226–232. doi: 10.1051/parasite/2008153226

Steverding, D. (2014). The history of chagas disease. *Parasit Vectors* 7, 1–8. doi: 10.1186/1756-3305-7-317

Strasen, J., Williams, T., Ertl, G., Zoller, T., Stich, A., and Ritter, O. (2013). Epidemiology of chagas disease in Europe: many calculations, little knowledge. *Clin. Res. Cardiol.* 103, 1–10. doi: 10.1007/s00392-013-0613-y

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell* 140, 805–820. doi: 10.1016/j.cell.2010.01.022

Teixeira, A., Vinaud, M., and Castro, A. M. (2009). *Emerging Chagas Disease*. Brasília: Bentham.

Terrazas, C. A., Huitron, E., Vazquez, A., Juarez, I., Camacho, G. M., Calleja, E. A., et al. (2011). MIF synergizes with *Trypanosoma cruzi* antigens to promote efficient dendritic cell maturation and IL-12 production via p38 MAPK. *Int. J. Biol. Sci.* 7, 1298–1310. doi: 10.7150/ijbs.7.1298

Terrazas, C. A., Terrazas, L. I., and Gómez-García, L. (2010). Modulation of dendritic cell responses by parasites: a common strategy to survive. *J. Biomed. Biotechnol.* 2010:357106. doi: 10.1155/2010/357106

Tibayrenc, M. (1998). Genetic epidemiology of parasitic protozoa and other infectious agents: the need for an integrated approach. *Int. J. Parasitol.* 28, 85–104. doi: 10.1016/S0020-7519(97)00180-X

Tomlinson, S., Pontes de Carvalho, L. C., Vandekerckhove, F., and Nussenzweig, V. (1994). Role of sialic acid in the resistance of *Trypanosoma cruzi* trypomastigotes to complement. *J. Immunol.* 153, 3141–3147.

Tonelli, R. R., Silber, A. M., Almeida-de-Faria, M., Hirata, I. Y., Colli, W., and Alves, J. M. (2004). L-proline is essential for the intracellular differentiation of *Trypanosoma cruzi*. *Cell. Microbiol.* 6, 733–741. doi: 10.1111/j.1462-5822.2004.00397.x

van Driel, B. J., Liao, G., Engel, P., and Terhorst, C. (2016). Responses to microbial challenges by SLAMF receptors. *Front. Immunol.* 7:4. doi: 10.3389/fimmu.2016.00004

Van Overtvelt, L., Andrieu, M., Verhasselt, V., Connan, F., Choppin, J., Vercruysse, V., et al. (2002). *Trypanosoma cruzi* down-regulates lipopolysaccharide-induced MHC class I on human dendritic cells and impairs antigen presentation to specific CD8 + T lymphocytes. *Int. Immunol.* 14, 1135–1144. doi: 10.1093/intimm/dxf077

Van Overtvelt, L., Vanderheyde, N., Verhasselt, V., Ismaili, J., De Vos, L., Goldman, M., et al. (1999). *Trypanosoma cruzi* infects human dendritic cells and prevents their maturation: inhibition of cytokines, HLA-DR, and costimulatory molecules. *Infect. Immun.* 67, 4033–4040.

Vasta, G. R. (2009). Roles of galectins in infection. *Nat. Rev. Microbiol.* 7, 424–438. doi: 10.1038/nrmicro2146

Verginis, P., Li, H. S., and Carayanniotis, G. (2005). Tolerogenic semimature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4+CD25+ T cells. *J. Immunol.* 174, 7433–7439. doi: 10.4049/jimmunol.174.11.7433

Verhasselt, V., Buelens, C., Willems, F., De Groote, D., Haeffner-Cavaillon, N., and Goldman, M. (1997). Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. *J. Immunol.* 158, 2919–2925.

Vray, B., Camby, I., Vercruysse, V., Mijatovic, T., Bovin, N. V., Ricciardi-Castagnoli, P., et al. (2004). Up-regulation of galectin-3 and its ligands by *Trypanosoma cruzi* infection with modulation of adhesion and migration of murine dendritic cells. *Glycobiology* 14, 647–657. doi: 10.1093/glycob/cwh068

Vyas, J. M., Van der Veen, A. G., and Ploegh, H. L. (2008). The known unknowns of antigen processing and presentation. *Nat. Rev. Immunol.* 8, 607–618. doi: 10.1038/nri2368

Watanabe Costa, R., da Silveira, J. F., and Bahia, D. (2016). Interactions between *Trypanosoma cruzi* secreted proteins and host cell signaling pathways. *Front. Microbiol.* 7:388. doi: 10.3389/fmicb.2016.00388

Wilkinson, S. R., Bot, C., Kelly, J. M., and Hall, B. S. (2011). Trypanocidal activity of nitroaromatic prodrugs: current treatments and future perspectives. *Curr. Top. Med. Chem.* 11, 2072–2084. doi: 10.2174/156802611796575894

World Health Organization (2015). *World Health Organization: Chagas Disease*. Available at: http://www.who.int/mediacentre/factsheets/fs340/en/

Yoshida, N. (2006). Molecular basis of mammalian cell invasion by *Trypanosoma cruzi*. *An. Acad. Bras. Cienc.* 78, 87–111. doi: 10.1590/S0001-37652006000100010

Zingales, B., Andrade, S. G., Briones, M. R. S., Campbell, D. A., Chiari, E., Fernandes, O., et al. (2009). A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Mem. Inst. Oswaldo Cruz* 104, 1051–1054. doi: 10.1590/S0074-02762009000700021

Zingales, B., Miles, M. A., Campbell, D. A., Tibayrenc, M., Macedo, A. M., Teixeira, M. M. G., et al. (2012). The revised *Trypanosoma cruzi* subspecific nomenclature: rationale, epidemiological relevance and research applications. *Infect. Genet. Evol.* 12, 240–253. doi: 10.1016/j.meegid.2011.12.009

Zúñiga, E., Gruppi, A., Hirabayashi, J., Kasai, K. I., and Rabinovich, G. A. (2001a). Regulated expression and effect of galectin-1 on *Trypanosoma cruzi*-infected macrophages: modulation of microbicidal activity and survival. *Infect. Immun.* 69, 6804–6812. doi: 10.1128/IAI.69.11.6804-6812.2001

Zúñiga, E., Rabinovich, G. A., Iglesias, M. M., and Gruppi, A. (2001b). Regulated expression of galectin-1 during B-cell activation and implications for T-cell apoptosis. *J. Leukoc. Biol.* 70, 73–79.

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Leishmania infantum and *Leishmania braziliensis:* Differences and Similarities to Evade the Innate Immune System

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Visceral leishmaniasis is a severe form of the disease, caused by *Leishmania infantum* in the New World. Patients present an anergic immune response that favors parasite establishment and spreading through tissues like bone marrow and liver. On the other hand, *Leishmania braziliensis* causes localized cutaneous lesions, which can be self-healing in some individuals. Interactions between host and parasite are essential to understand disease pathogenesis and progression. In this context, dendritic cells (DCs) act as essential bridges that connect innate and adaptive immune responses. In this way, the aim of this study was to compare the effects of these two *Leishmania* species, in some aspects of human DCs' biology for better understanding of the evasion mechanisms of *Leishmania* from host innate immune response. To do so, DCs were obtained from monocytes from whole peripheral blood of healthy volunteer donors and from those infected with *L. infantum* or *L. braziliensis* for 24 h. We observed similar rates of infection (around 40%) as well as parasite burden for both *Leishmania* species. Concerning surface molecules, we observed that both parasites induced CD86 expression when DCs were infected for 24 h. On the other hand, we detected a lower surface expression of CD209 in the presence of both *L. braziliensis* and *L. infantum*, but only the last one promoted the survival of DCs after 24 h. Therefore, DCs infected by both *Leishmania* species showed a higher expression of CD86 and a decrease of CD209 expression, suggesting that both enter DCs through CD209 molecule. However, only *L. infantum* had the ability to inhibit DC apoptotic death, as an evasion mechanism that enables its spreading to organs like bone marrow and liver. Lastly, *L. braziliensis* was more silent parasite, once it did not inhibit DC apoptosis in our *in vitro* model.

Keywords: apoptosis, dendritic cells, *Leishmania infantum*, *Leishmania braziliensis*, CD209

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INTRODUCTION

Leishmaniasis is a vector-borne disease that commits millions of people around the world. In 2013, around 215,000 new cases were reported in the world. Of these, around 21,000 were reported in Brazil (1). Around 0.2–0.4 and 0.7–1.2 million visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), respectively, occur each year. Over 90% of new cases of VL occur in six countries all

over the world, including Brazil. About 95% of CL cases occur in the Americas, the Mediterranean basin, and the Middle East and Central Asia (2).

In the sandfly, the flagellated motile forms of *Leishmania* spp. called promastigotes progress through several morphological stages of differentiation, regulating the vector midgut environment (3). Finally, it becomes the non-dividing, infectious metacyclic promastigotes that are transmitted during a sandfly bite, when they are able to infect or be phagocyted by professional phagocytes as macrophages (4) and dendritic cells (DCs) (5). The parasite inside host cells becomes amastigote, a stage without an externalized flagellum that is capable of multiplication in antigen-presenting cells (6). CL is the most common clinical form of leishmaniasis and causes localized skin lesions, especially in arms and legs. In the New World, *Leishmania braziliensis* is able to cause from localized self-limited lesions to tissue destructive mucosal forms (7) that can worsen with age (8). On the other hand, visceral forms are caused by *Leishmania infantum* (9), and the disease, characterized by fever, weight loss, enlargement of the spleen and liver, and anemia, is fatal if left untreated (10).

Host–parasite interactions during innate immune responses determine the fate of adaptive immunity, contributing to healing or parasite persistence in leishmaniasis (11). DCs are professional antigen-presenting cells that interact with pathogens in peripheral tissues and stimulate T lymphocytes after migration to secondary lymphoid organs (12). In the periphery, DCs are in an immature state, with high potential to perform phagocytosis through many receptors that recognize pathogen-associated molecular patterns, as DC-SIGN (CD209) (13–15). Toll-like receptors (TLRs) are also involved in innate response to *Leishmania* parasites. During murine *Leishmania major* infection, data in literature showed that TLR9 was required for the induction of IL-12 in bone marrow-derived DCs (BMDCs) by intact *L. major* parasites or *L. major* DNA. This IL-12 production was essential for early interferon-gamma expression and NK cell activation (16). After pathogen internalization, DC is able to migrate from periphery to secondary tissues, rising MHC expression as well as other co-stimulatory molecules CD86 and CD83. After all these events, DCs are able to properly perform antigen presentation to T cells and determinate the fate of adaptive immune response (17). DCs can also become tolerogenic cells, as observed in the physiologic regulation of apoptosis (18). When *L. braziliensis* were in contact with murine $CD11c^+$ DCs, they were able to produce high levels of IL-12p70 as well as stimulate a significant expression of CD40 and CD83 in the surface of these DCs (19). Another study from the literature, working with mice BMDCs, showed that *L. infantum* could infect and survive inside these cells. Besides, they observed that *L. infantum* promastigotes were not able to upregulate CD40 and CD86 surface expression. The authors also observed that *L. infantum* was able to induce some level of IL-12p40 and IL-10, with no differences in TNF-α levels (20). Another group showed that bystander BMDCs from Balb/c mice increased IL-12p40 and expressed more CD40, CD86, and MHC class II in the cell surface than infected or not exposed cells. These bystander DCs induced a protective $CD4^+$ IFN-γ T cells response, while *L. infantum*-infected DCs polarizing to T-bet+IFN-γ+IL-10+ double producer T cells phenotype (21). On the other hand, human DCs infected with *L. major* showed an increase of HLA-DR, CD86, and CD40. When *L. major*-infected DCs were cocultured with T lymphocytes and treated with anti-CD40 ligand (CD40L), IL-12p70 and IFN-γ production decreased, concluding that IL-12p70 and IFN-γ production are CD40L-dependent (22). When CD86 $(B7\text{-}2)^{-/-}$ mice were infected with *L. major*, they presented a resistance, differently CD80 $(B7\text{-}1)^{-/-}$, and wild-type Balb/c mice maintained its susceptibility. Moreover, $CD86^{-/-}$ produced more IL-4 than wild-type mice, suggesting that CD86 induced a Th2 response (23). Infection of BALB/c mice by *Leishmania amazonensis* led to higher accumulation of Langerhans cells, and $CD4^+$ and $CD8^+$ T cells were found that produced IL-4 and IL-10. Nevertheless, *L. braziliensis* infection induced dermal DCs accumulation, increased effectors, and activated memory $CD4^+$ T cells and IL-4, IL-10, IFN-γ production by $CD4^+$ and $CD8^+$ T cells (24).

Many pathogens alter DC biology, favoring its persistence in the infected host. Data in literature showed that *L. amazonensis* is able to inhibit human DC differentiation from monocytes (25) and several intracellular signaling pathways (26), altering DC biology and function. Data from murine models of leishmaniasis show that inhibitory pathways are also involved in disease pathogenesis. One example is the OX40L–OX40 pathway that enhances Th2 responses in *L. major*-infected mice (27). Working with $Ox40l^{-/-}$ mice, the authors observed that they were very susceptible to *L. major* and *Leishmania mexicana*. Interestingly, only lymphnode cells from *L. major* $Ox40l^{-/-}$ mice produced less IL-4 and IL-10 than $Ox40l^{+/+}$. In this way, this pathway is relevant to Th2 development only in the context of *L. major* infection, but it does not alter *L. major* outcome of infection (28, 29). Other surface receptors, the programmed death ligand 1 (PD-L1) and 2 (PD-L2), were studied in susceptible mice infected with *L. mexicana*. PD-L1 knockout mouse demonstrated resistance, decrease of lesion size, as well as parasitic load and IL-4 production after infection. Although PD-L2 knockout mouse showed increase of lesion size, parasitic load, and antibodies production, no difference on IFN-γ and IL-4 production was observed. Both receptors play different roles in response to *L. mexicana* (30).

Another way that pathogens have to adapt to the host is to alter cell death. It is well known that *Mycobacterium tuberculosis* induces cell death through apoptosis, but this event favors antigen presentation (31). Moreover, pathogens as *Leishmania* delay cell death as a way to survive inside host cell. For example, apoptosis of monocyte-derived dendritic cells (moDCs) induced by treatment with camptothecin was downregulated by infection with *L. mexicana* amastigotes, detected by annexin V binding to phosphatidylserine (32).

It is not clear how the regulation of human DC biology is affected in different ways by species of *Leishmania*. In this way, the aim of this study is to analyze the expression of surface molecules relevant to antigen processing and presentation as well as DCs survival after interaction with *L. infantum* or *L. braziliensis*.

MATERIALS AND METHODS

Parasites

Leishmania infantum (MHOM/BR/1974/PP75) and *L. braziliensis* (MHOM/BR/01/BA788) promastigotes were maintained

in Schneider's medium (SIGMA) with 20% of calf fetal serum (GIBCO) and gentamicin (50 μm/mL). *Leishmania* stationary-phase promastigotes were quantified in Neubauer chamber. After counting, promastigotes were harvested from culture bottles, washed three times with cold PBS (800 g for 10 min), and adjusted in RPMI medium to be added in DC culture.

Dendritic Cell Culture

Human DCs were differentiated from monocytes. Briefly, venous blood was collected from volunteer healthy donors ($n = 8$), and peripheral blood mononuclear cells (PBMC) were obtained by passage over a Ficoll gradient (GE Healthcare). Cells were harvested, washed, and stained with antibodies, anti-CD14, conjugated with microbeads (Miltenyi Biotec). Stained cells (monocytes) were purified after positive selection in a magnetic field. Monocytes were then counted and cultivated in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (GIBCO), IL-4 (800 IU/mL), and GM-CSF (50 μg/mL) (both from PeproTech) with 10% of fetal serum bovine (GIBCO) in 24-wells plates (5×10^5 cells/wells) for 7 days at 37°C and 5% CO_2. New medium (200 μL) supplemented with cytokines was added on days 3 and 6 of the culture. On day 7, DCs were infected (1 DC:10 parasites), cultivated in 24-wells plates for 24 h at 37°C and 5% CO_2, harvested, and characterized by flow cytometry (FACS Verse BD Biosciences). All experimental conditions and measurements were performed with the same donors.

Flow Cytometry and Antibodies

Dendritic cells were cocultured with *Leishmania* species for 24 h, and control cells were harvested and washed with PBS. Cells were then resuspended in FACS buffer (5×10^6 cells/mL) and incubated at 4°C for 20 min. The cells were stained with CD1a FITC-conjugated (clone HI149), HLA-DR PE-conjugated (clone LN3), CD86 PE-Cy5-conjugated (clone IT2-2), and CD209 PE-conjugated (clone eB-h209) (all eBioscience) and incubated for 30 min at 4°C. Finally, cells were washed, resuspended in FACS buffer, and acquired on a flow cytometer. For cell death experiments, after infection time, the cells were washed and resuspended in 195 μL of binding buffer to each $2–5 \times 10^5$ cells, added 5 μL of annexin V, and incubated for 10 min at room temperature. The cells were washed with PBS 1×, resuspended in 190 μL of binding buffer, added 10 μL propidium iodide (PI) (annexin V and propidium iodide kit, eBioscience), and acquired on a flow cytometer. Stained cells were acquired on a Verse flow cytometer (BD Bioscience) and analyzed using FlowJo Software (Tree Star, Inc.).

Cytokine Assay

Dendritic cells were infected with both *Leishmania* species. After 24 h of infection, supernatant was collected and level of TNF-α was measured by ELISA. TNF-α was quantified (test detection range from 20 to 1000 pg/mL) with purified antihuman TNF-α, antihuman TNF-α biotin, and streptavidin–alkaline phosphatase, according to the instruction of suppliers (Novex, Life Technologies, Invitrogen). Absorbance was measured and analyzed with the program SoftMax Pro (Molecular Devices).

Ethical Statement

Human blood samples were collected after the signature of an informed consent signed by all volunteers, and the project was approved by The Ethical Committee for Human Beings from the Medicine Faculty of University of Brasilia (approval no. 072/2009). Samples were collected by venous puncture by a trained and specialized laboratory technician in the Universidade de Brasília.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism Software. Samples were tested by Shapiro–Wilk normality test, and the significance of the results was calculated using parametrical paired *t* test, and a *p*-value of <0.05 was considered significant.

RESULTS

L. braziliensis- or *L. infantum*-Exposed Dendritic Cells Upregulate CD86 and Downregulate CD209 Expression

After 24 h, the parasite load of DCs was accessed by optic microscopy, and we observed 40.67 ± 5.8% *L. infantum* (Li)-infected DCs and 42.13 ± 3.1% *L. braziliensis* (Lb)-infected DCs (**Figure 1A**). The amastigotes number per 100 cells was 249.8 ± 44.23 amastigotes in *L. infantum* (Li)-infected DCs and 185.3 ± 32.73 in

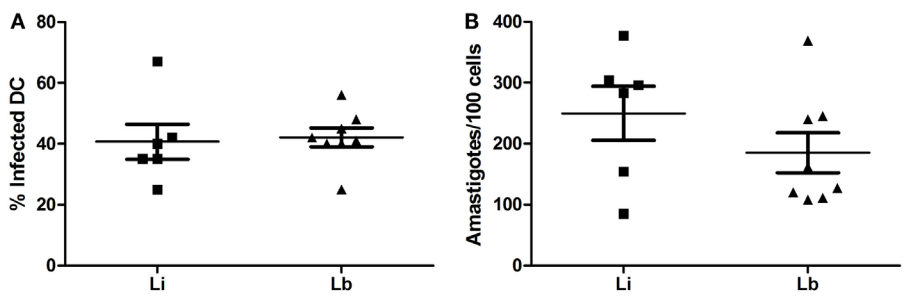

FIGURE 1 | Dendritic cells' infection by *L. infantum* (Li) or *L. braziliensis* (Lb) – immature DCs were infected in a rate of 10 *Leishmania* to 1 DC. Infection after 24 h, in hematoxylin–eosin (HE)-stained slides. Median ± SD. Each point represents one donor. *$p < 0.01$. **(A)** Percentage of infected DCs. **(B)** Amastigotes per 100 cells.

L. braziliensis (Lb)-infected cells (**Figure 1B**). Later, we decided to observe the co-stimulatory and surface molecules expression by DCs after the exposure to *L. infantum* or *L. braziliensis*. Then, we stained the cultured DCs with medium or each parasites species with anti-CD1a, anti-HLA-DR, anti-CD86, and anti-CD209 antibodies. The cells were gated by granularity and size, and the gated cells were further analyzed for expression of CD1a/HLA-DR/CD86/CD209 surface molecules (**Figure 2**). Control DCs, differentiated with media only (M), showed 56.53 ± 8.2% of CD1a expression. This was very similar in DCs exposed to *L. infantum* (60.40 ± 8.1%) as well as exposed to *L. braziliensis* (57.8 ± 8.3%) (**Figure 3A**).

In the same way, we observed a consistent higher expression of HLA-DR in control cells (73.81 ± 5.55%)-, in *L. infantum* (68.27 ± 4.8%)-, and *L. braziliensis* (80.89 ± 2.7%)-exposed DCs, with no significant differences between the groups (**Figure 3B**).

However, both *Leishmania* were able to induce a significant raise in CD86 expression. We observed that CD86 expression was 73.23 ± 5.4% in control cells and increased to 78.16 ± 4.3% in the presence of *L. infantum* and to 92.24 ± 1.9% in the presence of *L. braziliensis* (**Figure 3C**). Moreover, this increase was significantly higher in *L. braziliensis*-infected DCs than in *L. infantum*-infected DCs (**Figure 3C**).

On the other hand, we observed that both *Leishmania* were able to inhibit significantly CD209 expression. Its expression was 80.09 ± 6.8% in control cells, while in the presence of *L. infantum*, it was down modulated to 62.17 ± 9.88%. The presence of *L. braziliensis* also led to a lower CD209 expression (75.36 ± 7.3%) (**Figure 3D**). This decrease was significantly lower in *L. infantum*-infected DCs than *L. braziliensis*-infected DCs (**Figure 3D**).

When we analyzed the mean intensity of fluorescence (MFI) of these molecules, we observed the same effects that we observed in the percentage of expression. The MFI was similar in all experimental conditions for CD1a and HLA-DR (**Figures 4A,B**). On the other hand, we observed a significantly higher CD86 MFI induced by *L. braziliensis* (2060 ± 351.9), but no significant difference was found in the presence of *L. infantum* (1537 ± 169.4) when compared to control cells (1227 ± 152.2) (**Figure 4C**). Also, as we observed in surface expression, the CD209 MFI was significantly lower in the presence of *L. infantum* (856.1 ± 72.8) or *L. braziliensis* (1033 ± 112.6), compared to control cells (1127 ± 116.7) (**Figure 4D**).

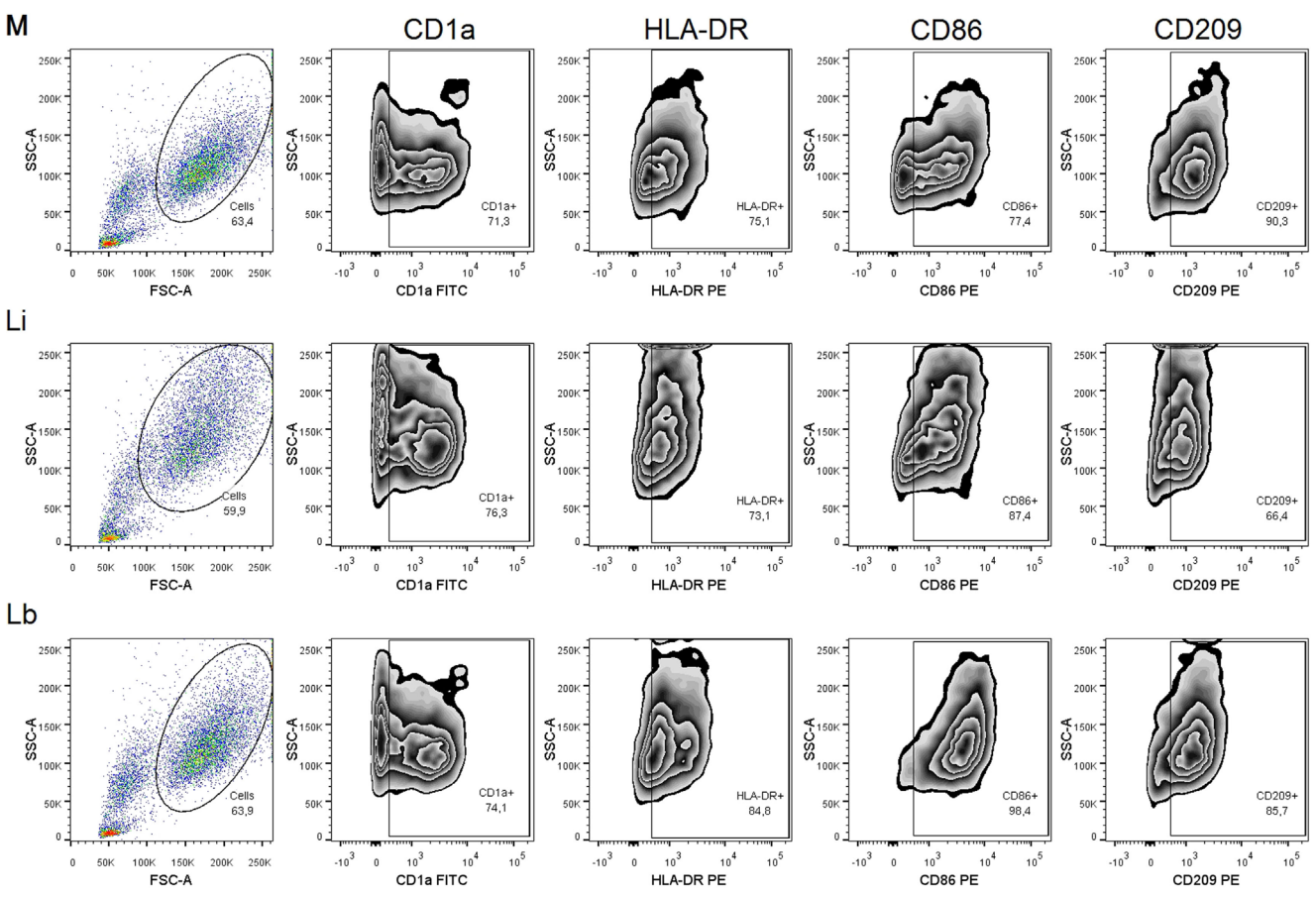

FIGURE 2 | Gating strategy to analyze DCs' regulation by *Leishmania* parasites – DCs in control and infected conditions were gated by granularity and size and further analyzed for expression of CD1a/HLA-DR/CD86/CD209 surface molecules. M, medium, Li, *Leishmania infantum*, Lb, *Leishmania braziliensis*.

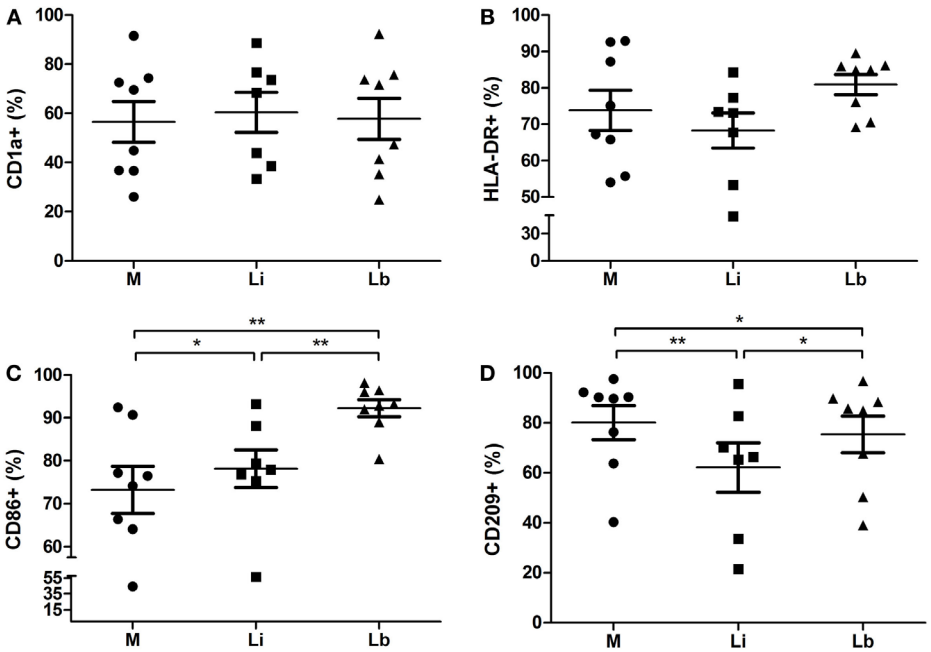

FIGURE 3 | *L. braziliensis*- or *L. infantum*-exposed dendritic cells upregulate CD86 and downregulate CD209 expression – flow cytometry analysis of surface molecule expression in immature DCs differentiated from human monocytes with cytokine-conditioned media (M) or conditioned media in the presence of viable *L. infantum* (Li) or *L. braziliensis* (Lb). CD1a **(A)**, HLA-DR **(B)**, CD86 **(C)**, and CD209 **(D)**. Median ± SD. Each point represents one donor. $^{*}p < 0.05$, $^{**}p < 0.01$.

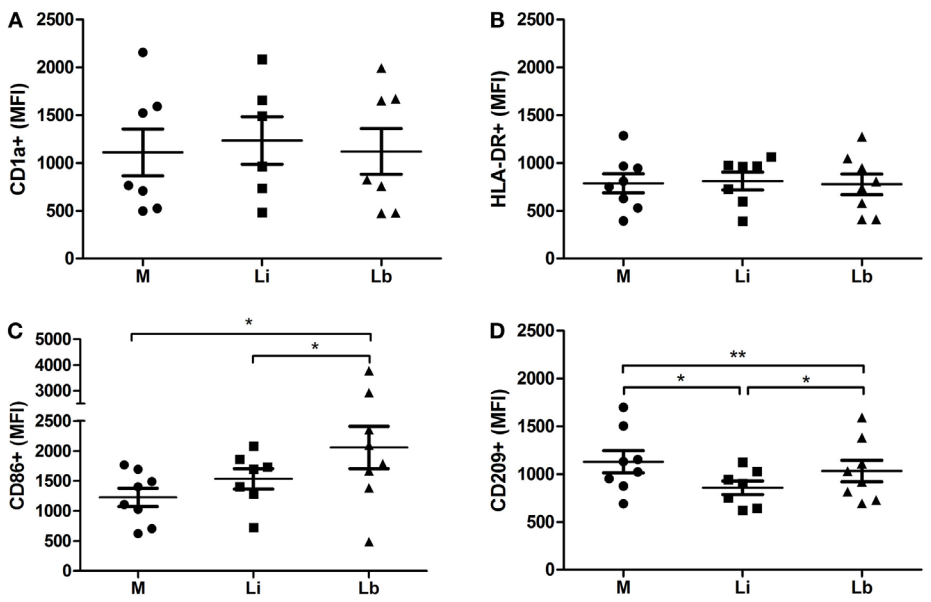

FIGURE 4 | *L. braziliensis*-exposed DCs increase the mean of fluorescence intensity (MFI) of CD86 and decrease CD209 – flow cytometry analyzes of the MFI in immature DCs differentiated from human monocytes with cytokine-conditioned media (M) or conditioned media in the presence of viable *L. infantum* (Li) *or L. braziliensis* (Lb). CD1a **(A)**, HLA-DR **(B)**, CD86 **(C)**, and CD209 **(D)**. Median ± SD. Each point represents one donor. $^{*}p < 0.05$, $^{**}p < 0.01$.

Exposure to *L. infantum* but Not *L. braziliensis* Decreases the Dendritic Cells' Cellular Death

Next, we decided to evaluate if these two *Leishmania* species were able to induce or not cell death of DCs. To do so, after a coculture of the DCs with the two parasite species, we stained the cells with annexin V and PI (**Figure 5A**). We observed no significant differences between DCs only and *L. braziliensis*-exposed DCs in the annexin V+PI− and annexin V+PI+ populations (**Figures 5B,C**, respectively). Nevertheless, the cell population that was annexin V+PI− population significantly decreased in *L. infantum*-exposed DCs, from 5.7 ± 1.0% in control cells to 2.9 ± 0.6%, that is a 50.8% decrease in DC death, showing that the *L. infantum* is able to inhibit cellular death of DCs (**Figure 5B**). However, no significant decrease was observed in the annexin V+PI+ population, 11.36 ± 2% in control cells and 8.3 ± 2.4% in *L. infantum*-exposed DCs (**Figure 5C**).

L. infantum- and *L. braziliensis*-Exposed Dendritic Cells Produced TNF-α

Coculture with *L. infantum* or *L. braziliensis* triggered a significant secretion of TNF-α by DCs. Control cells produced 17.6 ± 4.4 pg/mL, while DCs cocultured with *L. infantum* induced 61.9 ± 10.4 pg/mL, a 3.5-fold significant increase in this cytokine secretion. On the other hand, *L. braziliensis* stimulated significantly even more TNF-α secretion (133.2 ± 43.7 pg/mL), almost an eightfold increase (**Figure 6**).

DISCUSSION

Many data in literature show the relevant role of DCs in diverse pathogen–host interactions, especially in the initial events of innate immunity. These initial events lead the course of adaptive response (33). For a better understanding of *Leishmania* and infected host regulation, we initially cocultured DCs with stationary non-opsonized *L. braziliensis* and *L. infantum* promastigotes for 24 h. The infection rate was similar in our experiments between the two *Leishmania* species (around 40%), and we found no significant difference of internalized parasites number. Concerning infection data, non-opsonized *L. mexicana* promastigotes presented higher phagocytosis than opsonized promastigotes or amastigotes by human moDCs after 3 h. Six hours later, opsonized promastigotes showed higher phagocytosis than non-opsonized promastigotes and amastigotes, but no difference was observed after 24 h of infection (5). Infection of human DCs with *L. amazonensis* showed a similar rate of infection, around 45%, and a parasite burden median of six amastigotes per DC (25).

We observed that both *L. braziliensis* and *L. infantum* were able to induce an increase in CD86 expression and also caused a decrease in CD209 (DC-SIGN) expression in human DCs. A study with *L. amazonensis* showed that the parasite modulates the co-stimulatory molecules expression during the differentiation of human moDCs, affecting the mixed leukocyte reaction and the specific T lymphocytes responses. Similar to our data, *L. amazonensis* also caused an increase in CD86 expression after a 24-h parasite DCs contact (25). On the other hand, murine

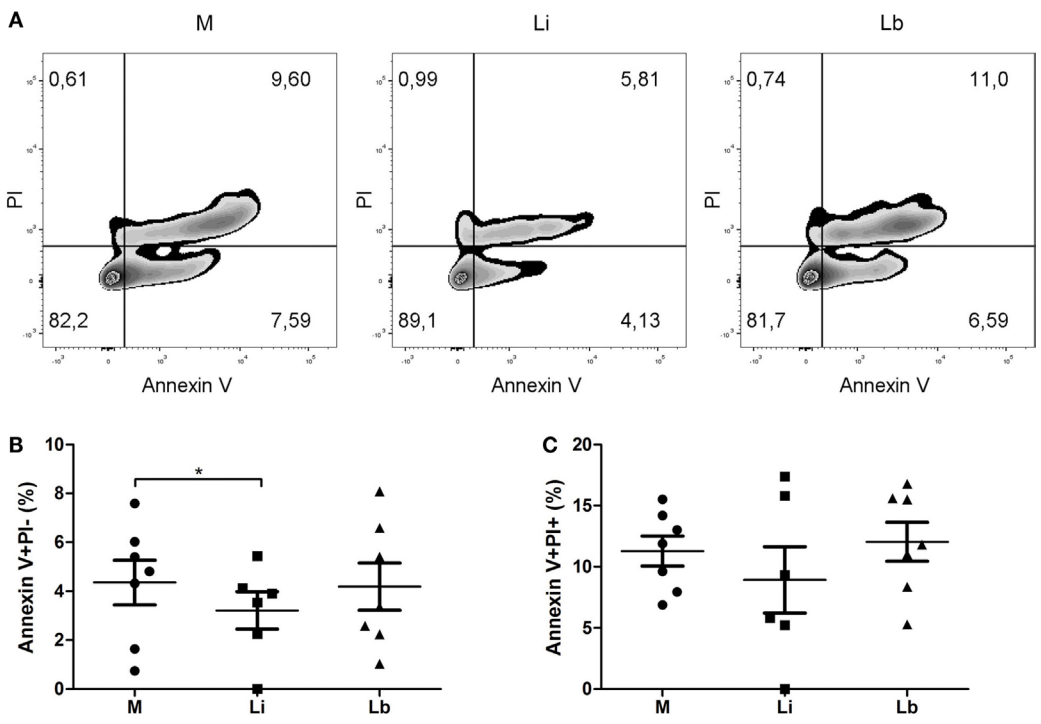

FIGURE 5 | *L. infantum* delayed the cellular death of dendritic cells – flow cytometry gating strategy (A) to perform analyses of annexin V and propidium iodide (PI) staining of control DCs (M) or treated with viable *L. infantum* (Li) or *L. braziliensis* (Lb). Annexin V positive cells **(B)** and annexin V and PI positive cells **(C)**. Median ± SD. Each point represents one donor. *$p < 0.05$, **$p < 0.01$.

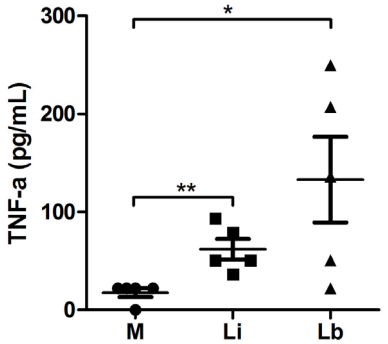

FIGURE 6 | *L. braziliensis* induced TNF-α secretion on immature DCs – cytokine levels measured on culture supernatants after 24 h of infection. Control DCs were cultured in cytokine-conditioned media only or treated with viable *L. infantum* or *L. braziliensis* parasites. Median ± SD. Each point represents one donor. $^{*}p < 0.05$, $^{**}p < 0.01$.

mature DCs infected with *L. amazonensis* amastigotes presented a decreased antigen presentation, CD40 and CD83 expression, and also a reduced $CD4^+$ T cells priming (34). Contreras et al. (35), showed that *L. mexicana* infection also interfered in the DC maturation process, decreasing co-stimulatory molecules expression and antigen presentation, injuring the presentation to T cells by human DCs. Macrophages cultured with peripheral blood leukocytes (PBL) exhibited CD40 and CD86 expression in the absence of *L. major*, but their expression levels were higher in the presence of the parasite. After blocking CD80, CD86, both, or CD40, macrophages cultured with PBL and *L. major* produced less IFN-γ, IL-5, and IL-12, showing that these co-stimulatory pathways were important to the development of an anti-*Leishmania*-efficient immune response (36). On the other hand, a study performed with VL patients showed that the subset of inflammatory monocytes $CD14^+CD16^+$ remained the same as in the control group. Similarly, the population of $CD54^+$HLA-DR^+ monocytes was significantly downregulated during active disease. Finally, they observed an increase in CD80 expression level, but a decreased CD86 expression (37). Concerning infection with *L. braziliensis*, data from literature showed that $CD14^+$ monocytes of peripheral blood from CL patients had a decreased expression of CD80 and CD86 following culture with media only or after restimulation with *Leishmania* antigen (38). It seems that monocytes and DCs have different roles in the regulation leishmaniasis. Monocytes seem to have a role in regulating the lesion site during CL pathogenesis, and the authors mentioned before Vieira et al. (38) observed a diminished expression of B7 molecules. In our model, we worked with moDCs from healthy donors' peripheral blood. In this way, it is possible that in these initial interactions, once our experiments were performed after a 24-h infection with *Leishmania* parasites, these DCs regulate CD86 expression positively, being able to activate naive lymphocytes. On the other hand, the effects seem to be opposite for monocytes that migrate to lesion sites, once in patients with CL it is observed a negative regulation of CD86 (38).

Concerning CD209 expression, the decrease of this surface molecule was also observed in *L. major*- and *L. donovani*-infected human DCs (39). It is well established that the receptor CD209 can be used for internalizing microrganisms as parasites (40). In this way, our data are in accordance with data in the literature, suggesting that *L. infantum* and *L. braziliensis* attach to CD209 to access host DCs, which would explain the decrease in CD209 in the surface, as it could be internalized. Nevertheless, CD209 was involved in the phagocytosis of *L. mexicana* promastigotes after 3 h, but not after 6 or 24 h (5). Excreted or secreted recombinant proteins of *L. infantum* interfered in the differentiation of DCs, decreasing the HLA-DR expression but increasing the CD86 expression (41). We observed the same effect concerning CD86, but we observed no significant differences in HLA-DR surface expression caused by *L. infantum* or *L. braziliensis* in our work. Regarding CD86 and CD209 expression, we found a higher expression of CD86 in the presence of *L. braziliensis* than in the presence of *L. infantum*; however, the decrease in CD209 expression was greater in cells infected with *L. infantum* than in *L. braziliensis*-infected ones. Though, the rates of infection as well as the number of internalized amastigotes were similar between both the species of *Leishmania*. This can be explained by the fact that DCs have other recognition receptors that are able to recognize and be involved in *Leishmania* internalization, which were not investigated in this work. We can mention the mannose–fucose receptor, which is present in DCs that can recognize lipophosphoglycan (LPG), GP63, and proteophosphoglicans (PPG) present in the membrane of *Leishmania* species (42, 43). This pathway could explain the DCs' infection rate by *L. braziliensis* and the number of amastigotes to be similar to the *L. infantum*-infection. However, macrophages of B6 mice mannose receptor (MR) knockout showed an increase of parasite load after infection with *L. infantum*. This group showed that MR and Dectin-1 signaling pathways are essential to trigger the oxidative burst in macrophages infected with *L. infantum*, enabling parasite control (44).

Many data in literature showed the relevance of cytokines regulation in leishmaniasis pathogenesis. The presence of *L. amazonensis* during the differentiation of human DC inhibited IL-6 and IL-10 secretion. Besides, these DCs cocultured with *L. amazonensis*, when in contact with autologous lymphocytes, caused a lower secretion of IFN-γ (25). Moreover, amastigotes of *L. amazonensis* suppressed IL-12+p40 and IL-10 production by murine DCs (34). However, we found a higher release of TNF-α after coculture of DCs and *L. braziliensis* or *L. infantum*. The excreted or secreted recombinant proteins of *L. infantum* also induced TNF-α production (41), as we saw for both *Leishmania* species in this work. Studies with fluorescent *L. braziliensis* parasites allowed the observation that the TNF-α production derived from infected DCs, but not from bystander DCs, had contact with *L. braziliensis* in the medium but were not infected. Moreover, soluble parasite products and the TNF-α released by infected DCs provided a more efficient antigen presentation by DCs (45). Data from literature evaluated TNF-α production from PBMC of patients and healthy individuals after stimulation with soluble *L. braziliensis* antigens. The results showed a range in the SD, as expected for human individuals. They showed that healthy donors produced 48.6 ± 77.18 pg/mL of TNF-α (46). Another study that compared PBMC responses from patients

with CL and mucosal leishmaniasis (ML), after stimulation with *L. braziliensis* soluble antigen, also detected a wide range of TNF-α levels. In CL, TNF-α levels ranged from 0 to 7440 pg/mL, while in ML patients from 892 to 9173 pg/mL (47). These differences in TNF levels are expected and may exist due to the genetic constitution of the host. On the other hand, data from literature that analyzed immune responses from patients with active VL detected some similarities among VL patients and healthy individuals, concerning cytokines regulation. Analysis *ex vivo* of patient's cells with active VL showed the production of high levels of anti-inflammatory cytokines and lower TNF levels, the last ones very similar to healthy controls (37). Another study with patients with active VL due to *L. infantum* also showed lower TNF-α levels, very similar to healthy controls (48). These observations are similar to our results in which we could not observe the individual variations in healthy donors' DCs infected with *L. infantum*. Another possibility is that even with similar rates of infection and parasites per cell, our results reflect the differences cited before concerning the immunogenicity of *L. braziliensis* and *L. infantum*.

We observed that *L. infantum* caused an inhibition of DC apoptosis. The same phenomenon was not observed for *L. braziliensis*. This demonstrates that possibly only *L. infantum* was able to stimulate the development of an environment adequate for the multiplication inside the cells and that favors a possible spread to other organs as bone marrow and liver. This surviving of DC was also seen in *L. mexicana* infection when apoptosis was induced by camptothecin (49). Indeed, DCs infected with *L. mexicana* amastigotes decreased the phosphorylation of MAP kinase p38 and JNK, causing a decreased DNA fragmentation in the camptothecin stimulated DCs. *L. mexicana* amastigotes activated antiapoptotic pathways, such as PI3K and AKT, allowing the inhibition of infected DCs' cell death (50). The opposite effect was observed with the bacteria *Brucella abortus* that induced apoptosis and necrosis of murine DCs. This apoptosis regulation was performed by caspase-2 in mouse model (51).

A very elegant paper showed that Dectin-1, MR, and DC-SIGN homolog SIGNR3 are able to recognize *L. infantum*, but only DC-SIGN (CD209) and MR triggered pro-IL1 beta processing in a caspase-1-dependent way, being crucial for microbicidal activity of macrophages. On the other hand, signaling through SIGNR3 seems to favor parasite survival because of its modulation of inflammasome activation (44). Once we found a possible correlation of CD209 and cell death, perhaps the internalization of *L. infantum* by CD209 in human DCs could also be interfering in the activation of caspase-1, causing the survival of DCs differently from the data observed on macrophages. On the other hand, *L. braziliensis* seems to not interfere in this pathway of cell death regulation, once we did not observe differences concerning DC survival.

In this way, we can conclude that both *L. infantum* and *L. braziliensis* possibly enter DCs through DC-SIGN, causing a positive regulation on CD86 expression as well as on TNF-α production after this initial interaction. Nevertheless, only *L. infantum* induced a survival in human DCs, probably favoring its establishment and spread in the host, differently from *L. braziliensis* that seems to be more silent in these initial events concerning DC survival. This work shed light in the differences between these two parasites and their interactions with human DCs and opens the field to study the development of intracellular signaling pathways responsible for these phenomena.

AUTHOR CONTRIBUTIONS

SF and CF: conceived and designed the experiments. SF, TJ, LF, DB, and CF: performed the experiments. SF, TJ, and CF: analyzed the data. SF, JS, and CF: contributed reagents/materials/analysis tools. SF and CF: wrote the paper.

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REFERENCES

1. WHO. *Accelerating Work to Overcome the Global Impact of Neglected Tropical*. Geneva: WHO (2012).
2. Alvar J, Vélez D, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* (2012) 7:e35671. doi:10.1371/journal.pone.0035671
3. Santos VC, Vale VF, Silva SM, Nascimento AA, Saab NA, Soares RP, et al. Host modulation by a parasite: how *Leishmania infantum* modifies the intestinal environment of *Lutzomyia longipalpis* to favor its development. *PLoS One* (2014) 9:e111241. doi:10.1371/journal.pone.0111241
4. Ramos PK, Brito MV, Silveira FT, Salgado CG, De Souza W, Picanço-Diniz CW, et al. In vitro cytokines profile and ultrastructural changes of microglia and macrophages following interaction with *Leishmania*. *Parasitology* (2014) 141:1052–63. doi:10.1017/S0031182014000274
5. Argueta-Donohué J, Wilkins-Rodríguez AA, Aguirre-García M, Gutiérrez-Kobeh L. Differential phagocytosis of *Leishmania mexicana* promastigotes and amastigotes by monocyte-derived dendritic cells. *Microbiol Immunol* (2015). 60:369–81. doi:10.1111/1348-0421.12325
6. Prina E, Abdi S, Lebastard M, Perret E, Winter N, Antoine J. Dendritic cells as host cells for the promastigote and amastigote stages of *Leishmania amazonensis*: the role of opsonins in parasite uptake and dendritic cell maturation. *J Cell Sci* (2004) 117:315–25. doi:10.1242/jcs.00860
7. Barral A, Barral-Netto M, Almeida R, de Jesus AR, Grimaldi Júnior G, Netto EM, et al. Lymphadenopathy associated with *Leishmania braziliensis* cutaneous infection. *Am J Trop Med Hyg* (1992) 47:587–92.
8. Carvalho AM, Amorim CF, Barbosa JL, Lago AS, Carvalho EM. Age modifies the immunologic response and clinical presentation of American tegumentary leishmaniasis. *Am J Trop Med Hyg* (2015) 92:1173–7. doi:10.4269/ajtmh.14-0631
9. Ready PD. Epidemiology of visceral leishmaniasis. *Clin Epidemiol* (2014) 6:147–54. doi:10.2147/CLEP.S44267
10. Bhattacharya SK, Dash AP. Treatment of visceral leishmaniasis: options and choice. *Lancet Infect Dis* (2016) 16:142–3. doi:10.1016/S1473-3099(15)00528-9
11. Gollob KJ, Viana AG, Dutra WO. Immunoregulation in human American leishmaniasis: balancing pathology and protection. *Parasite Immunol* (2014) 36:367–76. doi:10.1111/pim.12100

12. Cools N, Ponsaerts P, Van Tendeloo VF, Berneman ZN. Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. *J Leukoc Biol* (2007) 82:1365–74. doi:10.1189/jlb.0307166
13. Colmenares M, Puig-Kröger A, Pello OM, Corbí AL, Rivas L. Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for *Leishmania amastigotes*. *J Biol Chem* (2002) 277:36766–9. doi:10.1074/jbc.M205270200
14. Vannberg FO, Chapman SJ, Khor CC, Tosh K, Floyd S, Jackson-Sillah D, et al. CD209 genetic polymorphism and tuberculosis disease. *PLoS One* (2008) 3:e1388. doi:10.1371/journal.pone.0001388
15. Jin W, Li C, Du T, Hu K, Huang X, Hu Q. DC-SIGN plays a stronger role than DCIR in mediating HIV-1 capture and transfer. *Virology* (2014) 458-459:83–92. doi:10.1016/j.virol.2014.04.016
16. Liese J, Schleicher U, Bogdan C. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur J Immunol* (2007) 37:3424–34. doi:10.1002/eji.200737182
17. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J* (2014) 33:1104–16. doi:10.1002/embj.201488027
18. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* (2000) 191:411–6. doi:10.1084/jem.191.3.411
19. Vargas-Inchaustegui DA, Xin L, Soong L. *Leishmania braziliensis* infection induces dendritic cell activation, ISG15 transcription, and the generation of protective immune responses. *J Immunol* (2008) 180:7537–45. doi:10.4049/jimmunol.180.11.7537
20. Neves BM, Silvestre R, Resende M, Ouaissi A, Cunha J, Tavares J, et al. Activation of phosphatidylinositol 3-kinase/Akt and impairment of nuclear factor-kappaB: molecular mechanisms behind the arrested maturation/activation state of *Leishmania infantum*-infected dendritic cells. *Am J Pathol* (2010) 177:2898–911. doi:10.2353/ajpath.2010.100367
21. Resende M, Moreira D, Augusto J, Cunha J, Neves B, Cruz MT, et al. *Leishmania*-infected MHC class II high dendritic cells polarize CD4+ T cells toward a nonprotective T-bet+ IFN-γ+ IL-10+ phenotype. *J Immunol* (2013) 191:262–73. doi:10.4049/jimmunol.1203518
22. Marovich MA, McDowell MA, Thomas EK, Nutman TB. IL-12p70 production by *Leishmania major*-harboring human dendritic cells is a CD40/CD40 ligand-dependent process. *J Immunol* (2000) 164:5858–65. doi:10.4049/jimmunol.164.11.5858
23. Brown JA, Greenwald RJ, Scott S, Schweitzer AN, Satoskar AR, Chung C, et al. T helper differentiation in resistant and susceptible B7-deficient mice infected with *Leishmania major*. *Eur J Immunol* (2002) 32:1764–72. doi:10.1002/1521-4141(200206)32:6<1764::AID-IMMU1764>3.0.CO;2-V
24. Carvalho AK, Carvalho K, Passero LF, Sousa MG, da Matta VL, Gomes CM, et al. Differential recruitment of dendritic cells subsets to lymph nodes correlates with a protective or permissive T-cell response during *Leishmania* (*Viannia*) *braziliensis* or *Leishmania* (*Leishmania*) *amazonensis* infection. *Mediators Inflamm* (2016) 2016:7068287. doi:10.1155/2016/7068287
25. Favali C, Tavares N, Clarêncio J, Barral A, Barral-Netto M, Brodskyn C. *Leishmania amazonensis* infection impairs differentiation and function of human dendritic cells. *J Leukoc Biol* (2007) 82:1401–6. doi:10.1189/jlb.0307187
26. Soong L. Modulation of dendritic cell function by *Leishmania* parasites. *J Immunol* (2008) 180:4355–60. doi:10.4049/jimmunol.180.7.4355
27. Ishii N, Ndhlovu LC, Murata K, Sato T, Kamanaka M, Sugamura K. OX40 (CD134) and OX40 ligand interaction plays an adjuvant role during in vivo Th2 responses. *Eur J Immunol* (2003) 33:2372–81. doi:10.1002/eji.200324031
28. Akiba H, Miyahira Y, Atsuta M, Takeda K, Nohara C, Futagawa T, et al. Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *J Exp Med* (2000) 191:375–80. doi:10.1084/jem.191.2.375
29. Tuladhar R, Oghumu S, Dong R, Peterson A, Sharpe AH, Satoskar AR. Ox40L-Ox40 pathway plays distinct roles in regulating Th2 responses but does not determine outcome of cutaneous leishmaniasis caused by *Leishmania mexicana* and *Leishmania major*. *Exp Parasitol* (2015) 148:49–55. doi:10.1016/j.exppara.2014.11.002
30. Liang SC, Greenwald RJ, Latchman YE, Rosas L, Satoskar A, Freeman GJ, et al. PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis. *Eur J Immunol* (2006) 36:58–64. doi:10.1002/eji.200535458
31. Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, et al. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* (2006) 24:105–17. doi:10.1016/j.immuni.2005.12.001
32. Gutiérrez-Kobeh L, de Oyarzabal E, Argueta J, Wilkins A, Salaiza N, Fernández E, et al. Inhibition of dendritic cell apoptosis by *Leishmania mexicana* amastigotes. *Parasitol Res* (2013) 112:1755–62. doi:10.1007/s00436-013-3334-2
33. Bieber K, Autenrieth SE. Insights how monocytes and dendritic cells contribute and regulate immune defense against microbial pathogens. *Immunobiology* (2015) 220:215–26. doi:10.1016/j.imbio.2014.10.025
34. Xin L, Li K, Soong L. Down-regulation of dendritic cell signaling pathways by *Leishmania amazonensis* amastigotes. *Mol Immunol* (2008) 45:3371–82. doi:10.1016/j.molimm.2008.04.018
35. Contreras I, Estrada JA, Guak H, Martel C, Borjian A, Ralph B, et al. Impact of *Leishmania mexicana* infection on dendritic cell signaling and functions. *PLoS Negl Trop Dis* (2014) 8:e3202. doi:10.1371/journal.pntd.0003202
36. Brodskyn C, Beverley SM, Titus RG. Virulent or avirulent (dhfr-ts-) *Leishmania major* elicit predominantly a type-1 cytokine response by human cells in vitro. *Clin Exp Immunol* (2000) 119:299–304. doi:10.1046/j.1365-2249.2000.01122.x
37. Roy S, Mukhopadhyay D, Mukherjee S, Ghosh S, Kumar S, Sarkar K, et al. A defective oxidative burst and impaired antigen presentation are hallmarks of human visceral leishmaniasis. *J Clin Immunol* (2015) 35:56–67. doi:10.1007/s10875-014-0115-3
38. Vieira ÉL, Keesen TS, Machado PR, Guimarães LH, Carvalho EM, Dutra WO, et al. Immunoregulatory profile of monocytes from cutaneous leishmaniasis patients and association with lesion size. *Parasite Immunol* (2013) 35:65–72. doi:10.1111/pim.12012
39. Revest M, Donaghy L, Cabillic F, Guiguen C, Gangneux JP. Comparison of the immunomodulatory effects of *L. donovani* and *L. major* excreted-secreted antigens, particulate and soluble extracts and viable parasites on human dendritic cells. *Vaccine* (2008) 26:6119–23. doi:10.1016/j.vaccine.2008.09.005
40. Colmenares M, Corbí AL, Turco SJ, Rivas L. The dendritic cell receptor DC-SIGN discriminates among species and life cycle forms of *Leishmania*. *J Immunol* (2004) 172:1186–90. doi:10.4049/jimmunol.172.2.1186
41. Markikou-Ouni W, Drini S, Bahi-Jaber N, Chenik M, Meddeb-Garnaoui A. Immunomodulatory effects of four *Leishmania infantum* potentially excreted/secreted proteins on human dendritic cells differentiation and maturation. *PLoS One* (2015) 10:e0143063. doi:10.1371/journal.pone.0143063
42. Green PJ, Feizi T, Stoll MS, Thiel S, Prescott A, McConville MJ. Recognition of the major cell surface glycoconjugates of *Leishmania* parasites by the human serum mannan-binding protein. *Mol Biochem Parasitol* (1994) 66:319–28. doi:10.1016/0166-6851(94)90158-9
43. Liu D, Uzonna JE. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* (2012) 2:83. doi:10.3389/fcimb.2012.00083
44. Lefèvre L, Lugo-Villarino G, Meunier E, Valentin A, Olagnier D, Authier H, et al. The C-type lectin receptors dectin-1, MR, and SIGNR3 contribute both positively and negatively to the macrophage response to *Leishmania infantum*. *Immunity* (2013) 38:1038–49. doi:10.1016/j.immuni.2013.04.010
45. Carvalho K, Vallejo M, Camargo Z, Puccia R. Use of recombinant gp43 isoforms expressed in *Pichia pastoris* for diagnosis of paracoccidioidomycosis. *Clin Vaccine Immunol* (2008) 15:622–9. doi:10.1128/CVI.00437-07
46. de Assis Souza M, de Castro MC, de Oliveira AP, de Almeida AF, de Almeida TM, Reis LC, et al. Cytokines and NO in American tegumentary leishmaniasis patients: profiles in active disease, after therapy and in self-healed individuals. *Microb Pathog* (2013) 57:27–32. doi:10.1016/j.micpath.2013.02.004
47. Oliveira WN, Ribeiro LE, Schrieffer A, Machado P, Carvalho EM, Bacellar O. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of human tegumentary leishmaniasis. *Cytokine* (2014) 66:127–32. doi:10.1016/j.cyto.2013.12.016
48. Nateghi Rostami M, Seyyedan Jasbi E, Khamesipour A, Mohammadi AM. Tumour necrosis factor-alpha (TNF-α) and its soluble receptor type 1 (sTNFR I) in human active and healed leishmaniases. *Parasite Immunol* (2016) 38:255–60. doi:10.1111/pim.12305

49. Valdés-Reyes L, Argueta J, Morán J, Salaiza N, Hernández J, Berzunza M, et al. *Leishmania mexicana*: inhibition of camptothecin-induced apoptosis of monocyte-derived dendritic cells. *Exp Parasitol* (2009) 121:199–207. doi:10.1016/j.exppara.2008.10.020
50. Vázquez-López R, Argueta-Donohué J, Wilkins-Rodríguez A, Escalona-Montaño A, Aguirre-García M, Gutiérrez-Kobeh L. *Leishmania mexicana* amastigotes inhibit p38 and JNK and activate PI3K/AKT: role in the inhibition of apoptosis of dendritic cells. *Parasite Immunol* (2015) 37:579–89. doi:10.1111/pim.12275
51. Li X, He Y. Caspase-2-dependent dendritic cell death, maturation, and priming of T cells in response to *Brucella abortus* infection. *PLoS One* (2012) 7:e43512. doi:10.1371/journal.pone.0043512

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Scrutinizing the Biomarkers for the Neglected Chagas Disease: How Remarkable!

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Biomarkers or biosignature profiles have become accessible over time in population-based studies for Chagas disease. Thus, the identification of consistent and reliable indicators of the diagnosis and prognosis of patients with heart failure might facilitate the prioritization of therapeutic management to those with the highest chance of contracting this disease. The purpose of this paper is to review the recent state and the upcoming trends in biomarkers for human Chagas disease. As an emerging concept, we propose a classification of biomarkers based on plasmatic-, phenotype-, antigenic-, genetic-, and management-related candidates. The available data revisited here reveal the lessons learned thus far and the existing challenges that still lie ahead to enable biomarkers to be employed consistently in risk evaluation for this disease. There is a strong need for biomarker validation, particularly for biomarkers that are specific to the clinical forms of Chagas disease. The current failure to achieve the eradication of the transmission of this disease has produced determination to solve this validation issue. Finally, it would be strategic to develop a wide variety of biomarkers and to test them in both preclinical and clinical trials.

Keywords: Chagas disease, biomarkers for immune responsiveness, human experimentation, clinical forms, mini review

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INTRODUCTION

Population-based studies have identified a range of biomarkers that indicate exposure to, effects of, and genetic susceptibility for different pathogen-related diseases. These biomarkers could potentially be applied for diagnostic and prognostic purposes in human Chagas disease. The available data reveal the lessons learned to date and the current challenges that still remain to enable biomarkers to be employed reliably in risk evaluation for this disease. Our main purpose here is to revisit the current evidence and future trends in biomarker research for human Chagas disease. And due to recent, elegant systematic reviews focusing on this important topic (see below), we instead present here a literature review.

Chagas disease, or American trypanosomiasis, is caused by the etiological agent *Trypanosoma cruzi* and affects at least eight million people in Central and South America (1). The morbidity is high. The acute phase of infection is followed by an asymptomatic phase, but ~30% of infected patients develop a symptomatic, chronic phase that is characterized by either severe cardiac or digestive forms of Chagas disease (2, 3). Hence, the identification of consistent and reliable indicators

of Chagas disease pathology, namely biomarkers or biosignature profiles, might facilitate the prioritization of management to those with a chance of contracting Chagas disease. Biomarker candidates might be engaged for the determination of some forms of Chagas disease. It is difficult to predict features related to morbidity and mortality as well as disease evolution. This information would assist in the supervision, decision-making, and follow-up related to this complex disease. Hence, there is a need to discover simple and quantifiable biomarkers that are more reliable than conventional screening methods and can be applied to support the diagnosis and prognosis of patients with heart failure (4).

A total of two systematic reviews of prospective biomarker targets during the course of therapeutic chronic Chagas disease underlined the requirement for the development of unique biomarkers to assess prompt responses to therapeutic management of the disease (5, 6). There are several studies that are investing in new diagnostic approaches to cure Chagas disease, particularly regarding the identification of disease biomarkers. Data from forthcoming studies will assist the categorization of patients in terms of clinical aspects for initial follow-ups (6).

Some blood-derived biomarkers that have demonstrated the capacity to predict the progression of early Chagas disease cardiomyopathy have been engaged to assess the value of antiparasitic drugs and to identify initial cardiac and gastrointestinal injuries in asymptomatic patients. Nevertheless, future studies with extended follow-ups are required to establish biomarkers that are able to assess clinical or parasitological cures following therapy (6).

Biomarkers might be categorized based on biochemical configurations and major biological activities, such as inflammation and cellular injury biomarkers, metabolic biomarkers, prothrombotic biomarkers, and antigenic biomarkers (i.e., specific antigens of the parasite). Conversely, we propose a different classification. **Table 1** and the next sections of this review summarize the recent data related to biomarker research. Once the scientific data become more readily available, the future identification of critical and consistent biomarker candidates for human Chagas disease should be simplified.

PLASMATIC-RELATED CANDIDATES

Effectively treated and cured chagasic patients may be identified based on their patterns of circulating biomarkers (7). Recently, studies have indicated that serum markers, such as A- and B-type natriuretic peptides (ANP and BNP, respectively), N-terminal pro-BNP, troponin I, TGF-β, MMP-2, and TIMP-1 and -2, are higher during the severe stages of Chagas disease and represent cardiac damage and inflammation. However, several candidates are not disease specific. Hitherto, the levels of these aforementioned natriuretic peptides have been found to be higher in Chagas disease patients with cardiomyopathy than in those with different forms or other etiologies. Moreover, natriuretic peptide levels are increased in asymptomatic chagasic patients who exhibit no signs of ventricular dysfunction. Hence, natriuretic peptides showed a high predictive value for evaluated outcomes (4, 8). Both of the pioneering studies were performed in Brazilian cohorts. According to the data from these studies, BNP is comparable to echocardiogram in terms of the assessment of cardiological patients. To reinforce those prior findings, another study performed in an independent setting found that BNP, pro-BNP, creatine kinase (CK)-MB, and MMP-2 have high predictive values for short-term mortality even in the presence of a decreased ejection fraction and other clinical signs of congestive heart failure, which were all found to be associated with severe chagasic cardiomyopathy in a Bolivian cohort (9).

TABLE 1 | Summary of biomarker investigations related to Chagas disease.

Study	Source	Biomarker name	Result	Reference
Experimental, parasitemia-specific	Antigenic	Aptamer	Increased levels	(39, 40)
Chagasic cardiomyopathy	Genetic	CCL2 and MAL/TIRAP	Increased susceptibility	(41)
Chagasic cardiomyopathy	Genetic	CCR5	Protection	(41)
Chagasic cardiomyopathy	Phenotype	CD15s+ Treg cells	Protection	(35, 36)
Chagasic cardiomyopathy	Phenotype	CD27+CD28+CD8+ T cells	Protection	(37, 38)
Non-specific	Plasmatic	TIMP-1 and TIMP-2	Increased levels	(5, 6)
Non-specific	Plasmatic	Troponin I	Increased levels	(5, 6)
Non-specific	Plasmatic	TGF-β	Increased levels	(5, 6)
Asymptomatic	Plasmatic	IL-10	Increased levels	(11)
Non-specific	Plasmatic	APOA1	Decreased levels	(7)
Non-specific	Plasmatic	Fibronectin	Increased levels	(7)
Asymptomatic	Plasmatic	MMP-2	Increased levels	(14)
Chagasic cardiomyopathy	Plasmatic	MMP-9	Increased levels	(14)
Chagasic cardiomyopathy	Plasmatic	ANP, BNP, N-terminal pro-BNP, IFN-γ, TNF-α, IL-1β, and IL-6	Increased levels	(4, 8, 10, 11)
Chagasic cardiomyopathy	Plasmatic	miRNA-1, miRNA-133a and -133b, and miRNA-208a and -208b	Decreased levels	(13)
Experimental, chagasic cardiomyopathy	Plasmatic	PICP and PIIINP	Increased levels	(15)
Experimental, chagasic cardiomyopathy	Plasmatic	Syndecan-4, ICAM-1, and Galectin-3	Increased levels	(16)
Efficacy	Management	KMP11, HSP70, PAR2, and Tgp63	Increased Ab. levels	(5, 6)
Efficacy	Management	Antigen 13 and SAPA	Increased Ab. levels	(5, 6)
Efficacy	Management	Tc24	Increased Ab. levels	(43)

Because the BNP procedure is simple and quick, this biomarker can be used in endemic zones of Chagas disease with limited access to echocardiographic housing facilities. Finally, pro-BNP and *T. cruzi* DNA as detected by PCR are the only tests that have been found to have independent clinical value for disease staging in concert with electrocardiogram (ECG), echocardiogram, and clinical assessments (10). In a different study, the authors found that cytokine levels are related to cardiac injury in Chagas disease (11). Asymptomatic individuals exhibited high IL-10 levels that were associated with better prognosis. Conversely, IL-1β, IL-6, IFN-γ, and TNF-α reached the highest levels of expression in chagasic patients with cardiomyopathy. Overall, these findings sustain the perception that the balance between regulatory and inflammatory cytokines is associated with different forms of chronic Chagas disease (11, 12). Some micro (mi)RNAs, such as miRNA-1, miRNA-133, and miRNA-208, have been demonstrated to be involved with gene regulation properties and specific expression profiles, and imbalances can be found in chagasic cardiomyopathy (13). Recently, Santamaria and colleagues (7) pursued the identification of serum biomarkers that might be used as surrogates of therapeutic management in Chagas disease. APOA1 and specific fragments thereof and one fragment of fibronectin were uncovered. In chagasic samples, these biomarkers, excluding the full-length APOA1, are upregulated. These biomarkers revert to regular levels in 43% of cured patients. Notably, whenever serum MMP-9 levels are dominant, cardiac remodeling is strengthened and the advance of the cardiac form of Chagas disease is favored. Conversely, when serum MMP-2 levels prevail, patients persist as clinically asymptomatic. These processes might be IL-1β- and TNF-α dependent (14). In a particular model of infection, the cardiac levels of collagen I, III, and IV rise steadily and reach a peak during the chronic phase of Chagas disease. Thereafter, high serum levels of procollagen type I carboxy-terminal propeptide (PICP) and procollagen type III amino-terminal propeptide (PIIINP) are also observed. Given that increased PICP and PIIINP levels may indicate cardiac fibrosis, it is tempting to speculate that both biomarkers are suitable for detecting fibrosis during cardiac remodeling associated with *T. cruzi* infection (15). ICAM-1, galectin-3, and syndecan-4 have been found to be overexpressed in the hearts of mice chronically infected with *T. cruzi* (16). High levels of expression of galectin-3 in inflammatory cells have also been uncovered, and these levels are correlated with a decline in inflammation. A reduction in syndecan-4 and ICAM-1 might indirectly reduce cell migration into the myocardium and, thus, decrease inflammation (17). By contrast, in attempts to the uncover critical aspects of TGF-β as a candidate with prognostic value, several studies have demonstrated the influence of this anti-inflammatory cytokine on the development of chagasic cardiomyopathy by facilitating parasite cell invasion and its cycle (18, 19), improving parasite survival (20, 21), inducing exacerbated heart fibrosis and remodeling (22, 23), downregulating cardiac gap junctions (24), and mediating hypertrophy of the surviving cardiomyocytes (25). Increased circulating levels of TGF-β are observed in chronic Chagas disease patients (22, 26), and its active form is observed in the myocardia of chronic patients (27, 28). Moreover, due to the substantial involvement of TGF-β in the development of cardiac damage observed in Chagas disease, active compounds targeting TGF-β are currently under study as alternative treatments for the symptomatic cardiac form of Chagas disease (24, 29). Recently, the major cysteine protease from *T. cruzi*, cruzipain, has been observed to be capable of directly activating latent TGF-β, which favors parasite invasion into host cells (30). New therapeutic approaches for Chagas disease using anti-cruzipain compounds would be of beneficial not only due to their trypanocidal effect but also because they indirectly inhibit different TGF-β activities that are crucial for the development of Chagas disease. A retrospective study reported evidence supporting the clinical prognostic value of TGF-β as a biomarker for Chagas disease by demonstrating an association between its serological levels and clinical outcomes after 10 years of follow-up (31). Accordingly, TGF-β has demonstrated prognostic value as an independent predictor of all-cause mortality in patients without heart failure and with an ejection fraction above 45%. The optimal TGF-β cutoff for identifying patients who presented with all-cause mortality was 12.9 ng/ml. A further prospective study is clearly necessary to validate these data. Thus, the serological levels of TGF-β could be considered one potential biomarker for the outcome of Chagas disease and, moreover, could be used to follow the effects of treatments and interventions.

PHENOTYPE-RELATED CANDIDATES

First, we would like to propose the regulatory T cell (Treg) axis as a biomarker for Chagas disease progression. As discussed elsewhere, a malfunction of regulatory immune mechanisms may also be involved in the pathogenesis of Chagas disease (32). This malfunction may be due to the action of Treg cells that have the potential to curb effector responses, allow a partially effective anti-parasite immune response, and therefore enable the establishment and maintenance of chronic Chagas infection (32). By contrast, recent findings in humans have demonstrated an increased rate of Treg cells in chagasic patients in the indeterminate chronic phase (free of disease) compared with those with heart damage, which suggests an important role for Treg cells in the control of the inflammatory response during Chagas disease (33). Additionally, using a non-depleting monoclonal antibody to CD25, it was recently demonstrated that Treg cells bearing the CD4+CD25+Foxp3+ phenotype may also help to control the inflammatory immune response in mice that are chronically infected with *T. cruzi* (34). Therefore, there is a clear indication that the functional activity of Treg cells might be of crucial importance during the chronic phase of the infection due to their potential to decrease tissue damage and pathology. Recently, the expression of CD15s (Sialyl Lewis x) was described to identify the majority of suppressive Treg cells in humans (35). Thus, this biomarker discriminates suppressive from effector CD4+CD25+Foxp3+ T cells in humans (35). Interestingly, a previous study reported that the expression of CD15s is decreased in peripheral blood lymphocytes from patients with severe Chagas disease (36). Although additional studies are urgently required to uncover the critical aspects of both phenotypes, the expression of CD15s in Treg cells may be a reliable biomarker for the prediction of the progression to pathology of chagasic patients. Additionally,

another distinct phenotype, i.e., fully differentiated memory CD8 T cells (CD27−CD28−) bearing increased CCR7 expression, has been related to Chagas disease outcome (37). This study demonstrated an increase in total effector/memory CD8+ T cells in *T. cruzi*-infected individuals with mild heart disease compared with otherwise healthy controls. The study was based on the combined expression of CD27 and CD28 as previously proposed by Appay and colleagues (38), being related to a linear differentiation model for memory CD8+ T cells (38). This study suggested different fates of the T cell lineage, including early, intermediate, and late stages of cell-memory as follows: CD27+CD28+, CD27−CD28+ (or CD27+CD28−), and CD27−CD28− cells, respectively. As has previously been recognized, the proportion of fully differentiated memory (CD27−CD28−) in the total amount of CD8+ T cells is increased in mild Chagas disease. Conversely, the frequency of CD27+CD28+CD8+ T cells in the total memory CD8+ T cell population decreases as the disease becomes more severe. Albareda and colleagues (37) hypothesized that this pattern could be a consequence of the gradual clonal exhaustion of the CD8+ T cell population during infection. Analysis of the chemokine receptor CCR7 for lymph node homing (CCR7 expression) also revealed a significant increase in total effector/memory CD8 T cells in subjects with mild heart disease compared with healthy controls (37).

ANTIGENIC-RELATED CANDIDATES

A correlated ELISA approach to the detection of aptamers in mouse plasma that is highly specific for circulating parasite excreted-secreted antigens (TESA) has been developed for biomarkers of *T. cruzi* infection (39, 40). An aptamer exhibited specific binding to TESA and trypomastigote extract, but it did not bind to self-proteins or *Leishmania donovani* proteins. Infected mice have exhibited increased levels of aptamer binding compared with control littermates, which suggests this aptamer as a potential candidate for a future biomarker of *T. cruzi* infection. Furthermore, this aptamer might sense circulating biomarkers in both acute and chronic phases of Chagas disease (39). Recently, the same group confirmed that *T. cruzi*-infected mice have considerably higher biomarker levels than their non-infected counterparts. This study found that the biomarker levels are also diminished upon therapy (40). However, the biomarker levels in the infected and treated group did not decrease entirely and persisted above the assay cutoff point, which suggests that parasitemia was reduced, but a cure was not achieved. The test was capable of distinguishing circulating biomarkers in animals infected with several subpopulations of *T. cruzi*.

GENETIC-RELATED CANDIDATES

Genetic markers can provide evidence of the pathogenesis of Chagas disease and also have the potential to be utilized to identify new therapeutic targets. Frade and colleagues (41) studied genetic predispositions that influenced left ventricular ejection fractions in a Brazilian cohort. The authors found that CCL2 and MAL/TIRAP, but not CCR5, were linked to an increased susceptibility to chagasic cardiomyopathy.

MANAGEMENT-RELATED CANDIDATES

Regardless of most recent advances in drug development, there is virtually no consensus regarding the use of biomarkers to assess the efficacies of new drugs. Between the two main classes of recombinant proteins that are active during distinct ages and stages of Chagas disease, a 16-protein group and a combination of four recombinant proteins, namely KMP11, HSP70, PAR2, and Tgp63, have been identified (5). That combination could potentially serve as biomarkers candidates. Similarly, antibodies against antigen 13, among 5 others comprising the shed acute phase antigen (SAPA), have been demonstrated to be potential markers of cure efficacy [reviewed in Ref. (5)]. Additionally, a complement-mediated lysis test and an ELISA method based on Tc24 have also been created and the latter found to be a reliable candidate for a helpful parasite biomarker (42, 43).

FUTURE TRENDS

Researchers agree that the use of biomarkers in human Chagas disease will foster the progressing steps during the clinical assessment and also assist in the development of consistent diagnostic tools to lessen the time gap between the progression and detection of disease-relevant measures. Additionally, such biomarkers will allow for the prediction of both primary (genetic) and secondary (acquired factors) immunodeficiencies related to individual susceptibility. The deficiency of biomarkers in the prediction of parasitological outcome status and cure represents a main hurdle for the development of new drugs for Chagas disease. Thus, it is crucial to develop a reliable method to assess the cure of this disease. The aforementioned classes of biomarkers could yield uninterrupted longitudinal results related to Chagas disease management. Processes that are commonly used to identify biomarkers cannot be employed as endpoint evaluations in human clinical trials for ethical reasons. Some existing studies aim to develop alternative, emerging applications for biotechnologies based on the results of chagasic biomarker research. Furthermore, data from biomarker discovery research, such as the presence of TESA, could be used in vaccine development. In parasite-challenged vaccinated animals, TESA positivity could be an indication that the immune response was not appropriate to control the infection (39, 40). As a result of these struggles, the success of biomarker research in Chagas disease has not yet allowed for a better understanding of the disease risk from the clinical perspective. A range of issues exacerbates this frustration. One such issue is the incomplete validations of many biomarker candidates. Before conducting human trials, it is essential to identify and validate biomarkers that indicate cured patients. It is also necessary to evaluate the risks associated with the use of new agents in larger cohorts; thus, high-throughput biomarker procedures are needed. Ideally, approaching an accurate, rapid, and reliable point-of-care diagnostic tool in resource-limited, high-burden settings for Chagas disease through evaluation of biomarkers across that clinical spectrum in order to detect relevant pathogen-specific fingerprints will be of communal benefits, which undoubtedly outweigh the costs. The WHO predicted the eradication of

Chagas disease transmission by the year 2010, but this goal has not yet been achieved. Indeed, the disease is spreading beyond locations in which it was originally endemic (1). It is crucial to understand the partial corroboration of simple biomarker technologies to avoid generating data that exceeds our ability to analyze the applications of novel technologies that are emerging from population studies. Finally, it would be strategic to develop a wide variety of biomarkers and to test them during preclinical and clinical trials.

AUTHOR CONTRIBUTIONS

RP and PA conceived and participated in the design and coordination of the manuscript. MW, FC, and JM provided helpful discussions and edited the manuscript. All authors wrote, read, and approved the final manuscript.

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REFERENCES

1. WHO. *Chagas Disease (American trypanosomiasis) Fact Sheet 340*. World Health Organization (2012). Available from: http://www.who.int/mediacentre/factsheets/fs340/en/index.html
2. Punukollu G, Gowda RM, Khan IA, Navarro VS, Vasavada BC. Clinical aspects of the Chagas' heart disease. *Int J Cardiol* (2007) 115:279–83. doi:10.1016/j.ijcard.2006.03.004
3. Pinazo MJ, Canas E, Elizalde JI, Garcia M, Gascon J, Gimeno F, et al. Diagnosis, management and treatment of chronic Chagas' gastrointestinal disease in areas where *Trypanosoma cruzi* infection is not endemic. *Gastroenterol Hepatol* (2010) 33:191–200. doi:10.1016/j.gastrohep.2009.07.009
4. Wang Y, Moreira Mda C, Heringer-Walther S, Ebermann L, Schultheiss HP, Wessel N, et al. Plasma ACE 2 activity is an independent prognostic marker in Chagas' disease and equally potent as BNP. *J Card Fail* (2010) 16(2):157–63. doi:10.1016/j.cardfail.2009.09.005
5. Requena-Mendez A, Lopez MC, Angheben A, Izquierdo L, Ribeiro I, Pinazo MJ, et al. Evaluating Chagas' disease progression and cure through blood-derived biomarkers: a systematic review. *Expert Rev Anti Infect Ther* (2013) 11(9):957–76. doi:10.1586/14787210.2013.824718
6. Pinazo MJ, Thomas MC, Bua J, Perrone A, Schijman AG, Viotti RJ, et al. Biological markers for evaluating therapeutic efficacy in Chagas' disease, a systematic review. *Expert Rev Anti Infect Ther* (2014) 12(4):479–96. doi:10.1586/14787210.2014.899150
7. Santamaria C, Chatelain E, Jackson Y, Miao Q, Ward BJ, Chappuis F, et al. Serum biomarkers predictive of cure in Chagas' disease patients after nifurtimox treatment. *BMC Infect Dis* (2014) 14:302. doi:10.1186/1471-2334-14-302
8. Ribeiro AL, Dos Reis AM, Barros MV, Rocha MOC. Brain natriuretic peptide and left ventricular dysfunction in Chagas' disease. *Lancet* (2002) 360(9331):461–2. doi:10.1016/S0140-6736(02)09638-1
9. Sherbuk JE, Okamoto EE, Marks MA, Fortuny E, Clark EH, Galdos-Cardenas G, et al. Biomarkers and mortality in severe Chagas cardiomyopathy. *Glob Heart* (2015) 10(3):173–80. doi:10.1016/j.gheart.2015.07.003
10. Keating SM, Deng X, Fernandes F, Cunha-Neto E, Ribeiro AL, Adesina B, et al. Inflammatory and cardiac biomarkers are differentially expressed in clinical stages of Chagas disease. *Int J Cardiol* (2015) 15(199):451–9. doi:10.1016/j.ijcard.2015.07.040
11. Sousa GR, Gomes JA, Fares RC, Damasio MP, Chaves AT, Ferreira KS, et al. Plasma cytokine expression is associated with cardiac morbidity in Chagas' disease. *PLoS One* (2014) 9(3):e87082. doi:10.1371/journal.pone.0087082
12. Guedes PM, Gutierrez FR, Silva GK, Dellalibera-Joviliano R, Rodrigues GJ, Bendhack LM, et al. Deficient regulatory T cell activity and low frequency of IL-17-producing T cells correlate with the extent of cardiomyopathy in human Chagas' disease. *PLoS Negl Trop Dis* (2012) 6(4):e1630. doi:10.1371/journal.pntd.0001630
13. Ferreira LR, Frade AF, Santos RH, Teixeira PC, Baron MA, Navarro IC, et al. MicroRNAs miR-1, miR-133a, miR-133b, miR-208a and miR-208b are dysregulated in chronic Chagas disease cardiomyopathy. *Int J Cardiol* (2014) 175(3):409–17. doi:10.1016/j.ijcard.2014.05.019
14. Fares RC, Gomes Jde A, Garzoni LR, Waghabi MC, Saraiva RM, Medeiros NI, et al. Matrix metalloproteinases 2 and 9 are differentially expressed in patients with indeterminate and cardiac clinical forms of Chagas' disease. *Infect Immun* (2013) 81(10):3600–8. doi:10.1128/IAI.00153-13
15. Castro-Sesquen YE, Gilman RH, Paico H, Yauri V, Angulo N, Ccopa F, et al. Cell death and serum markers of collagen metabolism during cardiac remodeling in *Cavia porcellus* experimentally infected with *Trypanosoma cruzi*. *PLoS Negl Trop Dis* (2013) 7(2):e1996. doi:10.1371/journal.pntd.0001996
16. Soares MB, de Lima RS, Rocha LL, Vasconcelos JF, Rogatto SR, dos Santos RR, et al. Gene expression changes associated with myocarditis and fibrosis in hearts of mice with chronic chagasic cardiomyopathy. *J Infect Dis* (2010) 202(3):416–26. doi:10.1086/653481
17. Vasconcelos JF, Souza BS, Lins TF, Garcia LM, Kaneto CM, Sampaio GP, et al. Administration of granulocyte colony-stimulating factor induces immunomodulation, recruitment of T regulatory cells, reduction of myocarditis and decrease of parasite load in a mouse model of chronic Chagas disease cardiomyopathy. *FASEB J* (2013) 27(12):4691–702. doi:10.1096/fj.13-229351
18. Waghabi MC, Keramidas M, Bailly S, Degrave W, Mendonça-Lima L, Soeiro MN, et al. Uptake of host cell transforming growth factor-beta by *Trypanosoma cruzi* amastigotes in cardiomyocytes: potential role in parasite cycle completion. *Am J Pathol* (2005) 167(4):993–1003. doi:10.1016/S0002-9440(10)61189-3
19. Waghabi MC, Keramidas M, Feige JJ, Araujo-Jorge TC, Bailly S. Activation of transforming growth factor beta by *Trypanosoma cruzi*. *Cell Microbiol* (2005) 7(4):511–7. doi:10.1111/j.1462-5822.2004.00481.x
20. Silva JS, Twardzik DR, Reed SG. Regulation of *Trypanosoma cruzi* infections in vitro and in vivo by transforming growth factor beta (TGF-beta). *J Exp Med* (1991) 174(3):539–45. doi:10.1084/jem.174.3.539
21. dos Reis GA. Evasion of immune responses by *Trypanosoma cruzi*, the etiological agent of Chagas disease. *Braz J Med Biol Res* (2011) 44:84–90. doi:10.1590/S0100-879X2011007500005
22. Araújo-Jorge TC, Waghabi MC, Hasslocher-Moreno AM, Xavier SS, Higuchi ML, Keramidas M, et al. Implication of transforming growth factor-beta1 in Chagas disease myocardiopathy. *J Infect Dis* (2002) 186(12):1823–8. doi:10.1086/345882
23. Rossi MA. The pattern of myocardial fibrosis in chronic Chagas' heart disease. *Int J Cardiol* (1991) 30(3):335–40. doi:10.1016/0167-5273(91)90012-E
24. Waghabi MC, Coutinho-Silva R, Feige JJ, Higuchi Mde L, Becker D, Burnstock G, et al. Gap junction reduction in cardiomyocytes following transforming growth factor-beta treatment and *Trypanosoma cruzi* infection. *Mem Inst Oswaldo Cruz* (2009) 104(8):1083–90.
25. Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res* (2004) 63(3):423–32. doi:10.1016/j.cardiores.2004.04.030
26. Perez AR, Silva-Barbosa SD, Berbert LR, Revelli S, Beloscar J, Savino W, et al. Immunoneuroendocrine alterations in patients with progressive forms of chronic Chagas disease. *J Neuroimmunol* (2011) 235(1–2):84–90. doi:10.1016/j.jneuroim.2011.03.010

27. Araujo-Jorge TC, Waghabi MC, Soeiro MN, Keramidas M, Bailly S, Feige JJ. Pivotal role for TGF-beta in infectious heart disease: the case of *Trypanosoma cruzi* infection and consequent chagasic myocardiopathy. *Cytokine Growth Factor Rev* (2008) 19(5–6):405–13. doi:10.1016/j.cytogfr.2008.08.002
28. Araujo-Jorge TC, Waghabi MC, Bailly S, Feige JJ. The TGF-β pathway as an emerging target for Chagas disease therapy. *Clin Pharmacol Ther* (2012) 92(5):613–21. doi:10.1038/clpt.2012.102
29. de Oliveira FL, Araujo-Jorge TC, de Souza EM, de Oliveira GM, Degrave WM, Feige JJ, et al. Oral administration of GW788388, an inhibitor of transforming growth factor beta signaling, prevents heart fibrosis in Chagas disease. *PLoS Negl Trop Dis* (2012) 6(6):e1696. doi:10.1371/journal.pntd.0001696
30. Ferrao PM, d'Avila-Levy CM, Araujo-Jorge TC, Degrave WM, Gonçalves AS, Garzoni LR, et al. Cruzipain activates latent TGF-β from host cells during *T. cruzi* invasion. *PLoS One* (2015) 10(5):e0124832. doi:10.1371/journal.pone.0124832
31. Saraiva RM, Waghabi MC, Vilela MF, Madeira FS, Sperandio da Silva GM, Xavier SS, et al. Predictive value of transforming growth factor-β1 in Chagas disease: towards a biomarker surrogate of clinical outcome. *Trans R Soc Trop Med Hyg* (2013) 107(8):518–25. doi:10.1093/trstmh/trt050
32. Cardillo F, de Pinho RT, Antas PR, Mengel J. Immunity and immune modulation in *Trypanosoma cruzi* infection. *Pathog Dis* (2015) 73(9):ftv082. doi:10.1093/femspd/ftv082
33. de Araujo FF, Vitelli-Avelar DM, Teixeira-Carvalho A, Antas PR, Assis Silva Gomes J, Sathler-Avelar R, et al. Regulatory T cells phenotype in different clinical forms of Chagas disease. *PLoS Negl Trop Dis* (2011) 5(5):e992. doi:10.1371/journal.pntd.0000992
34. Nihei J, Cardillo F, Dos Santos WL, Pontes-de-Carvalho L, Mengel J. Administration of a nondepleting anti-CD25 monoclonal antibody reduces disease severity in mice infected with *Trypanosoma cruzi*. *Eur J Microbiol Immunol (Bp)* (2014) 4(2):128–37. doi:10.1556/EuJMI.4.2014.2.6
35. Miyara M, Chader D, Sage E, Sugiyama D, Nishikawa H, Bouvry D, et al. Sialyl Lewis x (CD15s) identifies highly differentiated and most suppressive FOXP3high regulatory T cells in humans. *Proc Natl Acad Sci U S A*. (2015) 112(23):7225–30. doi:10.1073/pnas.1508224112
36. Laucella SA, Riarte A, Prado N, Zapata J, Segura EL. alpha 4 integrins and sialyl Lewis x modulation in chronic Chagas disease: further evidence of persistent immune activation. *Scand J Immunol* (2001) 53(5):514–9. doi:10.1046/j.1365-3083.2001.00916.x
37. Albareda MC, Laucella SA, Alvarez MG, Armenti AH, Bertochi G, Tarleton RL, et al. *Trypanosoma cruzi* modulates the profile of memory CD8 T cells in chronic Chagas disease patients. *Int Immunol* (2006) 18(3):465–71. doi:10.1093/intimm/dxh387
38. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* (2006) 8:379. doi:10.1038/nm0402-379
39. Nagarkatti R, de Araujo FF, Gupta C, Debrabant A. Aptamer based, non-PCR, non-serological detection of Chagas' disease biomarkers in *Trypanosoma cruzi* infected mice. *PLoS Negl Trop Dis* (2014) 8(1):e2650. doi:10.1371/journal.pntd.0002650
40. de Araujo FF, Nagarkatti R, Gupta C, Marino AP, Debrabant A. Aptamer-based detection of disease biomarkers in mouse models for Chagas drug discovery. *PLoS Negl Trop Dis* (2015) 9(1):e3451. doi:10.1371/journal.pntd.0003451
41. Frade AF, Pissetti CW, Ianni BM, Saba B, Lin-Wang HT, Nogueira LG, et al. Genetic susceptibility to Chagas disease cardiomyopathy: involvement of several genes of the innate immunity and chemokine-dependent migration pathways. *BMC Infect Dis* (2013) 13:587. doi:10.1186/1471-2334-13-587
42. Galvao LM, Nunes RM, Cançado JR, Brener Z, Krettli AU. Lytic antibody titre as a means of assessing cure after treatment of Chagas' disease: a 10 years follow-up study. *Trans R Soc Trop Med Hyg* (1993) 87(2):220–3. doi:10.1016/0035-9203(93)90501-G
43. Krautz GM, Galvão LM, Cançado JR, Guevara-Espinoza A, Ouaissi A, Krettli AU. Use of a 24-kilodalton *Trypanosoma cruzi* recombinant protein to monitor cure of human Chagas' disease. *J Clin Microbiol* (1995) 33(8):2086–90.

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Immune Escape Strategies of Malaria Parasites

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Malaria is one of the most life-threatening infectious diseases worldwide. Immunity to malaria is slow and short-lived despite the repeated parasite exposure in endemic areas. Malaria parasites have evolved refined machinery to evade the immune system based on a range of genetic changes that include allelic variation, biomolecular exposure of proteins, and intracellular replication. All of these features increase the probability of survival in both mosquitoes and the vertebrate host. *Plasmodium* species escape from the first immunological trap in its invertebrate vector host, the *Anopheles* mosquitoes. The parasites have to pass through various immunological barriers within the mosquito such as anti-microbial molecules and the mosquito microbiota in order to achieve successful transmission to the vertebrate host. Within these hosts, *Plasmodium* species employ various immune evasion strategies during different life cycle stages. Parasite persistence against the vertebrate immune response depends on the balance among virulence factors, pathology, metabolic cost of the host immune response, and the parasites ability to evade the immune response. In this review we discuss the strategies that *Plasmodium* parasites use to avoid the vertebrate host immune system and how they promote successful infection and transmission.

Keywords: plamosdium, evasion strategies, liver stage, blood stage, malaria, microbiology, immulogy

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INTRODUCTION

Malaria still remains one of the biggest global health burdens and causes of mortality in the world. Malaria mainly occurs in tropical and subtropical parts of the world, being a serious cause of mortality and morbidity in Sub-Saharan Africa. There are five species of *Plasmodium* that cause disease in humans: *P. ovale, P. malariae, P. vivax, P. falciparum,* and the primate species *P. knowlesi*. In a previous report by the WHO, there were 215 million cases and 438 thousand deaths due to malaria in 2015 (WHO, 2015). Malaria is transmitted by the female *Anopheles* mosquito during a feeding. As the mosquito feeds on its host, sporozoites are released into the blood stream, and they migrate to the liver (Cirimotich et al., 2010). The complete life cycle of *Plasmodium* requires two hosts: the mosquito vector and the vertebrate host (Crompton et al., 2014). In the vertebrate host it undergoes two stages: an asymptomatic pre-erythrocytic stage (liver stage), and a symptomatic erythrocytic stage (blood stage) (Haque and Engwerda, 2014). The journey of sporozoites to the liver is asymptomatic (Zheng et al., 2014), but various reports have described that an initial immune response occurs during this stage. Some of the immunological responses described to occur during the liver stage are: apoptosis of infected cells (Meslin et al., 2007), isolation and targeting of

parasites in specific compartments for elimination (Yano and Kurata, 2011), type I IFN production induced by parasite RNA (Liehl et al., 2015) and LC3-mediated autophagy targeting of sporozoites (Risco-Castillo et al., 2015). Nevertheless, several mechanisms such as immune evasion, immune exploitation, and molecular piracy are employed by the parasites to promote their survival in the host (Hisaeda et al., 2005). After the first immunological attack during liver stage, the majority of parasites that survived will replicate within the hepatocytes and amplify their number exponentially leading to the release of hundreds of thousands of merozoites into circulation (Prado et al., 2015). The released merozoites will subsequently initiate the symptomatic blood stage cycle, which is responsible for the clinical features and pathologies associated with malaria (Ocaña-Morgner et al., 2003).

During the blood stage some parasites will differentiate into male and female gametocytes. The gametocytes will be ingested by other feeding mosquitoes where they will further develop inside the mosquito gut (Cirimotich et al., 2010). Within the mosquito, ineffective sporozoites will be kept until the next blood meal, where they will be transmitted to a mammalian host continuing the parasite life cycle (Molina-Cruz et al., 2012). The complexity of the parasite life cycle allows for the use of various immune evasion strategies by *Plasmodium*, which pose a challenge to the development of a malaria vaccine (Hisaeda et al., 2005). In this review, we discuss some of the immune evasion strategies that have been described for *Plasmodium* at both the liver and the blood stages. We also highlight how these immune evasion strategies can promote successful infection, overcoming many of the challenges that human pathogens face with the host immune response.

IMMUNE EVASION DURING THE PRE-ERYTROCYTIC STAGES

Mechanical Strategies to Overcome the First Barrier: the Skin

An infected mosquito bite transmits around 100–200 sporozoites in the human skin and despite innate immune destruction of most of them; some parasites are still able to establish successful infection at very low numbers (Risco-Castillo et al., 2015). The skin is the first barrier that parasites encounter after transmission into the vertebrate host (Cirimotich et al., 2010). Thus, sporozoites have evolved to overcome this barrier by various mechanical strategies such as motility and cell transversal (Tavares et al., 2013). To achieve this, sporozoites are equipped with specialized mechanical proteins that help them achieve successful passage. This has been evidenced by studies that show how the transmission of sporozoites deficient in SPECT-1 (spororozoite microneme protein essential for cell traversal) and SPECT-2 (also called perforin-like protein 1 or PLP1) are blocked in the dermis and are ingested by phagocytes preventing their progression. These proteins were reported to be necessary for cell traversal and for migration to the liver (Patarroyo et al., 2011). The sporozoites can tranverse various kind of cells, wich includes immune cells. The transversal of immune cells can lead to inactivation of immune cell defenses and prevent the clearance of sporozoites (exocytosis) before crossing the barrier (Sinnis and Zavala, 2012). Another protein responsible for motility of sporozoites is TRAP (Thrombospondin-Related Anonymous Protein). This protein is found on the micronemes and the surface of sporozoites. TRAP allows the parasite to interact with surface host molecules that provide gliding motility to exit the dermis. Additionally, TRAP can bind to sulfated glycoconjugate motifs that can aid in recognition and entry to hepatocytes (Müller et al., 1993). Regardless, some of the sporozoites will enter the lymphatic system where they can be recognized and destroyed by immune cells such as dendritic cells (DCs) (Wilson et al., 2016).

Overcoming the Hepatic Immune Defenses to Establish Liver Stage Infection

A successful infection in great part is due to the parasite evading the first attacks of the host immune response, that despite its strength, it is not enough to block the development of the blood stage (Singh et al., 2010). After the sporozoites migrate from the skin, crossing all cellular barriers, they will reach the blood, and consequently the lymphatic system (Crompton et al., 2014). Once inside the circulatory system, sporozoites rapidly reach the sinusoid cavity of the liver. The exoerythrocytic (liver) stage has been described as asymptomatic because the liver is an immunoprivileged organ that is protected against a strong immune response (Liehl et al., 2015). Even though initially the liver stage was considered an unresponsive phase, various reports have showed that the liver immune cells are present and active in different rodent malaria models, suggesting an initial immune response during the liver stage (Liehl et al., 2014). Some reports have described how *Plasmodium* RNA could activate the type I interferon (IFN) pathway via the cytosolic Pattern Recognition Receptor (PRR), MDA5 (Liehl et al., 2014). Type I interferons are potent inflammatory cytokines that are known to inhibit growth of exoerythrocytic forms (Hisaeda et al., 2005). Some of the cells that mediate these anti-parasitic effects include Natural Killer and Natural Killer T cells (NK, NKT), and γδT cells. These cells mainly inhibit parasite growth through secretion of interferon, including both type I interferons and IFN-γ (Ocaña-Morgner et al., 2003; Risco-Castillo et al., 2015). Other molecules such as hepcidin have been implicated in growth inhibition of exoerytrhrocytic phases (Spottiswoode et al., 2014).

Modulation on of Kupffer Cells

To invade hepatocytes, sporozoites must cross the barrier lined by endothelial cells (ECs) and immune phagocytic cells called Kupffer Cells (KCs) (Tavares et al., 2013). Sporozoites will have to interact with these resident cells in order to establish a successful infection (Meslin et al., 2007).

When the sporozoites reach the liver they are attracted to sulfated molecules that are present in ECs and KCs. This interaction is mainly mediated between the circumsporozoite protein (CSP) and sulfated heparin sulfate proteogycans (HSPGs) in the surface of the host cells. Other molecules that are involved in this process include P39 and CD38 (Cha et al., 2015). Previous studies employing intravital and electron microscopy

suggested KCs, but not the ECs are the preferred passage used by sporozoites (Cha et al., 2015). Recent studies proposed that sporozoites exploit multiple paths to cross the sinusoidal barrier. Also, there have been reports that sporozoites pass through gaps between ECs and KCs, not engaging in traversal capacity (Meis et al., 1983). For the sporozoites passing through KCs, it would be interesting to know why the KCs do not degrade parasites even though they efficiently kill other microorganisms that invade hepatocytes (Sinnis and Zavala, 2012). Additionally, it has been described in a mice model that sporozoites can go through KCs and modulate their cytokine profile, leading to the down-regulation of Th1 cytokines (TNF-α, IL-6, and MCP-1) and the upregulation of Th2 cytokines (IL-10) for ensuring safe passage (Klotz and Frevert, 2008). Additionally, CSP can interact with LRP-1 (low-density lipoprotein receptor-related protein) and proteoglycans on the KC surface, which increases the levels of intracellular cAMP/EPAC and prevents the formation of reactive oxygen species (ROS). ROS is a natural byproduct that is produced during environmental stress and can cause cellular damage and it can kill the parasite (Ikarashi et al., 2013). In some cases, the parasite forces the KCs into apoptosis. Lastly, sporozoites can also negatively affect the antigen-presentation capacity of KCs, which display reduced expression of MHC-class I and IL-12 (Steers et al., 2005). Some evidence suggests that sporozoites are able to manipulate the KCs functions and immunosupress the microenvironment for its own advantage.

Modulation of Hepatocytes

After successful penetration of the sinusoidal cell layer, sporozoites enter the hepatocytes and start intra-hepatic development. Sporozoites actively invade the host cells (hepatocytes) employing the cholesterol uptake pathway, which is different from other microorganisms that exploit phagocytic activity of host cells for invasion (Itoe et al., 2014). Additionally, released CSP supports parasite development through the suppression of the NF-κB signaling pathway (Ding et al., 2012) and the up regulation of host heme oxygenase-1 (HO-1), which further promotes the parasite development in the liver by modulating the host inflammatory response (Pamplona et al., 2007). Sporozoite infection of hepatocytes also interferes with the mTOR pathway, which alters the levels of proteins involved in cell survival, proliferation, autophagy, anabolism, and cell growth (Hanson et al., 2013). After invading the final hepatocyte, sporozoites are enclosed in a parasitophorous vacuole (PVM), which physically separates it from the host cytoplasm thereby avoiding degradation by the endocytic/lysosome system. This isolation keeps the parasitophorous vacuole isolated from cell intrinsic defenses such as apoptosis and selective autophagy (Thieleke-Matos et al., 2016). The transition of parasites from liver stage merozoites to initiate blood stage represents another key point of the cycle for immune evasion. In order to start the blood stage, liver stage parasites must exit from the hepatocytes through hepatic spaces where they are exposed to resident phagocytic cells such as KCs and DCs. Merozoites avoid being killed by liver phagocytes by covering themselves in membranes derived from the host known as merosomes (Sturm et al., 2006). The merosomes are derived from infected hepatocytes when they bud off from the cells and manage to avoid recognition by phagocytes to initiate blood stage (Garg et al., 2013). The initiation of blood stage could take days after exiting the liver. Collectively, all of this data demonstrates the long journey that parasites travel to overcome the immune system.

IMMUNE EVASION BY ERYTHROCYTIC PARASITE STAGES

The blood stage is where the clinical symptoms that are characteristic of malaria appear. Blood stage is initiated when merozoites released from hepatocytes invade RBCs to develop into ring shaped, and young and mature trophozoites, undergoing schizogony. In schizogony each one leads to six to 32 daughter clones that will burst out into the bloodstream and reinvade new RBCs (Garg et al., 2013). An effective anti-parasitic immunity has been proven to be complex and involving various elements of the immune system. Antibodies and T-cells have proven to be important components in achieving parasite clearance (Chotivanich et al., 2002). Antibodies can have anti-parasitic activity by binding to infected erythrocytes and mediating their phagocytosis (opsonization) by circulating macrophages (Dups et al., 2014). They can also prevent invasion of red blood cells by binding to extracellular merozoites and marking them for clearance or lysis by complement. T-cells play an essential role by both direct and indirect mechanisms. Direct mechanisms include production of pro-inflammatory cytokines such as IFN-γ and TNF-α that prime macrophages and other components of the anti-parasite immune response (Nasr et al., 2014). They also mediate an indirect mechanism by activating specific B-cell clones that will produce anti-parasite antibodies (Kafuye-Mlwilo et al., 2012). Other innate immune components that have been attributed to play a role include: NK cells, γδT cells (Inoue et al., 2013), host microbiota and natural antibodies (Crompton et al., 2014). Circulating infected blood cells can be targeted for destruction in the spleen but the parasite avoids this by developing several mechanisms to evade the host immune response (Chotivanich et al., 2002). As an example for complement evasion, inhibition of the membrane attack complex (MAC) formation has been described in *P. falciparum*-infected patients and it even correlated with severe malaria cases (Schmidt et al., 2015).

The intracellular survival is the most primitive immune escape mechanism of parasites, which avoid the direct interaction of parasites with the immune cells. Furthermore, RBCs do not express MHC class I molecules on their surface thereby escaping from recognition by $CD8^+$ T cells (Bowen and Walker, 2005). Another feature that parasites use to avoid clearance is the formation of rosettes. Rossettes is a phenomenon where infected erythrocytes cluster with uninfected erythrocytes helping the parasites bind to RBC epitopes and avoid immune recognition. Blood type is one factor correlated with the rosette formation. For example parasites that bind blood type A are more virulent than the ones that bind blood type O because their capacity of forming rosettes is stronger (Moll et al., 2015).

Overall the evasion mechanisms are divided in two fields of strategies. One of them is the expression of variable antigenic proteins at the surface in different life cycle stages of parasites

that help camouflage them from the host immune system (Escalante et al., 1998). The evasion of immune clearance is due the highly polymorphic proteins that mediate antigenic variation by changing and adapting to host immune response, promoting long-lasting infections (Wilson et al., 2016). The second is sequestration, which is mediated by genes products of the *PfEMP-1, Var, Rifin* (Mwakalinga et al., 2012), and *Stevor* multigene families (Kraemer and Smith, 2006). These proteins allow iRBC adherence in vascular endothelium hence avoiding clearance, and sequestering them in the microvasculature of various organs. They also exploit host components such as platelets and inflammation that can lead agglutination of uninfected RBCs with iRBCs and promote the appropriate sequestration microenvironment (Helmby et al., 1993).

In the blood stream, merozoites utilize complex set of proteins in order to invade uninfected red blood cells (Helmby et al., 1993). The parasite locates closely its proteins to proteins of the host erythrocytes, mediating reorientation and tight binding allowing them to invade the host RBCs. MSP-1 (merozoite surface proteins) anchors in the host erythrocyte membrane through GPI (glicosilfosfatilinositol) anchors. (Nosjean et al., 1997). MSPs along with other merozoite proteins called erythrocyte binding-like (EBL) proteins exist in several alleles or copies in the genome, showing a high degree of polymorphism (Holder et al., 1999). Several studies about new vaccine candidates against malaria are based in interactions between surface proteins, such as MSPs, GPI and PfEMP-1 (Boyle et al., 2014). The EBL protein family has a sophisticated profile with redundant functions that evolved to promote antigenic variation and immune evasion (Souza-Silva et al., 2014). EBL proteins are proteins that contain a Duffy-binding-like (DBL) which have been shown to mediate host receptor binding, in the case of *P.vivax* infection (VanBuskirk et al., 2004).

All *Plasmodium* species use to the same principle for invasion of the cell through interaction of parasite proteins to host erythrocyte receptor. The most studied invasion mechanisms are the ones used by *P.vivax* and *P.falciparum*. The merozoites of *P.falciparum* have at least five mechanisms of invasion, characterized by its respective receptor in erythrocytes (Baum et al., 2003). *P.vivax* shows great preference to invade immature red blood cell forms, such as reticulocytes. Some studies have reported that people who have the DARC (Duffy antigen receptor chemokines) in erythrocytes are more susceptible to infection by *P. vivax*. Conversely, the lack of this receptor (Souza-Silva et al., 2014) confers resistance to infection by *P. vivax*. However, other studies from west Kenya and Brazil have reported *P.vivax* cases, despite the lack of DARC expression in RBCs, suggesting alternative pathways for *P.vivax* infection (Ryan et al., 2006).

P. falciparum has evolved more adapt ways of immune evasion. One of the most studied strategies is the exposure PfEMP1 on infected red blood cells. The PfEMP-1 expression is a sophisticated apparatus that promotes the ability to bind different host endothelial cells, such as human brain microvascular endothelial cells (HBMEC). This binding helps the parasites avoid clearance by the spleen and it's directly related to complicated falciparum malaria such as cerebral malaria (Abdi et al., 2016).

The alterations in the iRBC membrane created by exposure of these proteins are denominated Knobs. These knobs will mediate cytoadhesion to the endothelium. Some of the targeted endothelium receptors include EPCR, CSA, CD36, and ICAMs (Yipp et al., 2000). Condroitin sulfate A (CSA) is the receptor targeted by the specific variant that leads to placental malaria in pregnant women (Kraemer and Smith, 2006). Each PfEMP variant can promote binding to different receptors in several organs (Hviid and Jensen, 2015). In both iRBC and uninfected RBCs in severe malaria, the erythrocytes become rigid following impediment to their flow through capillaries that have midpoint diameters that are all smaller than the erythrocyte itself (Dondorp et al., 2000). This can block parasite recirculation or recruitment of non-infected RBCs leading to rosettes (Uyoga et al., 2012) and has been associated with severe malaria anemia (Uyoga et al., 2012). Knobs sequestration in post-capillary venules occurs in mature blood stages such trophozoite and schizonts (Sharma, 1991). The presence of the parasite leads to activation of the immune response and production of cytokines, enhancing expression of receptors in endothelial cells targeted by the parasite adhesins (Rowe et al., 2009). This promotes the cyclical fevers associated with malaria while the constant presence of parasite exacerbates the inflammatory immune response (Gazzinelli et al., 2014).

Inadequate Innate and Adaptive Responses against *Plasmodium* Blood Stage Parasites

Even though the parasite has evolved various immune evasion strategies, the host immune response along with its genetic background, are essential for parasite control and prevention of clinical malaria. The human immune system is equipped with both innate and adaptive responses with great anti-parasitic activity. One major component of the innate immune system are Patter Recognition Receptors (PRRs) that recognize Pathogen-associated molecular patterns (PAMPs). In the case of malaria, three PAMPs that have been proposed for Plasmodium parasites are: GPI anchors, Hemozoin and immunostimulatory nucleic acid motifs (Gazzinelli et al., 2014).

Release array of pro and anti-inflammatory can result in either successful parasite control or alternatively an exacerbated immune response leading to pathology (Perkins et al., 2011). Even though mounting a potent immune response is not always completely efficient, it is necessary for controlling parasitemia. An important "bridge" between the innate and adaptive immune response are the dendritic cells (DCs). The DCs have a function of antigen presentation, stimulation of T-cells, and are major mediators of the adaptive immune response (Gowda et al., 2012). During malaria, CD4+ T− helper cells have been implicated for pathogenesis, protection and immune evasion of parasites (Wykes et al., 2014). The role of regulatory T cells (expressing FOXP3 CD4+ CD25+) in immunity has been controversial. For example, lack of T-regs during experimental cerebral malaria mice models renders them more susceptible to disease; while in human cerebral malaria correlates with higher parasitemia (Walther et al., 2005).

The role of CD8+ T cells-mediated immunity in blood stage has been less studied. This is mainly due to the fact that iRBCs don't express MHC class I rendering them resistant to the cytotoxic effect of CD8+ T-cells. Additionally, the high-level of mutations of epitopes in blood stage lead to immune evasion of the cytotoxic T lymphocyte (CTL) response, and hence fail to generate highly effective malaria vaccines (Templeton, 2009). Regardless, experiments using depletion both CD8+ and CD4+ T-cells during blood stage of *P.chabaudi* infections in mice resulted in a delayed clearance of the infection (Podoba and Stevenson, 1991), suggesting a possible role for CD8+ T-cells during blood stage malaria.

B cells have been attributed to an essential protective role during malaria. Antibody responses as immune agents alone can provide sufficient protection to control clinical disease (Wykes et al., 2014). The functions of antibodies can act on limiting the growth of blood stage parasites and the development of clinical symptoms for several mechanisms, like blocking erythrocyte invasion (Blackman et al., 1990), act as opsonins on parasitized erythrocytes (Osier et al., 2014), monocyte-mediated antibody-dependent cellular killing (Bouharoun-Tayoun et al., 1995), and complement-mediated lysis (Boyle et al., 2015), as well as interfering with the adherence of infected erythrocytes to vascular endothelium (Beeson et al., 2004). Unfortunately, the antibody responses to malaria infection from children and adults have been reported to be short-lived and rapidly lost in the absence of continued parasite exposure (Ryg-Cornejo et al., 2016). Collectively, these findings demonstrate that the immune system is efficient at reducing parasite burden but in most cases is not enough to prevent the progress of disease showing the high capacity of immune evasion by the parasite.

CONCLUDING REMARKS

Plasmodium has evolved a range of biomolecular strategies in order to escape the immune response and to guarantee the survival within the host. These parasites have great immune evasion ability across their whole life cycle. From an asymptomatic liver stage to a sophisticated system of proteins (such as PfEMP proteins) that is utilized by the parasite to avoid immune recognition and establish a successful infection. Hence further studies requiring close interactions of biomolecular, genetic, and immune strategies in both host and parasites are needed in order to understand better a protective anti-Plasmodium immunity. Thereby, in this review we summarize some of the important mechanisms that Plasmodium parasites utilize to facilitate evasion of the vertebrate host immune system across its life cycle. The challenges for the development of more effective therapeutics will have to overcome the various parasite immune evasion strategies and as several species cause malaria, several antigenic variations have to be considered. Recently we have a good candidate malaria vaccine in phase III (RST,S - GSK) of implanting, but even with a good initial response, it was also proved to be incomplete in points of recurrence of the disease (Morrison, 2015). Thus, the well-studied avoidance schemes propose an opening of new possibilities for further studies to consolidate the burden that malaria causes to hundreds of thousands of people annually.

AUTHOR CONTRIBUTIONS

PG, JB, JR, CF, and AM wrote the paper. All authors read and approved the final version of the manuscript.

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REFERENCES

Abdi, A. I., Warimwe, G. M., Muthui, M. K., Kivisi, C. A., Kiragu, E. W., Fegan, G. W., et al. (2016). Global selection of *Plasmodium falciparum* virulence antigen expression by host antibodies. *Sci. Rep*. 6:19882. doi: 10.1038/srep19882

Baum, J., Pinder, M., and Conway, D. J. (2003). Erythrocyte invasion phenotypes of *Plasmodium falciparum* in The Gambia. *Infect. Immun.* 71, 1856–1863. doi: 10.1128/IAI.71.4.1856-1863.2003

Beeson, J. G., Mann, E. J., Elliott, S. R., Lema, V. M., Tadesse, E., Molyneux, M. E., et al. (2004). Antibodies to variant surface antigens of *Plasmodium falciparum*-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. *J. Infect. Dis.* 189, 540–551. doi: 10.1086/381186

Blackman, M. J., Heidrich, H. G., Donachie, S., McBride, J. S., and Holder, A. A. (1990). A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J. Exp. Med.* 172, 379–382. doi: 10.1084/jem.172.1.379

Bouharoun-Tayoun, H., Oeuvray, C., Lunel, F., and Druilhe, P. (1995). Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. *J. Exp. Med.* 182, 409–418. doi: 10.1084/jem.182.2.409

Bowen, D. G., and Walker, C. M. (2005). Mutational escape from CD8+ T cell immunity: HCV evolution, from chimpanzees to man. *J. Exp. Med*. 201, 1709–1714. doi: 10.1084/jem.20050808

Boyle, M. J., Langer, C., Chan, J., Hodder, A. N., Coppel, R. L., Anders, R. F., et al. (2014). Sequential processing of merozoite surface proteins during and after erythrocyte invasion by *Plasmodium falciparum*. *Infect. Immun*. 82, 924–936. doi: 10.1128/IAI.00866-13

Boyle, M. J., Reiling, L., Feng, G., Langer, C., Osier, F. H., Aspeling-Jones, H. et al. (2015). Human antibodies fix complement to inhibit *Plasmodium falciparum* invasion of erythrocytes and are associated with protection against malaria. *Immunity* 42, 580–590. doi: 10.1016/j.immuni.2015.02.012

Cha, S. J., Srinivasan, P., Schindler, C. W., van Rooijen, N., van Rooijen, N., Stins, M., et al. (2015). CD68 acts as a major gateway for malaria sporozoite liver infection. *J. Exp. Med.* 212, 1391–1403. doi: 10.1084/jem.20110575

Chotivanich, K., Udomsangpetch, R., McGready, R., Proux, S., Newton, P., Pukrittayakamee, S., et al. (2002). Central role of the spleen in malaria parasite clearance. *J. Infect. Dis.* 185, 1538–1541. doi: 10.1086/340213

Cirimotich, C. M., Dong, Y., Garver, L. S., Sim, S., and Dimopoulous, G. (2010). Mosquito immune defenses against Plasmodium infection. *Dev. Comp. Immunol*. 34, 387–395. doi: 10.1016/j.dci.2009.12.005

Crompton, P. D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L. S., et al. (2014). Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu. Rev. Immunol.* 32, 157–187. doi: 10.1146/annurev-immunol-032713-120220

Ding, Y., Huang, X., Liu, T., Fu, Y., Tan, Z., Zheng, H. et al. (2012). The Plasmodium circumsporozoite protein, a novel NF-κB inhibitor, suppresses the growth of SW480. *Pathol. Oncol. Res.* 18, 895–902. doi: 10.1007/s12253-012-9519-7

Dondorp, A. M., Kager, P. A., Vreeken, J., and White, N. J. (2000). Abnormal blood flow and red blood cell deformability in severe malaria. *Parasitol. Today* 16, 228–232. doi: 10.1016/S0169-4758(00)01666-5

Dups, J. N., Pepper, M., and Cockburn, I. A. (2014). Antibody and B cell responses to Plasmodium sporozoites. *Front. Microbiol.* 5:625. doi: 10.3389/fmicb.2014.00625

Escalante, A. A., Lal, A. A., and Ayala, F. J. (1998). Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. *Genetics* 149, 189–202.

Garg, S., Agarwal, S., Kumar, S., Yazdani, S. S., Chitnis, C. E., and Singh, S. (2013). Calcium-dependent permeabilization of erythrocytes by a perforin-like protein during egress of malaria parasites. *Nat. Commun.* 4, 1736. doi: 10.1038/ncomms2725

Gazzinelli, R. T., Kalantari, P., Fitzgerald, K. A., and Golenbock, D. T. (2014). Innate sensing of malaria *parasites. Nat. Rev. Immunol.* 14, 744–757. doi: 10.1038/nri3742

Gowda, N. M., Wu, X., and Gowda, D. C. (2012). TLR9 and MyD88 are crucial for the development of protective immunity to malaria. *J. Immunol.* 188, 5073–5085. doi: 10.4049/jimmunol.1102143

Hanson, K. K., Ressurreicao, A. S., Buchholz, K., Prudencio, M., Herman-Ornelas, J. D., Rebelo, M., et al. (2013). Torins are potent antimalarials that block replenishment of Plasmodium liver stage parasitophorous vacuole membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2838–E2847. doi: 10.1073/pnas.1306097110

Haque, A., and Engwerda, C. (2014). Hepatocytes break the silence during liver-stage malaria. *Nat. Med.* 20, 17–19. doi: 10.1038/nm.3446

Helmby, H., Cavelier, L., Pettersson, U., and Wahlgren, M. (1993). Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. *Infect. Immun.* 61, 284–288.

Hisaeda, H., Yasutomo, K., and Himeno, K. (2005). Malaria: immune evasion by parasites. *Int. J. Biochem. Cell Biol.* 37, 700–706. doi: 10.1016/j.biocel.2004.10.009

Holder, A. A., Guevara Patino, J. A., Uthaipibull, C., Syed, S. E., Ling, I. T., Scott-Finnigan, T., et al. (1999). Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria. *Parassitologia* 41, 409–414.

Hviid, L., and Jensen, A. T. (2015). PfEMP1 - A Parasite Protein Family of Key Importance in *Plasmodium falciparum* malaria immunity and pathogenesis. *Adv. Parasitol.* 88, 51–84. doi: 10.1016/bs.apar.2015.02.004

Ikarashi, M., Nakashima, H., Kinoshita, M., Sato, A., Nakashima, M., Miyazaki, H. et al. (2013). Distinct development and functions of resident and recruited liver Kupffer cells/macrophages. *J. Leukoc. Biol.* 94, 1325–1336. doi: 10.1189/jlb.0313144

Inoue, S., Niikura, M., Mineo, S., and Kobayashi, F. (2013). Roles of IFN-γ and γδ T Cells in protective immunity against blood-stage malaria. *Front. Immunol.* 4:258. doi: 10.3389/fimmu.2013.00258

Itoe, M. A., Sampaio, J. L., Cabal, G. G., Real, E., Zuzarte-Luis, V., March, S. et al. (2014). Host cell phosphatidylcholine is a key mediator of malaria parasite survival during liver stage infection. *Cell Host Microbe* 16, 778–786. doi: 10.1016/j.chom.2014.11.006

Kafuye-Mlwilo, M. Y., Mukherjee, P., and Chauhan, V. S. (2012). Kinetics of humoral and memory B cell response induced by the *Plasmodium falciparum* 19-kilodalton merozoite surface protein 1 in mice. *Infect. Immun.* 80, 633–642. doi: 10.1128/IAI.05188-11

Klotz, C., and Frevert, U. (2008). Plasmodium yoelii sporozoites modulate cytokine profile and induce apoptosis in murine Kupffer cells. *Int. J. Parasitol.* 38, 1639–1650. doi: 10.1016/j.ijpara.2008.05.018

Kraemer, S. M., and Smith, J. D. (2006). A family affair: var genes, PfEMP1 binding, and malaria disease. *Curr. Opin. Microbiol.* 9, 374–380. doi: 10.1016/j.mib.2006.06.006

Liehl, P., Meireles, P., Albuquerque, I. S., Pinkevych, M., Baptista, F., Mota, M. M. et al. (2015). Innate immunity induced by Plasmodium liver infection inhibits malaria reinfections. *Infect. Immun.* 83, 1172–1180. doi: 10.1128/IAI.02796-14

Liehl, P., Zuzarte-Luis, V., Chan, J., Zillinger, T., Carapau, D., Carapau, D., et al. (2014). Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection. *Nat. Med.* 20, 47–53. doi: 10.1038/nm.3424

Meis, J. F., Verhave, J. P., Jap, P. H., and Meuwissen, J. H. (1983). An ultrastructural study on the role of Kupffer cells in the process of infection by *Plasmodium berghei* sporozoites in rats. *Parasitology* 86(Pt 2), 231–242. doi: 10.1017/S003118200005040X

Meslin, B., Barnadas, C., Boni, V., Latour, C., De Monbrison, F., Kaiser, K., et al. (2007). Features of apoptosis in *Plasmodium falciparum* erythrocytic stage through a putative role of PfMCA1 metacaspase-like protein. *J. Infect. Dis.* 195, 1852–1859. doi: 10.1086/518253

Molina-Cruz, A., DeJong, R. J., Ortega, C., Haile, A., Abban, E., Rodrigues, J. et al. (2012). Some strains of *Plasmodium falciparum*, a human malaria parasite, evade the complement-like system of *Anopheles gambiae* mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1957–E1962. doi: 10.1073/pnas.1121183109

Moll, K., Palmkvist, M., Ch'ng, J., Kiwuwa, M. S., and Wahlgren, M. (2015). Evasion of immunity to *Plasmodium falciparum:* rosettes of blood group a impair recognition of pfemp1. *PLoS ONE* 10:e0145120. doi: 10.1371/journal.pone.0145120

Morrison, C. (2015). Landmark green light for Mosquirix malaria vaccine. *Nat Biotechnol.* 33, 1015–1016. doi: 10.1038/nbt1015-1015

Müller, H. M., Reckmann, I., Hollingdale, M. R., Bujard, H., Robson, K. J., and Crisanti, A. (1993). Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *EMBO J.* 12, 2881–2889.

Mwakalinga, S. B., Wang, C. W., Bengtsson, D. C., Turner, L., Dinko, B., Lusingu, J. P. et al. (2012). Expression of a type B RIFIN in *Plasmodium falciparum* merozoites and gametes. *Malar. J.* 11:429. doi: 10.1186/1475-2875-11-429

Nasr, A., Allam, G., Hamid, O., and Al-Ghamdi, A. (2014). IFN-gamma and TNF associated with severe *falciparum malaria* infection in Saudi pregnant women. *Malar. J.* 13:314. doi: 10.1186/1475-2875-13-314

Nosjean, O., Briolay, A., and Roux, B. (1997). Mammalian GPI proteins: sorting, membrane residence and functions. *Biochim. Biophys. Acta* 1331, 153–186. doi: 10.1016/S0304-4157(97)00005-1

Ocaña-Morgner, C., Mota, M. M., and Rodriguez, A. (2003). Malaria blood stage suppression of liver stage immunity by dendritic cells. *J. Exp. Med.* 197, 143–151. doi: 10.1084/jem.20021072

Osier, F. H., Feng, G., Boyle, M. J., Langer, C., Zhou, J., Richards, J. S. et al. (2014). Opsonic phagocytosis of *Plasmodium falciparum* merozoites: mechanism in human immunity and a correlate of protection against malaria. *BMC Med.* 12:108. doi: 10.1186/1741-7015-12-108

Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., et al. (2007). Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat. Med.* 13, 703–710. doi: 10.1038/nm1586

Patarroyo, M. E., Alba, M. P., and Curtidor, H. (2011). Biological and structural characteristics of the binding peptides from the sporozoite proteins essential for cell traversal (SPECT)-1 and -2. *Peptides* 32, 154–160. doi: 10.1016/j.peptides.2010.09.026

Perkins, D. J., Were, T., Davenport, G. C., Kempaiah, P., Hittner, J. B., and Ong'echa, J. M. (2011). Severe malarial anemia: innate immunity and pathogenesis. *Int. J. Biol. Sci.* 7, 1427–1442. doi: 10.7150/ijbs.7.1427

Podoba, J. E., and Stevenson, M. M. (1991). CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. *Infect. Immun.* 59, 51–58.

Prado, M., Eickel, N., De Niz, M., Heitmann, A., Agop-Nersesian, C., Wacker, R. et al. (2015). Long-term live imaging reveals cytosolic immune responses of host hepatocytes against Plasmodium infection and parasite escape mechanisms. *Autophagy* 11, 1561–1579. doi: 10.1080/15548627.2015.1067361

Risco-Castillo, V., Topcu, S., Marinach, C., Manzoni, G., Bigorgne, A. E., Briquet, S., et al. (2015). Malaria sporozoites traverse host cells within transient vacuoles. *Cell Host Microbe* 18, 593–603. doi: 10.1016/j.chom.2015.10.006

Rowe, J. A., Claessens, A., Corrigan, R. A., and Arman, M. (2009). Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular

mechanisms and therapeutic implications. *Expert Rev. Mol. Med.* 11, e16. doi: 10.1017/S1462399409001082

Ryan, J. R., Stoute, J. A., Amon, J., Dunton, R. F., Mtalib, R., Koros, J., et al. (2006). Evidence for transmission of Plasmodium vivax among a duffy antigen negative population in Western Kenya. *Am. J. Trop. Med. Hyg.* 75, 575–581.

Ryg-Cornejo, V., Ly, A., and Hansen, D. S. (2016). Immunological processes underlying the slow acquisition of humoral immunity to malaria. *Parasitology* 143, 199–207. doi: 10.1017/S0031182015001705

Schmidt, C. Q., Kennedy, A. T., and Tham, W. H. (2015). More than just immune evasion: hijacking complement by *Plasmodium falciparum*. *Mol. Immunol.* 67, 71–84. doi: 10.1016/j.molimm.2015.03.006

Sharma, Y. D. (1991). Knobs, knob proteins and cytoadherence in falciparum malaria. *Int. J. Biochem.* 23, 775–789. doi: 10.1016/0020-711X(91)90061-Q

Singh, S., Alam, M. M., Pal-Bhowmick, I., Brzostowski, J. A., and Chitnis, C. E. (2010). Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog.* 6:e1000746. doi: 10.1371/journal.ppat.1000746

Sinnis, P., and Zavala, F. (2012). The skin: where malaria infection and the host immune response begin. *Semin. Immunopathol.* 34, 787–792. doi: 10.1007/s00281-012-0345-5

Souza-Silva, F. A., Torres, L. M., Santos-Alves, J. R., Tang, M. L., Sanchez, B. A., Sousa, T. N., et al. (2014). Duffy antigen receptor for chemokine (DARC) polymorphisms and its involvement in acquisition of inhibitory anti-duffy binding protein II (DBPII) immunity. *PLoS ONE* 9:e93782. doi: 10.1371/journal.pone.0093782

Spottiswoode, N., Duffy, P. E., and Drakesmith, H. (2014). Iron, anemia and hepcidin in malaria. *Front. Pharmacol.* 5:125. doi: 10.3389/fphar.2014.00125

Steers, N., Schwenk, R., Bacon, D. J., Berenzon, D., Williams, J., and Krzych, U. (2005). The immune status of Kupffer cells profoundly influences their responses to infectious *Plasmodium berghei* sporozoites. *Eur. J. Immunol.* 35, 2335–2346. doi: 10.1002/eji.200425680

Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., et al. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* 313, 1287–1290. doi: 10.1126/science.1129720

Tavares, J., Formaglio, P., Thiberge, S., Mordelet, E., Van Rooijen, N., Medvinsky, A., et al. (2013). Role of host cell traversal by the malaria sporozoite during liver infection. *J. Exp. Med.* 210, 905–915. doi: 10.1084/jem.20121130

Templeton, T. J. (2009). The varieties of gene amplification, diversification and hypervariability in the human malaria parasite, *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 166, 109–116. doi: 10.1016/j.molbiopara.2009.04.003

Thieleke-Matos, C., Lopes da Silva, M., Cabrita-Santos, L., Portal, M. D., Rodrigues, I. P., Zuzarte-Luis, V., et al. (2016). Host cell autophagy contributes to Plasmodium liver development. *Cell. Microbiol.* 18, 437–450. doi: 10.1111/cmi.12524

Uyoga, S., Skorokhod, O. A., Opiyo, M., Orori, E. N., Williams, T. N., Arese, P., et al. (2012). Transfer of 4-hydroxynonenal from parasitized to non-parasitized erythrocytes in rosettes. Proposed role in severe malaria anemia. *Br. J. Haematol.* 157, 116–124. doi: 10.1111/j.1365-2141.2011.09015.x

VanBuskirk, K. M., Sevova, E., and Adams, J. H. (2004). Conserved residues in the Plasmodium vivax Duffy-binding protein ligand domain are critical for erythrocyte receptor recognition. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15754–15759. doi: 10.1073/pnas.0405421101

Walther, M., Tongren, J. E., Andrews, L., Korbel, D., King, E., Fletcher, H. et al. (2005). Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287–296. doi: 10.1016/j.immuni.2005.08.006

WHO (2015). *World Malaria Report*. Geneva: World Health Organization.

Wilson, K. L., Xiang, S. D., and Plebanski, M. (2016). A model to study the impact of polymorphism driven liver-stage immune evasion by malaria parasites, to help design effective cross-reactive vaccines. *Front. Microbiol.* 7:303. doi: 10.3389/fmicb.2016.00303

Wykes, M. N., Horne-Debets, J. M., Leow, C. Y., and Karunarathne, D. S. (2014). Malaria drives T cells *to exhaustion. Front. Microbiol.* 5:249. doi: 10.3389/fmicb.2014.00249

Yano, T., and Kurata, S. (2011). Intracellular recognition of pathogens and autophagy as an innate immune host defence. *J. Biochem.* 150, 143–149. doi: 10.1093/jb/mvr083

Yipp, B. G., Anand, S., Schollaardt, T., Patel, K. D., Looareesuwan, S., and Ho, M. (2000). Synergism of multiple adhesion molecules in mediating cytoadherence of *Plasmodium falciparum*-infected erythrocytes to microvascular endothelial cells under flow. *Blood* 96, 2292–2298.

Zheng, H., Tan, Z., and Xu, W. (2014). Immune evasion strategies of pre-erythrocytic malaria parasites. *Mediators Inflamm.* 2014:362605. doi: 10.1155/2014/362605

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Interleukin-27 Early Impacts *Leishmania infantum* Infection in Mice and Correlates with Active Visceral Disease in Humans

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The complexity of *Leishmania*–host interactions, one of the main leishmaniasis issues, is yet to be fully understood. We detected elevated IL-27 plasma levels in European patients with active visceral disease caused by *Leishmania infantum*, which returned to basal levels after successful treatment, suggesting this cytokine as a probable infection mediator. We further addressed this hypothesis recurring to two classical susceptible visceral leishmaniasis mouse models. BALB/c, but not C57BL/6 mice, showed increased IL-27 systemic levels after infection, which was associated with an upregulation of IL-27p28 expression by dendritic cells and higher parasite burdens. Neutralization of IL-27 in acutely infected BALB/c led to decreased parasite burdens and a transient increase in IFN-γ^+ splenic T cells, while administration of IL-27 to C57BL/6 promoted a local anti-inflammatory cytokine response at the site of infection and increased parasite loads. Overall, we show that, as in humans, BALB/c IL-27 systemic levels are infection dependently upregulated and may favor parasite installation by controlling inflammation.

Keywords: IL-27, *Leishmania infantum*, human, mouse models, immune regulation

INTRODUCTION

The 2013 Global Burden of Disease reveals leishmaniasis as one of the deadliest neglected tropical infectious diseases, affecting millions of people worldwide (1). It is caused by the sandfly transmitted protozoan parasite *Leishmania*. The infection can be asymptomatic or manifest in cutaneous, mucosal, and visceral forms, the last one being fatal if left untreated (2). These forms depend mostly on the infecting parasite species but also on the efficiency of the host immune response, especially during the first moments after parasite inoculation (3). Phagocytic cells are rapidly recruited upon *Leishmania* deposition in the skin by the sandfly, but the parasite is able to

manipulate them using different strategies. One of the effects of this immunomodulation is the alteration of cytokines secretion in the infection microenvironment. Interferences with receptors and signaling pathways prevent the production and the function of pro-inflammatory cytokines such as IL-12 and IFN-γ and favors anti-inflammatory IL-10 and TGF-β contributing to parasite survival [reviewed in Ref. (4)].

Interleukin-27 is composed of the subunits p28 and EBV-induced protein 3 (EBI3) that interacts with a receptor comprising the glycoprotein 130 (gp130) and IL-27Rα or WSX-1. Interleukin-27 is mainly produced by macrophages and dendritic cells (DCs) after the stimulation of surface receptors such as TLRs or CD40 and following IFNs signaling (5, 6). Although this cytokine was initially described as pro-inflammatory, numerous anti-inflammatory and immunomodulatory properties are recognized today [reviewed in Ref. (5, 6)].

In the past few years, several reports have contributed to the understanding of the IL-27 role during *Leishmania* infection. Active visceral leishmaniasis (VL) patients from India (7) and Brazil (8) present increased IL-27 in plasma. In mice, IL-27 appears to be essential to prevent severe immunopathology after infection with both cutaneous (9) and visceral (10) strains, mainly through the effects of IL-10. However, whether IL-27 directly affects parasite burdens (9, 11) or if IL-10 appears as a consequence of inflammatory (7, 12) or suppressor (13) events is still unclear.

In the present study, we report elevated IL-27 plasma levels in *Leishmania infantum*-infected European patients with active VL, which normalized after successful treatment. Curiously, IL-27 increased early after *L. infantum* infection in the serum of BALB/c, but not in C57BL/6 mice. We took advantage of this dichotomy to understand the mechanisms underlying the effects of IL-27 in visceral *Leishmania* infection. Early blocking of IL-27 in BALB/c mice decreased parasite loads, while IL-27 administration in C57BL/6 augmented parasite burdens. Immunological studies suggested that IL-27 is produced as a consequence of parasite subversion of the host immune response, resulting in a control of inflammation beneficial for parasite installation.

MATERIALS AND METHODS

Ethics Statement

Human sample collection was in accordance with Good Clinical Practice guidelines. The study was approved by the Ethics Committee of the University Hospital of Fuenlabrada (Madrid, Spain). All subjects provided their written informed consent.

Animal experiments were performed in accordance with the IBMC.INEB Animal Ethics Committees and the Portuguese National Authorities for Animal Health guidelines (directive 2010/63/EU). Begoña Pérez-Cabezas and Anabela Cordeiro-da-Silva are accredited for animal research (Portuguese Veterinary Direction, Ministerial Directive 113/2013).

Human Studies

Diagnosed VL patients were treated with liposomal Amphotericin B (21 mg/kg). Cure was 100% in 3 months after diagnosis. Subjects without previous VL symptomology were included as negative controls. Plasma was recovered from heparinized blood and stored at −20°C for posterior IL-27 determination using MILLIPLEX MAP (Millipore, Germany) and analyzed on a Bio-Plex-200 Luminex (Bio-Rad, CA, USA) (minimum detectable concentration 0.063 ng/mL).

Parasites and Mice

A clone of virulent *L. infantum* (MHOM/MA/67/ITMAP-263) was maintained by weekly subpassages at 26°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 microgram/mL streptomycin, and 20 mM HEPES buffer (all from BioWhittaker, Lonza, Switzerland). Promastigotes from 4 to 10 passages were used in these experiments.

Six- to eight-week-old male BALB/c and C57BL/6 mice (Charles River Laboratories, France) were maintained under specific pathogen-free conditions at the IBMC facilities. Animals were infected i.p. with 1×10^8 stationary promastigotes from 5 culture days.

Mice Sera Collection

Blood from mice was collected through intracardiac puncture under isoflurane anesthesia. Serum was collected and stored at −80°C for posterior analysis.

Splenic Dendritic Cells and Macrophages Cell Sorting and Hepatic Kupffer Cell Enrichment Procedure

Spleen and Liver were aseptically collected from 24 h infected mice. Splenocytes were obtained and labeled to distinguish DCs ($CD11b^+/CD11c^{high}$) and macrophages ($CD11b^+/Ly6G^-/Ly6C^+/F4\text{-}80^+$), and sorted using a FACSAria and the FACSDiva software (BD Biosciences, NJ, USA) for posterior RNA analysis. Sorted cells purity was >95%.

Enriched Kupffer cells were obtained after hepatic collagenase digestion, followed by a Percoll density gradient centrifugation (Sigma-Aldrich, MO, USA) and a brief adhesion step, as described elsewhere (14). The percentage of Kupffer cells was determined by flow cytometry ($F4/80^+/CD11b^{variable}$). Cells were resuspended in lysis buffer and frozen for posterior RNA analysis.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted (RNeasy Mini Kit, Qiagen, CA, USA), quantified (NanoDrop1000, Thermo Scientific, MA, USA), and reversely transcribed (NZY-First-Strand cDNA synthesis Kit, NZYTech, Portugal). Quantitative RT-PCRs were run on an iQ5 iCycler thermal cycler (Bio-Rad) (15). Results were analyzed (built-in iQ5 v2, Bio-Rad) and normalized using the reference gene GAPDH. The sequences of the primers used were *IL-27p28* Forward TCGATTGCCAGGAGTGAACC, Reverse CGAAG TGGTAGCGAGGAAG; *EBI3* Forward AGCAGCAGCCTCC TAGCCT, Reverse ACGCCTTCCGGAGGGTC; and *GAPDH* Forward CTGGTCCTGAGTGTAGCCCAA, Reverse CATGGC CTTCCGTGTTCCTA.

In Vitro Differentiation and Infection of Bone Marrow-Derived DCs and Macrophages

For bone marrow-derived DCs (BMDCs) differentiation, 6×10^6 bone marrow cells were seeded in 25 mL of complete RPMI supplemented with 50 μM 2-mercaptoethanol (Sigma-Aldrich) and 10% of GM-CSF-containing supernatant from J558 cell conditioned medium (DC medium) (16). Cells were cultured at 37°C and 5% CO_2 for 3 days, after which the same amount of DC medium was added to each flask. At day 6, half of the culture supernatant was replaced with fresh DC medium. At day 8, cells were thoroughly resuspended, recovered, counted, and, finally, plated in 96 round-bottom culture plates at 1×10^5 cells/well in 200 μL of DC medium. Bone marrow-derived macrophages (BMMØ) were obtained from non-adherent bone marrow cells collected after an initial overnight incubation (37°C and 5% CO_2) in complete Dulbecco's modified Eagle's medium (DMEM) (Lonza). Non-adherent cells were counted and distributed in 96 flat-bottom well culture plates at 1×10^5 cells/well in 100 μL of complete DMEM supplemented with 5% of L-929 cell conditioned medium (LCCM). After 3 days of culture, 100 μL of DMEM + 5% LCCM were added per well. Half of the media volume was renewed at day 6 of culture. BMMØ acquired a definitive differentiation status at day 8.

Stationary promastigotes from 5 culture days (cultured as described in Section "Parasites and Mice") were added to BMDC or BMMØ at a 10:1 ratio. Non-internalized parasites were washed after 4 h incubation (37°C and 5% CO_2). Non-infected cells were stimulated with 1 μg/mL LPS (Sigma-Aldrich) as positive control, or received complete medium as negative control. Supernatants were recovered 24 h post-infection for cytokine quantification.

In Vivo IL-27 Modulation

Twenty-four hours after infection, BALB/c mice were i.p. treated with 20 μg of goat anti-mouse IL-27p28 neutralizing antibody (a-IL-27) and C57BL/6 with 1 μg of mouse recombinant IL-27 (rIL-27) (both from R&D Systems), as previously reported (17, 18). As controls, BALB/c and C57BL/6 received 20 μg of goat IgG (isotype control) and PBS (mock), respectively.

Determination of Parasite Burdens

Spleen and liver were aseptically collected, weighted, and disrupted. Splenic and hepatic parasite burdens were assessed by the limit dilution method. The parasite titer was considered as the last dilution with >1 motile parasite. The number of parasites per gram of organ was calculated, as discussed previously (19). Peritoneal cells were recovered and 2×10^5 cells from each exudate were subjected to cytospin in 200 μL of PBS during 5 min at 1000 rpm using a Shandon Cytospin II (GMI, MN, USA). Cell preparations were fixed with 2% paraformaldehyde (PFA) for 20 min. Afterward, an adapted staining protocol was performed (20) by 2 min immersion in Kaplow modified solution, followed by 45 s immersions in Hemacolor reagent 1 and reagent 2 (Merk Millipore, Germany). Finally, slides were washed with distilled water, air dried, and observed by optical microscopy (100× magnification). For determination of the percentage of infected cells, 200 consecutive cells were differentially counted (infected versus non-infected) in 3 different areas of the same preparation and the mean of the 3 areas was calculated. The number of parasites per infected cells was assessed by counting 100 different infected cells from which the mean was calculated.

Flow Cytometry

The anti-mouse monoclonal antibodies used to perform this study were all purchased to BioLegend (CA, USA) except if otherwise stated: FITC-labeled anti-IgM (R6-60.2, BD Biosciences, NJ, USA), anti-MHC-II(I-Ad) (AMS-32.1, BD), anti-MHC-II(I-A/I-E) (M5114.15.2), anti-IFN-γ (XMG1.2), and anti-IL-17A (TC11-18H10.1); PE-labeled anti-CD8 (53-6.7, BD), anti-CD11b (M1/70), anti-Siglec-F (E50-2440, BD), anti-F4/80 (BM8), anti-IL-4 (11B11), and anti-IL-6 (MP5-20F3); PerCP-labeled anti-CD11b (M1/70); PerCP-Cy5.5-labeled anti-Ly6C (HK1.4), anti-F4-80 (BM8), and anti-TNFα (MP6-XT22); PE-Cy7-labeled anti-CD3 (HA2) and anti-CD11b (M1/70); APC-Cy7-labeled anti-CD11c (N418); APC-labeled anti-CD19 (6D5), anti-IL-5 (TRFK5), and anti-IL-10 (JES5-16E3); BV510-labeled anti-CD4 (RM4-5); and Pacific Blue™-labeled anti-Ly6G (1A8).

To analyze lymphoid and myeloid cell populations, two panels of antibodies were designed. The lymphoid panel was composed of anti-CD8, -CD3, -CD4, and -CD19. The Myeloid panel comprised anti-CD11b, -CD11c, -Siglec-F, -Ly6C, -Ly6G, and -MHC-II. Surface staining of peritoneal and splenic cells was performed in PBS + 0.5% BSA (20min, 4°C) followed by 15 min fixation using 1% PFA. For intracellular staining, splenocytes were cultured for 2 h with PMA/Ionomycin (50/500 ng/mL) and then for 2 h with Brefeldin A (10 μg/mL). Cells were surface stained and then intracellularly after fixation and permeabilization with 1% saponin (all from Sigma) (21). Samples were acquired in a FACSCanto (BD) and analyzed using the FlowJo software v10 (TreeStar, OR, USA).

An initial gate plotting FSC-A versus SSC-A was performed. Afterward, singlets were selected by plotting FSC-A versus FSC-H and the remaining cell populations were resolved. T lymphoid cell populations were defined as $CD3^+/CD4^+$ and $CD3^+/CD8^+$, while B cells were defined as $CD19^+$. Cytokine production by T cells was assessed within $CD3^+/CD4^+$ and $CD3^+/CD8^+$cells. Myeloid cell populations were gated as eosinophils (Siglec-F^+/SSC-$H^{int/high}$), neutrophils ($CD11b^{high}$/$Ly6G^{high}$/Siglec-F^-), DCs ($CD11c^+$/MHC-$II^{int/high}$), and macrophages ($CD11b^+$/$CD11c^-$/$Ly6G^-$/Siglec-F^-).

Determination of Mouse Cytokines by ELISA

Cytokines were quantified, according to the manufacturer's instructions, using the commercial kits: IL-27 ELISA Ready-SET-Go!® (eBiosciences, CA, USA) (detection limit 16 pg/mL); IL-27p28/IL-30 and IL-10 DuoSet ELISA (R&D Systems, MN, USA) (detection limits 15.6 and 31.2 pg/mL, respectively); IL-12p70 and IFN-γ ELISA MAX Deluxe (BioLegend, CA, USA) (detection limit 4 pg/mL for both cytokines).

Statistical Analysis

Results are expressed as mean ± SEM. Statistical differences were analyzed using GraphPad Prism v6.01 (CA, USA). Comparisons between human samples were performed using Mann–Whitney test for unpaired samples and Wilcoxon test for paired data. Mice experimental groups were compared using the unpaired *t*-test unless otherwise stated.

RESULTS

Systemic IL-27 Increases in *L. infantum*-Infected European Patients and BALB/c Mice

To address if the increase of systemic IL-27 is a common fact of VL among different endemic areas (7, 8), we determined this cytokine in the plasma of active *L. infantum* infected individuals from a current outbreak in Spain. Patients with active disease presented higher IL-27 levels than both cured ($p \leq 0.001$) and negative controls ($p \leq 0.001$) (**Figure 1A**). Some patients were re-sampled after cure, which confirmed that IL-27 decreases after successful treatment ($p \leq 0.01$) (**Figure 1A**).

The role of IL-27 during *Leishmania* infection was addressed using mouse models by several authors (10–13, 22). However, whether IL-27 is also increased in the serum of *Leishmania*-infected mice has never been shown. Therefore, we addressed if BALB/c and C57BL/6, two susceptible VL mouse species, displayed a similar systemic increase of IL-27 as observed in *L. infantum*-infected humans. While IL-27 remained unchanged in the serum of infected C57BL/6 mice, infected BALB/c showed an early increase that was significant 4 days after infection ($p \leq 0.05$) and always higher than the IL-27 levels of C57BL/6 mice ($p \leq 0.05$ comparing the day 2 and $p \leq 0.01$ the day 4 after infection) (**Figure 1B**).

Dendritic Cells Are the Main Cellular Source of IL-27 in Infected BALB/c Mice

As the main sources of IL-27 are myeloid cell populations, and macrophages and DCs play critical roles during *Leishmania* infection, we decided to discriminate their contribution to IL-27 response after *L. infantum* infection. For that, the expression of IL-27 subunits in splenic DCs and macrophages sorted from infected BALB/c and C57BL/6 was evaluated. While the expression of EBI3 remained always comparable to basal levels (Figure S1 in Supplementary Material), IL-27p28 expression was significantly upregulated but only in DCs from BALB/c 24 h after infection ($p \leq 0.05$) (**Figure 2A**). The contribution of Kupffer cells, the resident liver macrophages, for the IL-27 response to *L. infantum* was also addressed. The expression of IL-27 subunits was analyzed in Kupffer cells from non-infected and infected BALB/c mice. However, no increase in the RNA levels of IL-27p28 and EBI3 was detected in these cells 24 h after infection (Figure S2 in Supplementary Material), suggesting that DCs, and not macrophages, are the main cell source responsible for the increase of IL-27 in our model.

To confirm our data at protein level, we determined IL-27 and IL-27p28 in supernatants from 24 h *L. infantum*-infected BMMØ and BMDCs. Although after infection the production of IL-27p28 was greater in BMMØ from C57BL/6 than from BALB/c ($p \leq 0.05$), IL-27 concentration was similar in both mouse strains. No difference was found after LPS activation (**Figure 2B**). In line with *ex vivo* results, both IL-27p28 and IL-27 secretion by BMDCs were significantly higher in cells

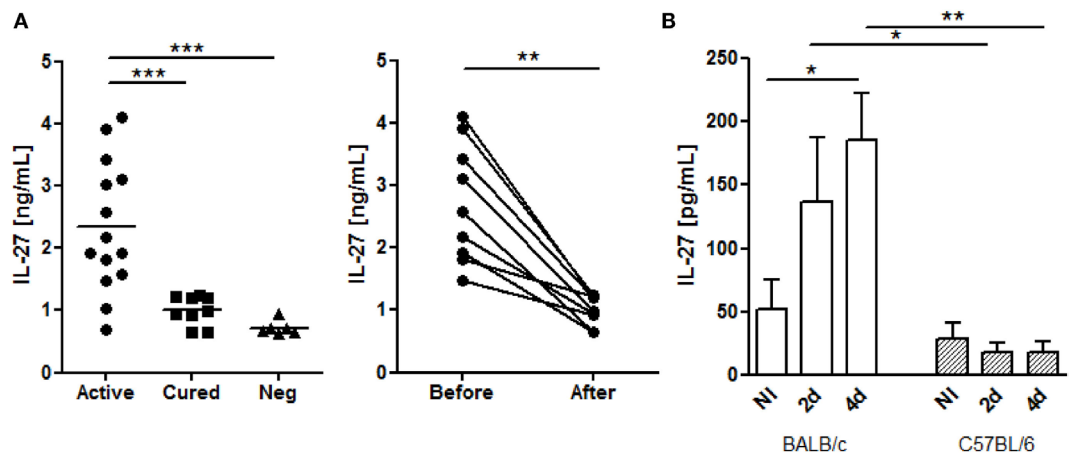

FIGURE 1 | *L. infantum* infection increases systemic IL-27 in European VL patients and in BALB/c mice. (A) Interleukin-27 levels in the plasma of European patients with active VL caused by *L. infantum* (black dots, $n = 14$), cured patients (black squares, $n = 9$), and negative controls (black triangles, $n = 6$). The cytokine was also determined in VL patients before and after treatment ($n = 9$). Each symbol represents one individual; bars represent the mean of the experimental groups. Mann–Whitney test was used to compare clinical groups and Wilcoxon matched pairs test for paired comparisons. **(B)** Interleukin-27 levels in the serum of BALB/c (white columns) and C57BL/6 (patterned columns) mice, infected i.p. with 1×10^8 *L. infantum* promastigotes for 2 and 4 days. Bars represent mean ± SEM of three independent experiments with a minimum of four animals per group and experiment. Unpaired *t*-test was used to assess statistical significances (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

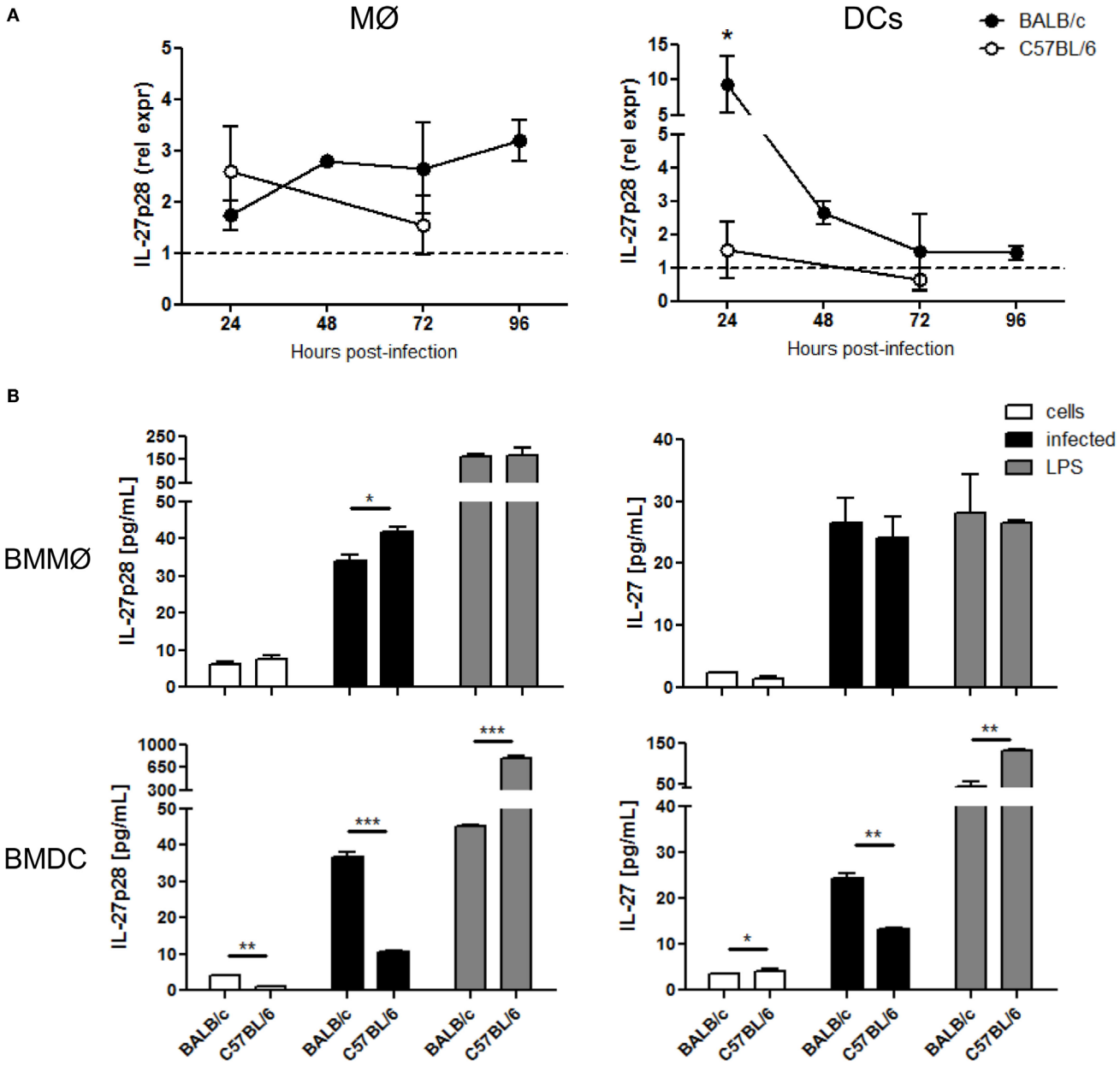

FIGURE 2 | Dendritic cells are the main source of IL-27 after *L. infantum* infection in BALB/c mice. **(A)** Splenic MØ and DCs were sorted from infected BALB/c (black symbols) and C57BL/6 (open symbols) mice at the indicated time points. The transcription levels of IL-27p28 were quantified by qRT-PCR. Results are relativized to non-infected mice values (=1). Every symbol and bars represent the mean and SEM, respectively, of four different animals per group analyzed in two different independent experiments. Unpaired *t*-test was used to assess statistical significances. **(B)** The secretion of IL-27p28 and IL-27 was quantified by ELISA in 24 h supernatants of BMMØ and BMDC infected with *L. infantum* promastigotes at a 1:10 ratio (black bars). Medium (white bars) and LPS (gray bars) were used as negative and positive controls, respectively. Bars represent the mean ± SEM of two independent experiments, every run in duplicate. Unpaired *t*-test was used to assess statistical significances (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

from BALB/c when compared to C57BL/6 ($p \leq 0.001$ and $p \leq 0.01$, respectively) (**Figure 2B**). Interestingly, the ability of BMDCs to produce both cytokines after LPS stimulation was higher in C57BL/6 than in BALB/c, indicating that these cells are really capable of producing IL-27, but not in response to *L. infantum* infection. These results suggest that the parasite can actively modulate the secretion of IL-27 and IL-27p28 in BMDCs obtained from BALB/c, but not from C57BL/6 mice.

IL-27 Favors *L. infantum* Infection in Mouse Models

To determine whether IL-27 contribute to the infection outcome, we neutralized IL-27 in BALB/c and administered

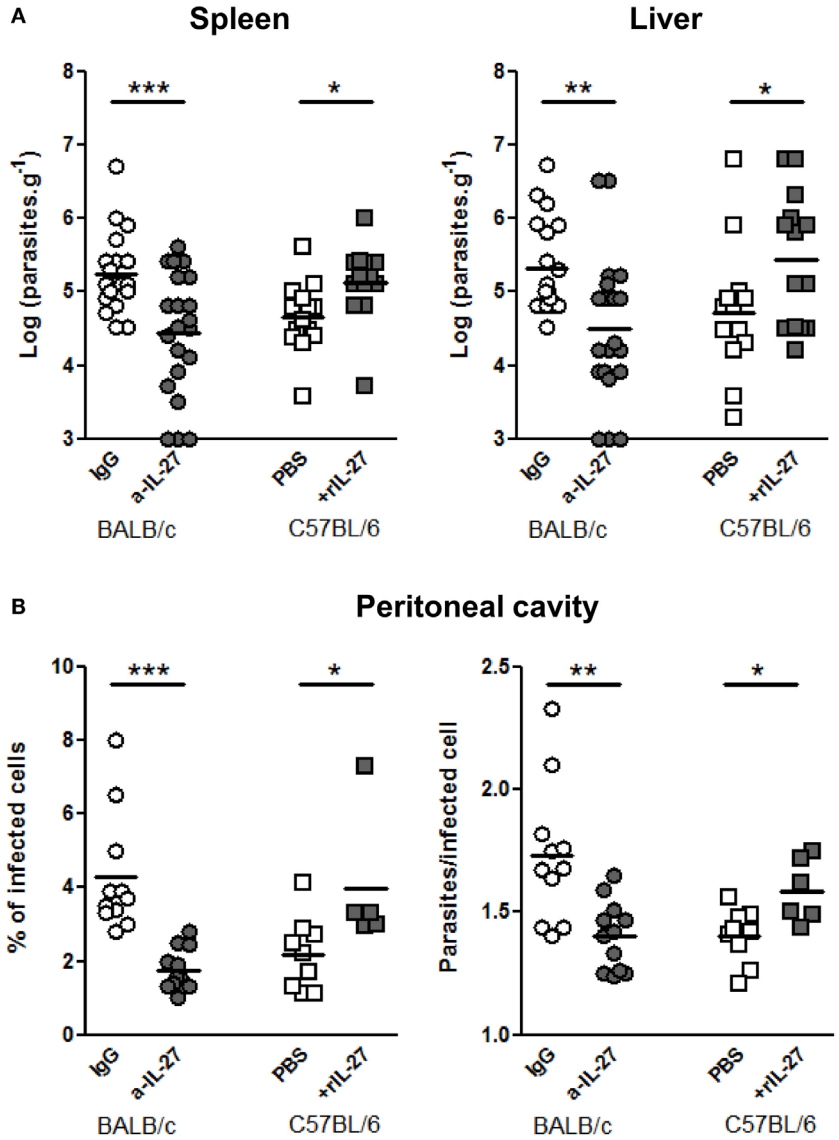

FIGURE 3 | IL-27 favors *L. infantum* infection in mice models. **(A)** BALB/c (circles) and C57BL/6 (squares) mice were i.p. infected with 1×10^8 promastigotes. Twenty-four hours later, BALB/c mice were treated i.p. with 20 μg of a-IL-27 (gray circles) or IgG isotype control (open circles), while C57BL/6 received i.p. 1 μg of mouse rIL-27 (gray squares) or the same volume of PBS (open squares). Three days after treatment, mice were euthanized and the parasite load of spleen and liver determined by limiting dilution. **(B)** In some animals, the peritoneal cavity was washed for collection of recruited cells. Approximately 2×10^5 cells from every exudate were cytospined, fixed, and stained with Kaplow modified solution and by the Giemsa method. Finally, slides were observed by optical microscopy at 100× for determination of the percentage of infected cells and the number of parasites/infected cell. All data represent results obtained in three independent experiments. Every symbol represents a mouse and the bars the mean of the group. Unpaired *t*-test was used to assess statistical significances (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

rIL-27 to C57BL/6 mice 24 h after infection, coinciding with the IL-27p28 RNA peak in the spleen and before the IL-27 increase observed in the serum, 2 days after the infection, in BALB/c mice. Seventy-two hours later, these treatments significantly decreased in BALB/c and increased in C57BL/6 splenic and hepatic parasite burdens (**Figure 3A**). We also observed that IL-27 significantly affected the percentage of infected cells and the number of parasites per infected cells in the peritoneal cavity (**Figure 3B**). These results revealed a relation between the levels of IL-27 and the establishment of the infection.

IL-27 Modulates the Cytokine Response in Infected Mice

To elucidate how IL-27 interferes at the early stages of *L. infantum* infection, we performed a kinetic study of the peritoneal and splenic compartments after IL-27 modulation. Interestingly, IL-27 neutralization in infected BALB/c mice

transiently decreased IL-10 in the peritoneal cavity ($p \leq 0.01$), and the administration of rIL-27 increased the presence of this cytokine in infected C57BL/6 mice 24 h ($p \leq 0.01$) and 72 h ($p \leq 0.05$) after treatment, always comparing with control infected animals (**Figure 4**). In addition, rIL-27 in C57BL/6 promoted a reduction of IFN-γ ($p \leq 0.05$) and IL-12p70 ($p \leq 0.01$) 24 h after treatment in comparison with infected non-treated animals (**Figure 4**). However, this shift in the cytokine profile determined by IL-27 almost did not alter cell recruitment to the site of infection, as when comparing treated and non-treated animals only the presence of B cells in the peritoneal cavity significantly increased 72 h after IL-27 blockage in BALB/c mice (**Figure 5**). In the spleen, the number of $CD4^+$ T cells increased in BALB/c mice 24 h after IL-27 blockage ($p \leq 0.05$), and the supply of the cytokine decreased the presence of the same cells in C57BL/6 mice after 72 h ($p \leq 0.05$) (**Figure 5**). The administration of rIL-27 also prevented the infiltration of neutrophils in the spleen of C57BL/6 mice ($p \leq 0.01$) (**Figure 6**). Analysis of the cytokine production revealed that IL-27 neutralization increased the numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells 24 h post-treatment (**Figure 7**) ($p \leq 0.01$). These differences were not detected at 72 h, likely indicating a reversible effect (**Figure 7**). The potential secretion of other cytokines was also analyzed but no differences between α-IL-27 and IgG receiving mice were observed (Figure S3 in Supplementary Material). In contrast, rIL-27 did not alter the cytokine response of C57BL/6 splenic T cells (**Figure 7**; Figure S3 in Supplementary Material).

DISCUSSION

Interleukin-27 is a cytokine with known immunomodulatory properties involved in the pathogenesis of numerous diseases (5, 6). Several types of infections also course with an increase of IL-27 (23–25). However, whether this fact is a simple host mechanism to control inflammation or a complex pathogen evasion strategy is still unknown. *Leishmania* spp. are expert in modulating immune activity through diverse strategies (4). Previous reports on human leishmaniasis indicate that IL-27 increases when the disease is active in both cutaneous (26–28) and visceral forms (7, 8). Here, for the first time, plasma levels of IL-27 were evaluated in *L. infantum*-infected individuals from Europe. This cytokine was significantly increased in the active phase of our VL patients returning to homeostatic levels after treatment. Interestingly, a relapsing case presented increased circulating IL-27, and positive IL-27 results were obtained even when immunofluorescence antibody test (IFAT) was doubtful (data not shown). Further analysis using a larger cohort of patients will help to better understand the value of IL-27 as a complementary biomarker for human VL diagnosis and even for treatment efficacy monitoring, as has been postulated for this disease (8) and other infections (29).

The role of IL-27 during *Leishmania* infection has been addressed using animal models by several groups (9, 10, 12, 30). The general conclusion was that IL-27 controls inflammation and pathology through limitation of IFN-γ (12) and IL-17 (9) production by $CD4^+$ T cells, resulting in a permissive environment for the infection. However, these results are based on experiments performed on WSX-$1^{-/-}$ or EBI$3^{-/-}$ mice, presenting two main limitations. First, both WSX-1 and EBI3 are subunits shared with the regulatory cytokine IL-35 (31), meaning that the over-inflammation observed in these knockout mice can be a result of the additive loss of the regulatory function of both cytokines. The second issue is that both mice models were generated in the C57BL/6 genetic background. Although both C57BL/6 and BALB/c strains are considered susceptible models for experimental VL (32), here we showed that only BALB/c and not C57BL/6 mice increased IL-27 in the serum early

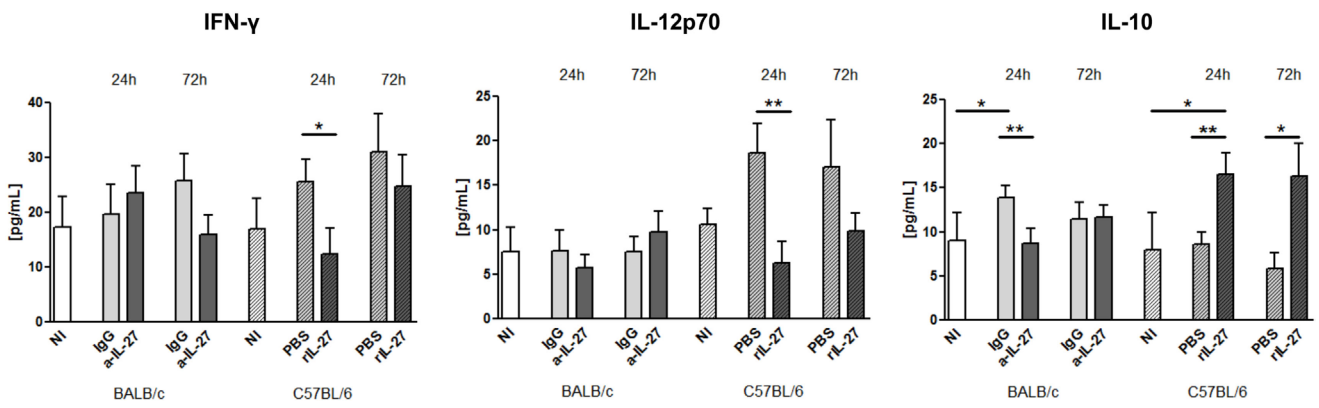

FIGURE 4 | IL-27 influences the cytokine responses in *L. infantum*-infected mice at the infection site. BALB/c and C57BL/6 mice were i.p. infected with 1×10^8 promastigotes. Twenty-four hours later, BALB/c mice were treated i.p. with 20 μg of a-IL-27 (clear dark-gray bars) or IgG isotype control (clear light-gray bars), while C57BL/6 received i.p. 1 μg of mouse rIL-27 (patterned dark-gray bars) or the same volume of PBS (patterned light-gray bars). Twenty-four or 72 h after treatment, mice were euthanized and the peritoneal cavity washed. Non-infected (NI) counterparts were always used as controls (white bars, non-patterned for BALB/c and patterned for C57BL/6 mice). The concentration of IFN-γ, IL-12p70, and IL-10 in the peritoneal exudates was determined by ELISA. Bars represent the mean ± SEM of three independent experiments, a minimum of four animals per condition and experiment was analyzed. Unpaired *t*-test was used to assess statistical significances (*$p \leq 0.05$ and **$p \leq 0.01$).

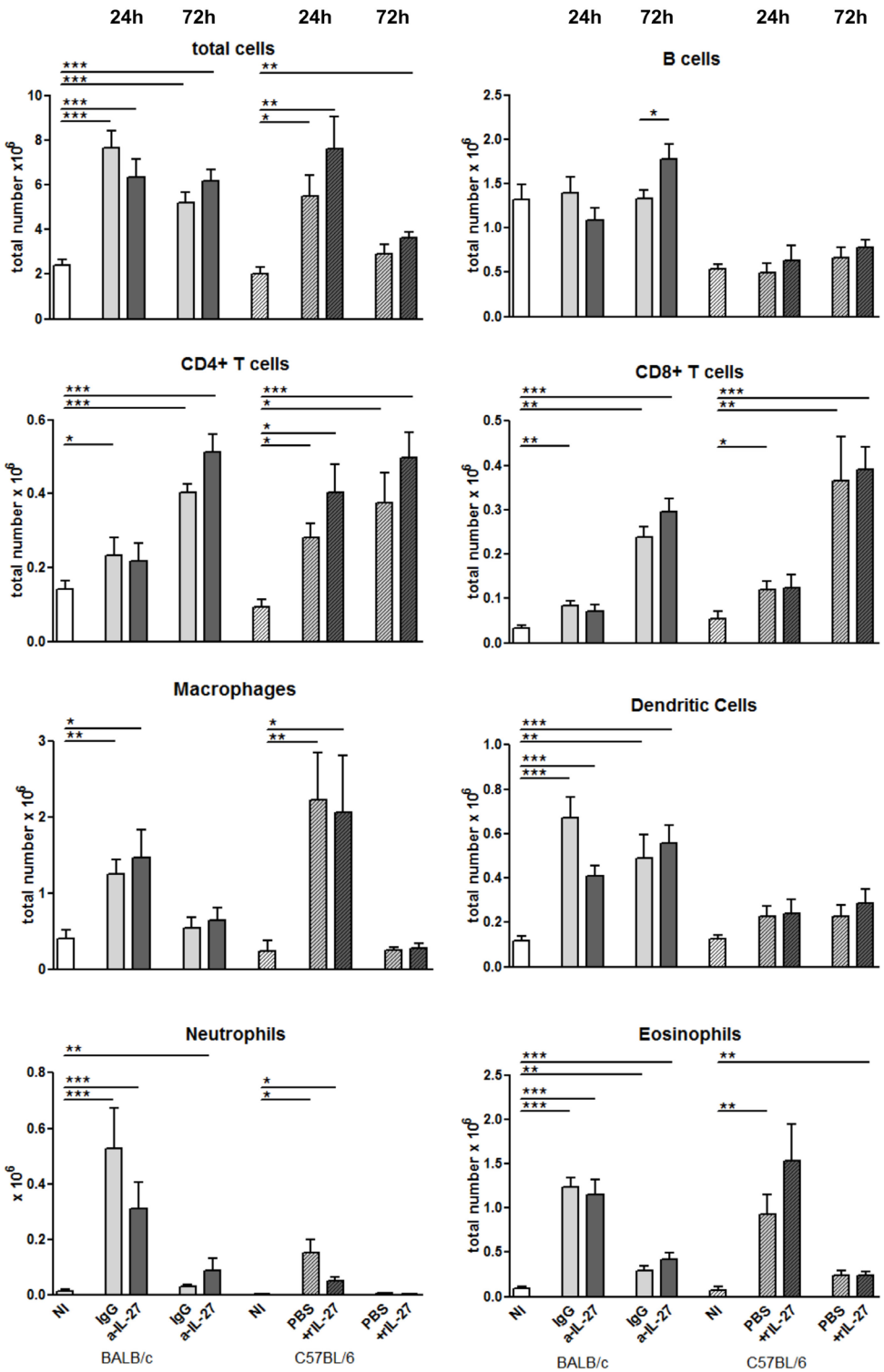

FIGURE 5 | Cell recruitment to the peritoneal cavity in response to *L. infantum* infection after IL-27 modulation. BALB/c and C57BL/6 mice were i.p. infected with 1×10^8 promastigotes. Twenty-four hours later, BALB/c mice were treated i.p. with 20 μg of a-IL-27 (clear dark-gray bars) or IgG isotype control (clear light-gray bars), while C57BL/6 received i.p. 1 μg of mouse rIL-27 (patterned dark-gray bars) or the same volume of PBS (patterned light-gray bars). Twenty-four or 72 h after treatment, mice were euthanized and the peritoneal cavity washed. Non-infected (NI) counterparts were always used as controls (white bars, non-patterned for BALB/c and patterned for C57BL/6 mice). Peritoneal cells were then extracellularly stained and acquired by flow cytometry. Bars represent the mean ± SEM of three independent experiments, a minimum of four animals per condition and experiment was analyzed. Unpaired *t*-test was always used to assess statistical significances (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

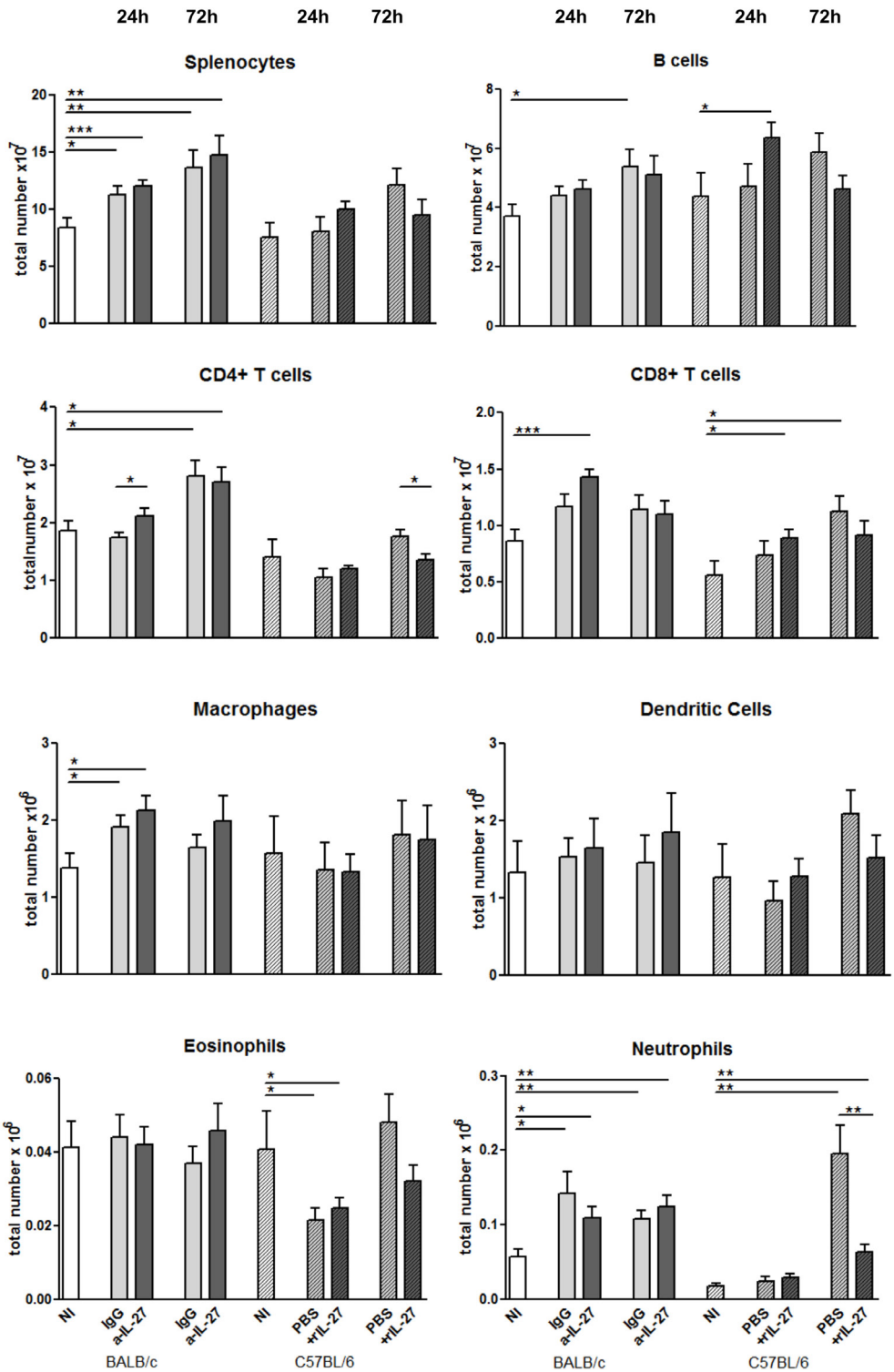

FIGURE 6 | **Cellular composition of the spleen from *L. infantum*-infected mice after IL-27 modulation**. BALB/c (clear bars) and C57BL/6 (patterned bars) mice were infected i.p. with 1×10^8 promastigotes. Twenty-four hours after infection, BALB/c mice were treated i.p. with 20 μg of IL-27 neutralizing antibody (a-IL-27, dark-gray bars) or IgG isotype control (IgG, light-gray bars), while C57BL/6 received i.p. 1 μg of mouse recombinant IL-27 (+rIL-27, dark-gray patterned bars) or the same volume of PBS (PBS, light-gray patterned bars). Non-infected (NI) counterparts were always used as controls (white bars, clear for BALB/c and patterned for C57BL/6 mice). Twenty-four or 72 h after treatment, mice were euthanized and the spleen collected and homogenized. Splenocytes were counted using an automatic cell counter, washed, and phenotyped by flow cytometry. Bars represent the mean and SEM of the three independent experiments, a minimum of four animals was analyzed per condition and experiment. Unpaired *t*-test was used to assess statistical significances (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

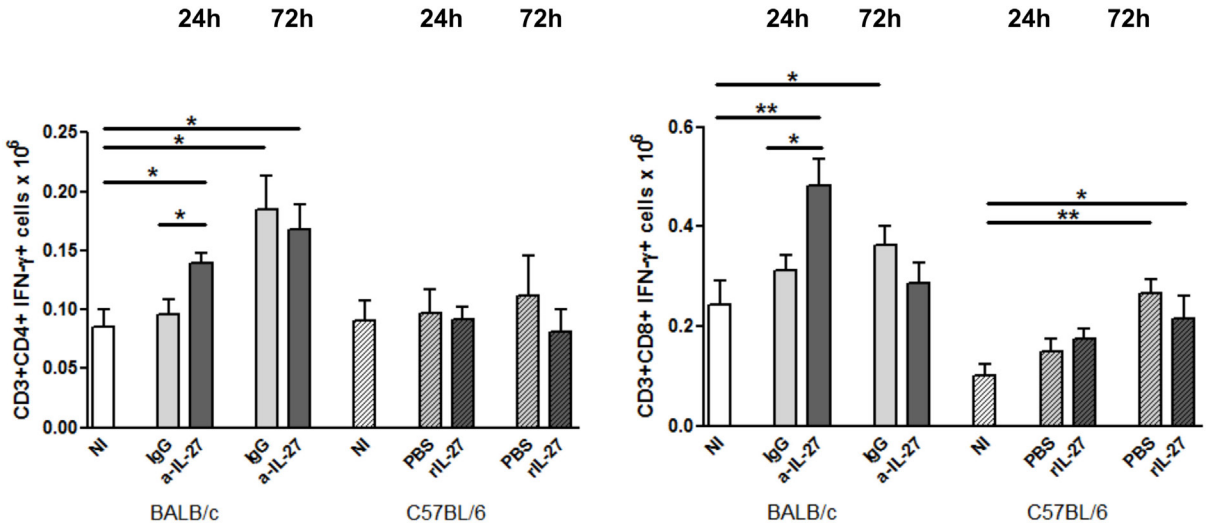

FIGURE 7 | IL-27 neutralization increases the IFN-γ response of splenic T cells in BALB/c mice (clear bars) and C57BL/6 (patterned bars) mice were infected i.p. with 1 × 10⁸ promastigotes. Twenty-four hours after infection, BALB/c mice were treated i.p. with 20 μg of IL-27 neutralizing antibody (a-IL-27, dark-gray bars) or IgG isotype control (IgG, light-gray bars), while C57BL/6 received i.p. 1 μg of mouse recombinant IL-27 (+rIL-27, dark-gray patterned bars) or the same volume of PBS (PBS, light-gray patterned bars). Non-infected (NI) counterparts were always used as controls (white bars, clear for BALB/c and patterned for C57BL/6 mice). Twenty-four or 72 h after treatment, mice were euthanized and the spleen collected and homogenized. Splenocytes were counted with an automatic cell counter, washed, and *in vitro* cultured during 4 h in the presence of PMA + Ionomycin and Brefeldin A. Cells were then extra- and intracellularly stained and acquired by flow cytometry. Bars represent the mean and SEM of the three independent experiments, a minimum of four animals was analyzed per condition and experiment. Unpaired *t*-test was used to assess statistical significances (*$p \leq 0.05$ and **$p \leq 0.01$).

after i.p. infection with *L. infantum*. The difference observed in the IL-27 production between the two mice strains in this study was confirmed at the cellular level, as splenic DCs from BALB/c but not from C57BL/6 mice upregulated the expression of IL-27p28 after infection. Upregulation of IL-27p28 in splenic DCs from BALB/c mice was previously shown after i.v. infection with *Leishmania donovani*, being CD8α⁺ DCs the major responsible subset (22). The production of IL-27 could restrict the typical Th1 polarization induced by these APCs, known to be essential for parasite limitation (33). In fact, in a conditional IL-27p28 knockout mice model restricted to DCs, CD4⁺ T cell IFN-γ response was exacerbated (34). Our data suggest that DCs are the main cell source responsible for the increase of IL-27 in BALB/c mice early after *L. infantum* infection probably as a result of active cell function modulation by the parasite. One explanation for the IL-27 divergence observed between the mice strains could be a differential expression of molecules involved in the recognition of parasite structures. An example is TLR-2, more expressed by BALB/c than by C57BL/6 mice (35). It has been demonstrated that TLR-2 signaling induces IL-27 production by respiratory epithelial cells (36). Therefore, this natural high expression of TLR-2 by BALB/c may favor the IL-27 increase early after infection, lowering inflammation, and promoting infection.

We exploited the IL-27 dichotomy in BALB/c and C57BL/6 mice to study the role of this cytokine during the early steps of infection, using an *in vivo* artificial-modulation approach. The resulting data revealed a relation between the levels of IL-27 and infection establishment, as the neutralization or the supply of IL-27 resulted in decreased or increased parasite burdens, respectively. In addition, the administration of rIL-27 significantly increased the production of IL-10 while decreased IFN-γ and IL-12p70 in the peritoneal cavity of C57BL/6 mice. Furthermore, the analysis of the cytokine production by splenic T cells revealed that IL-27 neutralization in BALB/c temporarily increased the numbers of IFN-γ producing CD4⁺ and CD8⁺ T cells. These results partially explain the higher parasite burdens quantified in the presence of IL-27. Addition of IL-27 *in vitro* exacerbates the infection of human macrophages by *Leishmania amazonensis via* IL-10 (11) and combined production of IL-27 and IL-10 by *L. donovani*-infected DCs is essential for IL-10 production by Th1 cells, resulting in parasite persistence (13). A recent work in EBI3$^{-/-}$ mice suggested a role for IL-27 in controlling IL-17 production and neutrophil infiltration during the chronic phase of *L. infantum* infection (30). However, whether the absence of the EBI3 subunit has an impact early after infection was not addressed in this work. We can also suggest that the IL-27 produced in response to infection shall not be a product of inflammation as BALB/c mice present higher parasite loads than C57BL/6 during the first days of infection, a difference that is counteracted by the treatments. Probably, the initial immune response of BALB/c is less inflammatory and less capable of limiting infection installation, suggesting again that the parasites directly induce the production of IL-27 for their own benefit. In fact, the absence of IL-27 can also prevent mycobacterial-induced phagosomal maturation arrest, favoring the elimination of the intracellular pathogen by macrophages depending on IFN-γ (37). In addition, IL-27 can impair the

protective immunity to *Mycobacterium tuberculosis* in mice, as WSX-1 deficient T cells accumulate more efficiently in the lesions, showing improved capacity to produce IL-2 and reduced expression of cell death markers (25), functions that may also be altered during *Leishmania* infection.

Altogether, our data demonstrate that IL-27 increases after *L. infantum* infection both in humans and in animal models. These results confirm the involvement of IL-27 in mice models of *Leishmania* visceral pathogenesis, limiting inflammation during the initial stages of the infection and favoring parasite persistence, suggesting that the presence of IL-27 early after infection could influence the host immune response and the progression of the disease. Finally, we would like to highlight that IL-27 has the potential to be a useful biomarker for active human VL and for treatment efficacy monitoring, independently of the etiological agent and the geographical region affected.

AUTHOR CONTRIBUTIONS

BP-C, RS, and AC-d-S conceived and designed the experiments; BP-C, PC, AR, and EC performed the experiments; BP-C, PC, RS, EC, JM, JVM, RV, and AC-d-S analyzed the data; JM and AC-d-S contributed with reagents/materials/analysis tools; and BP-C, PC, RV, and AC-d-S wrote the paper.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Institute for Health Metrics and Evaluation (IHME). *Age-Sex Specific All-Cause and Cause-Specific Mortality 1990-2013. Global Burden of Disease Study 2013*. Seattle, WA: GBD Publications (2014).
2. WHO. *Leishmaniasis Fact Sheet N°375*. (2015). Available from: http://www.who.int/mediacentre/factsheets/fs375/en/
3. Gollob KJ, Viana AG, Dutra WO. Immunoregulation in human American leishmaniasis: balancing pathology and protection. *Parasite Immunol* (2014) 36(8):367–76. doi:10.1111/pim.12100
4. Cecilio P, Perez-Cabezas B, Santarem N, Maciel J, Rodrigues V, Cordeiro da Silva A. Deception and manipulation: the arms of *Leishmania*, a successful parasite. *Front Immunol* (2014) 5:480. doi:10.3389/fimmu.2014.00480
5. Aparicio-Siegmund S, Garbers C. The biology of interleukin-27 reveals unique pro- and anti-inflammatory functions in immunity. *Cytokine Growth Factor Rev* (2015) 26(5):579–86. doi:10.1016/j.cytogfr.2015.07.008
6. Yoshida H, Hunter CA. The immunobiology of interleukin-27. *Annu Rev Immunol* (2015) 33:417–43. doi:10.1146/annurev-immunol-032414-112134
7. Ansari NA, Kumar R, Gautam S, Nylen S, Singh OP, Sundar S, et al. IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. *J Immunol* (2011) 186(7):3977–85. doi:10.4049/jimmunol.1003588
8. Dos Santos PL, de Oliveira FA, Santos ML, Cunha LC, Lino MT, de Oliveira MF, et al. The severity of visceral leishmaniasis correlates with elevated levels of serum IL-6, IL-27 and sCD14. *PLoS Negl Trop Dis* (2016) 10(1):e0004375. doi:10.1371/journal.pntd.0004375
9. Anderson CF, Stumhofer JS, Hunter CA, Sacks D. IL-27 regulates IL-10 and IL-17 from CD4+ cells in nonhealing *Leishmania major* infection. *J Immunol* (2009) 183(7):4619–27. doi:10.4049/jimmunol.0804024
10. Rosas LE, Satoskar AA, Roth KM, Keiser TL, Barbi J, Hunter C, et al. Interleukin-27R (WSX-1/T-cell cytokine receptor) gene-deficient mice display enhanced resistance to *Leishmania donovani* infection but develop severe liver immunopathology. *Am J Pathol* (2006) 168(1):158–69. doi:10.2353/ajpath.2006.050013
11. Barreto-de-Souza V, Ferreira PL, Vivarini Ade C, Calegari-Silva T, Soares DC, Regis EG, et al. IL-27 enhances *Leishmania amazonensis* infection via ds-RNA dependent kinase (PKR) and IL-10 signaling. *Immunobiology* (2015) 220(4):437–44. doi:10.1016/j.imbio.2014.11.006
12. Artis D, Johnson LM, Joyce K, Saris C, Villarino A, Hunter CA, et al. Cutting edge: early IL-4 production governs the requirement for IL-27-WSX-1 signaling in the development of protective Th1 cytokine responses following *Leishmania major* infection. *J Immunol* (2004) 172(8):4672–5. doi:10.4049/jimmunol.172.8.4672
13. Owens BM, Beattie L, Moore JW, Brown N, Mann JL, Dalton JE, et al. IL-10-producing Th1 cells and disease progression are regulated by distinct CD11c(+) cell populations during visceral leishmaniasis. *PLoS Pathog* (2012) 8(7):e1002827. doi:10.1371/journal.ppat.1002827
14. Smedsrod B, Pertoft H. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. *J Leukoc Biol* (1985) 38(2):213–30.
15. Neves BM, Silvestre R, Resende M, Ouaissi A, Cunha J, Tavares J, et al. Activation of phosphatidylinositol 3-kinase/Akt and impairment of nuclear factor-kappaB: molecular mechanisms behind the arrested maturation/activation state of *Leishmania infantum*-infected dendritic cells. *Am J Pathol* (2010) 177(6):2898–911. doi:10.2353/ajpath.2010.100367
16. Zal T, Volkmann A, Stockinger B. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp Med* (1994) 180(6):2089–99. doi:10.1084/jem.180.6.2089
17. Fujita H, Teng A, Nozawa R, Takamoto-Matsui Y, Katagiri-Matsumura H, Ikezawa Z, et al. Production of both IL-27 and IFN-gamma after the treatment with a ligand for invariant NK T cells is responsible for the suppression of Th2 response and allergic inflammation in a mouse experimental asthma model. *J Immunol* (2009) 183(1):254–60. doi:10.4049/jimmunol.0800520
18. Vasconcellos R, Carter NA, Rosser EC, Mauri C. IL-12p35 subunit contributes to autoimmunity by limiting IL-27-driven regulatory responses. *J Immunol* (2011) 187(6):3402–12. doi:10.4049/jimmunol.1100224
19. Silvestre R, Cordeiro-Da-Silva A, Santarem N, Vergnes B, Sereno D, Ouaissi A. SIR2-deficient *Leishmania infantum* induces a defined IFN-gamma/IL-10 pattern that correlates with protection. *J Immunol* (2007) 179(5):3161–70. doi:10.4049/jimmunol.179.5.3161
20. do Vale A, Afonso A, Silva MT. The professional phagocytes of sea bass (*Dicentrarchus labrax* L.): cytochemical characterisation of neutrophils and

macrophages in the normal and inflamed peritoneal cavity. *Fish Shellfish Immunol* (2002) 13(3):183–98. doi:10.1006/fsim.2001.0394
21. Resende M, Moreira D, Augusto J, Cunha J, Neves B, Cruz MT, et al. *Leishmania*-infected MHC class IIhigh dendritic cells polarize CD4+ T cells toward a nonprotective T-bet+ IFN-gamma+ IL-10+ phenotype. *J Immunol* (2013) 191(1):262–73. doi:10.4049/jimmunol.1203518
22. Maroof A, Kaye PM. Temporal regulation of interleukin-12p70 (IL-12p70) and IL-12-related cytokines in splenic dendritic cell subsets during *Leishmania donovani* infection. *Infect Immun* (2008) 76(1):239–49. doi:10.1128/IAI.00643-07
23. Zhu C, Zhang R, Liu L, Rasool ST, Mu Y, Sun W, et al. Hepatitis B virus enhances interleukin-27 expression both in vivo and in vitro. *Clin Immunol* (2009) 131(1):92–7. doi:10.1016/j.clim.2008.10.011
24. Liu L, Cao Z, Chen J, Li R, Cao Y, Zhu C, et al. Influenza A virus induces interleukin-27 through cyclooxygenase-2 and protein kinase A signaling. *J Biol Chem* (2012) 287(15):11899–910. doi:10.1074/jbc.M111.308064
25. Torrado E, Fountain JJ, Liao M, Tighe M, Reiley WW, Lai RP, et al. Interleukin 27R regulates CD4+ T cell phenotype and impacts protective immunity during *Mycobacterium tuberculosis* infection. *J Exp Med* (2015) 212(9):1449–63. doi:10.1084/jem.20141520
26. Novoa R, Bacellar O, Nascimento M, Cardoso TM, Ramasawmy R, Oliveira WN, et al. IL-17 and regulatory cytokines (IL-10 and IL-27) in *L. braziliensis* infection. *Parasite Immunol* (2011) 33(2):132–6. doi:10.1111/j.1365-3024.2010.01256.x
27. Tolouei S, Ghaedi K, Khamesipour A, Akbari M, Baghaei M, Hasheminia S, et al. IL-23 and IL-27 levels in macrophages collected from peripheral blood of patients with healing vs non-healing form of cutaneous leishmaniasis. *Iran J Parasitol* (2012) 7(1):18–25.
28. Costa DL, Cardoso TM, Queiroz A, Milanezi CM, Bacellar O, Carvalho EM, et al. Tr-1-like CD4+CD25-CD127-/lowFOXP3- cells are the main source of interleukin 10 in patients with cutaneous leishmaniasis due to *Leishmania braziliensis*. *J Infect Dis* (2015) 211(5):708–18. doi:10.1093/infdis/jiu406
29. Sandquist M, Wong HR. Biomarkers of sepsis and their potential value in diagnosis, prognosis and treatment. *Expert Rev Clin Immunol* (2014) 10(10):1349–56. doi:10.1586/1744666X.2014.949675
30. Quirino GF, Nascimento MS, Davoli-Ferreira M, Sacramento LA, Lima MH, Almeida RP, et al. Interleukin-27 mediates susceptibility to visceral leishmaniasis by suppressing the IL-17-neutrophil response. *Infect Immun* (2016) 84(8):2289–98. doi:10.1128/IAI.00283-16
31. Wang RX, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* (2014) 20(6):633–41. doi:10.1038/nm.3554
32. Lipoldova M, Demant P. Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. *Nat Rev Genet* (2006) 7(4):294–305. doi:10.1038/nrg1832
33. Blahoianu MA, Rahimi AAR, Kozlowski M, Angel JB, Kumar A. IFN-γ-induced IL-27 and IL-27p28 expression are differentially regulated through JNK MAPK and PI3K pathways independent of Jak/STAT in human monocytic cells. *Immunobiology* (2014) 219(1):1–8. doi:10.1016/j.imbio.2013.06.001
34. Zhang S, Liang R, Luo W, Liu C, Wu X, Gao Y, et al. High susceptibility to liver injury in IL-27 p28 conditional knockout mice involves intrinsic interferon-γ dysregulation of $CD4^+$ T cells. *Hepatology* (2013) 57(4):1620–31. doi:10.1002/hep.26166
35. Huang L, Hinchman M, Mendez S. Coinjection with TLR2 agonist Pam3CSK4 reduces the pathology of leishmanization in mice. *PLoS Negl Trop Dis* (2015) 9(3):e0003546. doi:10.1371/journal.pntd.0003546
36. Kim HS, Go H, Akira S, Chung DH. TLR2-mediated production of IL-27 and chemokines by respiratory epithelial cells promotes bleomycin-induced pulmonary fibrosis in mice. *J Immunol* (2011) 187(8):4007–17. doi:10.4049/jimmunol.1101654
37. Jung JY, Robinson CM. IL-12 and IL-27 regulate the phagolysosomal pathway in mycobacteria-infected human macrophages. *Cell Commun Signal* (2014) 12:16. doi:10.1186/1478-811X-12-16

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Malaria Parasites: The Great Escape

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Parasites of the genus *Plasmodium* have a complex life cycle. They alternate between their final mosquito host and their intermediate hosts. The parasite can be either extra- or intracellular, depending on the stage of development. By modifying their shape, motility, and metabolic requirements, the parasite adapts to the different environments in their different hosts. The parasite has evolved to escape the multiple immune mechanisms in the host that try to block parasite development at the different stages of their development. In this article, we describe the mechanisms reported thus far that allow the *Plasmodium* parasite to evade innate and adaptive immune responses.

Keywords: malaria, vaccine, escape mechanism, antibodies, T cells, immunosuppression, antigenic polymorphism, antigenic variation

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INTRODUCTION

Malaria, a disease caused by *Plasmodium* parasites and transmitted by *Anopheles* mosquitoes, remains one of the most deadly diseases. There are six species able to infect humans, namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the zoonotic monkey malaria species *Plasmodium knowlesi* (1) and *Plasmodium cynomolgi* (2). Around 300 million cases of clinical malaria are recorded every year. Approximately, half a million deaths occur in Africa and are mainly due to *P. falciparum* infections (3).

Plasmodium parasites have a complex life cycle. It starts when sporozoites are inoculated into the dermis of the mammalian host by infected mosquitoes. Sporozoites are highly motile and a majority of them migrate from the skin to the capillaries for dissemination by the bloodstream (4, 5). They are retained in the liver where they transmigrate through Kupffer cells and hepatocytes before seeding in final hepatocytes (6, 7). Depending on the species of their mammalian hosts, sporozoites mature in 2–14 days. At maturity, budding vesicles called merosomes are released and are ruptured in the lung circulation where the merozoites are released, ready to infect red blood cells (RBC) (8). During the erythrocytic cycle, a fraction of parasites differentiates into male and female gametocytes which can be taken up during the feeding of an *Anopheles* mosquito. In the mosquito midgut, ookinetes, generated by the gametocyte fusion, cross the mosquito midgut wall and develop into oocysts. Sporozoites released from mature oocyst then migrate to the salivary gland, ready for the next round of infection during the mosquito's next blood meal.

Malaria is a disease characterized by fever, headache, chills, sweating, and vomiting (9). Unlike viral or bacterial infections, the main indication of clinical malaria is the recurrent fever which varies between species. This is due to the release of parasite toxins into the bloodstream during the erythrocytic cycles of merozoite egress and reinvasion of erythrocytes. As the infection progresses, the number of RBC decreases and this may lead to severe anemia (10). In addition, RBC containing parasites such as *P. falciparum* can also sequester in deep tissues by cytoadhering to endothelial cells (11). This may cause organ failure, and is partly responsible for cerebral malaria. All these pathologies can eventually lead to death (9, 12, 13).

In the last two decades, the mortality to malaria has decreased substantially (3). This results from the combined efficacy of prevention measures, such as the use of insecticide-impregnated bednets, the development and use of rapid and easy to use diagnostic tools, and the potent artemisinin-based combinations therapies against the malaria parasites (14). However, this gain of lives might be temporary. In the recent years, all these interventions have shown some limitations. With the advent of decreased efficacy of artemisinin (15, 16), it is now clear that new drugs and other interventions should be developed (9, 17, 18). New drug families, such as spiroindolones (19, 20) and imidalopiperazines (21, 22) compounds, have shown promising results in phase II clinical trials in the recent years and have a great future ahead. However, a vaccine would be the most important tool in the armamentarium against malaria.

While vaccines have been readily developed for many bacterial and viral infections, there are currently no vaccines to protect against human parasites. The need to develop a vaccine to protect against malaria has been highlighted as early as the identification of the parasite in 1897 (23). There have been two schools of thought for the development an antimalarial vaccine. The first is based on the fact that naturally acquired immunity is often observed under field conditions. However, this immunity requires long period of time to develop. It first targets the disease and then the parasite (24). This immunity has been called premonition or relative immunity. It has been defined by Edmond Sergent in 1935 as "*a special type of immunity connected with the persistence of living germs in the organs of the immunized host*" (25). In other words, immunity is maintained as long as the host immunity is stimulated by the continuous or repeated parasite exposure. Understanding the mechanisms responsible for this premonition will help to develop a vaccine.

The other approach is based on the Jenner principle of vaccination, which was further exemplified by Louis Pasteur. Instead of letting Nature takes its course, this approach uses an inoffensive target as a formulation to induce an immune response in healthy individuals to protect against a subsequent infection. It might not be surprising, that the first attempt was reported by the same Sergent mentioned above, who was working in the Pasteur Institute in Algiers in Algeria. Sergent was able to partially protect birds from *Plasmodium lophurae*, an avian malaria parasite, using inactivated sporozoites (26). Decades later, in 1946, the first attempt in human was done by Heidelberger et al. using formalin-inactivated *P. vivax*-infected blood to immunize volunteers, however no protection was induced (27, 28). Jules Freund took it one step further by inventing the Freund adjuvant and combining the adjuvant with formalin-inactivated-blood infected with *P. lophurae* or *P. knowlesi*, a monkey malaria parasite. Freund was more successful in protecting ducks or rhesus monkeys, respectively (29, 30). However, because of its toxic side effects, the adjuvant could not be used in humans. Since then, various approaches and technologies have been used for vaccine development against the malaria parasites. Purified parasite preparation has been used as immunogens. Peptides, recombinant proteins, DNA plasmids, bacterial and viral vectors, and genetically modified malaria parasites, in combination with new adjuvants, have also been used as vaccine delivery systems (31). However, despite having more than thousands of pre-clinical trials in rodent and monkey models and more than 200 trials in humans, very few vaccine candidates have shown vaccine efficacy in human. The subunit vaccine, RTS,S, a chimeric molecule based on a large part of the circumsporozoite (CS) protein, the major surface protein of the sporozoite, fused to the S antigen of the hepatitis B virus, together with a strong adjuvant in the formulation, is the only candidate that has moved to Phase III clinical trials. However its efficacy was at best ~50% against infection or clinical disease (32, 33). So far, only whole-parasite based approaches have repeatedly shown high efficacy (34–36).

MECHANISMS OF IMMUNITY AGAINST THE MALARIA PARASITE

The parasite has a complex life cycle. Depending on the stage of development in its mammalian host, the parasite can be extracellular or intracellular. The parasite exists in different forms and shapes, possibly expressing different sets of its ~ 5000 gene pool (37) at a particular time. The parasite also has different localizations during development, infecting different cell types. Hence, various innate and adaptive immune mechanisms are involved in parasite control and elimination (38, 39). Thus, for any vaccine development, it is important to know the protective immune mechanisms to induce.

Immunity to the Pre-Erythrocytic Stage

During the pre-erythrocytic stage, antibodies can (i) inhibit sporozoite motility in the dermis or in the liver (40), (ii) bind to sporozoite and facilitate phagocytosis by monocytes or macrophages in the spleen or the liver (41), (iii) block sporozoite invasion into hepatocytes by preventing the sporozoite ligand to interact with the hepatocyte receptor(s) (42), and (iv) inhibit sporozoite development inside the hepatocytes (42). Antibodies can also recognize parasite neo-antigens such as heat-shock protein expressed at the surface of infected hepatocytes and induce liver parasite killing through an antibody-dependent cell-mediated mechanism likely involving Kuppfer cells or NK cells (43). The production of high levels of antibodies is dependent of CD4 T cell help, preferably by recognizing (an) epitope(s) present in the sporozoite to facilitate boosting of the antibody during natural infection with the parasite. When the parasite is inside the hepatocytes, it can become the target of CD4 or CD8 T cells (44–46). Hepatocytes express MHC Class I and Class II molecules that can be loaded with parasite antigen-derived epitopes following the TAP or the endosomal pathways (44–47). T cells kill the parasite either directly or through the action of cytokines, such as IFN-γ. IFN-γ induces the inducible nitric oxide enzyme to produce nitric oxide which directly kills the liver parasites (48–51). Innate immune mechanisms involving type I interferon pathway induced by the parasite infection and active against late schizonts or against reinfection have been recently uncovered (52–54).

Immunity against the Erythrocytic Stage

Adaptive immunity against the blood stage is more complex than in the liver stage. Merozoite-specific antibodies can (i) prevent

merozoites from invading RBC alone (55–57) or, in conjunction with complement factors, (ii) prevent merozoite egress from RBC, (iii) agglutinate released merozoites, (iv) promote phagocytosis of merozoites, and (v) facilitate clearance of infected RBC (iRBC) by phagocytic cells through a mechanism called antibody cell-dependent inhibition (ADCI) (58). In ADCI, anti-merozoite cryophilic (IgG1 or IgG3) antibodies bind to merozoites and the immune complexes stimulate phagocytes to release cytokines such as TNF-α, which in turn stimulate the phagocytes to produce mediators that lead to the killing of intra-erythrocytic parasites (59, 60). *Plasmodium* parasites express antigens at the surface of iRBC (61). These antigens are mainly encoded by multigene families such as the *var* (62), *stevor* (63), and *rifins* gene families (64, 65) for *P. falciparum* or the *pir* gene family for *P. vivax*, *P. knowlesi*, and the rodent malaria species (66). The antigens they encoded have been implicated in the cytoadherence phenotype to endothelial cells in deep tissues in order to avoid splenic clearance (67, 68). They are also involved in other adhesive phenomena, such as rosetting (the binding of an iRBC to non-infected RBC) (69–72) and agglutination (the binding to iRBC to iRBC through bridging by platelets) (73, 74). The cytoadherence abilities of the malaria parasites have been proposed to be responsible for some of the pathologies during malaria infection. Antibodies targeting the surface antigens are thought to act through preventing cytoadherence, promoting iRBC phagocytosis, or iRBC agglutination (74, 75).

Antibodies targeting the parasite toxins could also protect from disease. During infection, multiple parasite toxins are released at the time of iRBC rupture. These toxins include the malaria pigment, a by-product of heme degradation by the parasite (76–78), glycophosphatidylinositol (GPI) moieties that are present in many merozoite proteins, a TatD-like DNase (79), a tyrosine-tRNA synthase (80), or lipids extracted from *P. vivax* schizonts (81). Protection from disease by anti-toxins antibodies has been demonstrated experimentally using synthetic glycans mimicking GPI (82).

T cells are also critical effectors in the immunity against blood-stage malaria infections, despite the lack of MHC antigens on the surface of iRBC. First and foremost, blood-stage parasite-specific antibodies secreted by B cells depend on $CD4^+$ T helper cells enhancement for optimal production (39). Cytokines released by CD4 T cells are important for multiplication and maturation of B cells. The cytokines produced by malaria-specific T cells influence the isotype of the antibodies produced (83, 84) and thus possibly affecting the type of antibody-mediated responses induced. It has been shown that ADCI against *P. falciparum* is mediated by human IgG3 (induced by a Th1 response) and antagonized by IgG2 and IgG4 (induced by a Th2 response) (59, 85). Inducing the right isotype is thus important for an antibody-based vaccine. Recent studies have shown that inducing an immune response skewed toward the IgG3 could be achieved through the use of the right adjuvant (86). CD8 T cells were once thought to have only a minimal role in blood-stage immunity (87, 88). However, there is now evidence that these cells can inhibit blood-stage infection (89, 90). In particular, IFNγ-secreting CD8 T cells are important for preventing chronic blood-stage infection in mice (91).

Immunity against the Sexual Stages

The sexual forms of *Plasmodium* parasites, gametocytes, are also targets of the immunity against the disease. They are targeted by antibodies which can induce complement-mediated killing of the gametocytes in the host blood (92, 93). In the mosquito, antibodies can (i) prevent gamete fusion (94), (ii) induce complement-killing of gametes or ookinetes (95), and (ii) prevent ookinete motility, penetration of the midgut wall and formation of oocyst (96–99). Sexual-stage parasite-specific antibodies depend on CD4 T helper cells for optimal production. However, although antibodies specific for gametes or ookinetes can be produced by vaccination, the humoral immune response cannot be boosted by repeated infections since these forms are not present during infection in the mammalian hosts. Monocytes/macrophages stimulated during infection by the parasites produce cytokines such as TNF-α which in turn stimulates the monocytes/macrophages to produce nitric oxide, a potent inhibitor of gametocytes (100).

MECHANISMS OF IMMUNE EVASION

To avoid being eliminated by the host immunity, the parasite has developed many escape strategies (**Table 1**).

EVASION OF THE COMPLEMENT SYSTEM

The complement system is one of the first innate immune defense mechanisms against pathogens. Many proteins are involved in the activation or the regulation of its lytic activity (101). During the blood-stage, the parasite has developed multiple ways of evading the action of complement. *P. falciparum* merozoites and iRBC bind to the factor H (fH), a complement regulator factor, and its alternatively spliced form fH-like protein 1 through its surface molecule Pf92. In the mosquito, gametes bind the fH through PfGAP50 (102). In both situations, this allows protection against the activation of complement-mediated lysis. In addition, ookinetes express Pfs47, which disrupts the c-Jun N-terminal kinase pathway and prevents mosquito midgut epithelial nitration, making the parasite undetectable by complement system (103).

ANTIBODY-DEPENDENT ENHANCEMENT OF INFECTION

Antibody-dependent enhancement of infection was first described for viruses (104). Similarly, antibody-dependent enhancement of infection has been described for all stages of parasite development in the mammalian host. It was first reported in the early 1990s that antibodies against the repeat region of the CS protein enhance sporozoite entry and development in hepatocytes for both rodent (42, 43) and human parasites (105). Antibodies against the *P. falciparum* asparagine-rich protein enhance merozoite invasion of RBC *in vitro* (106). Merozoite-specific antibodies in conjunction with complement can also facilitate RBC invasion (107). Antibody-dependent enhancement of infection was also observed *in vivo* after immunization with a *Plasmodium berghei* parasite blood-stage preparation

TABLE 1 | Host immunity and parasite immune escape strategies.

Host immunity	Parasite stage	Parasite evasion strategy	Outcome
Complement	AS, S	Bind complement regulatory factor, factor H Disrupt c-Jun kinase pathway	Prevent complement activation system Avoid recognition by complement
Monocytes/macrophages	AS	Subvert or kill phagocytes by (1) inhibiting phagocytosis and (2) inducing apoptosis	Prevent parasite elimination by (1) inhibiting phagocyte function and (2) reducing phagocyte numbers
Dendritic cells (DC)	AS	Subvert or kill DC by (1) inhibiting DC maturation and (2) engaging apoptosis receptor, Fas	Decrease DC functions by (1) preventing T cell priming and expansion and (2) inducing immunosuppression through decreased pro-inflammatory (IL12) cytokine production and increased immunosuppressive (IL10) cytokine production
Antibodies/B cells	Spz, AS, S	Antibody enhancement invasion and/or growth	Expansion of parasite in host
	Spz, AS, S	Antigen polymorphism	Avoid recognition by antibody
	AS	Antigenic variation	Avoid recognition by antibody
	AS	Antigenic diversion	Prevent the action of neutralizing antibodies
	AS	Epitope masking	Prevent the action of neutralizing antibodies
	AS	Smoke-screen strategy	Avoid antibody recognition by diverting neutralizing antibody from their target
	AS	B cell dysregulation	Poor or limited B cell memory
	Spz, AS, S	Homology with host proteins	Poor or no antibody response by inducing immunological tolerance
	AS	B cell apoptosis	Poor antibody response
	AS	Redundancy in cell invasion pathways	Allow continued invasion and expansion of parasite even when one invasion pathway is inhibited by antibody
T cells	LS	T cell epitope polymorphism	Avoid T cell recognition prevent T cell priming and activity, and interfere with memory T cell development
	AS	Apoptosis	Poor T cell response
	AS	Induction of expression of check-point inhibitors	Anergy and/or T cell exhaustion
	AS	Regulatory T cells	Negative regulation of immune responses
Hepatocytes	LS	Cellular shelter	Avoid immune surveillance due to intracellular niche

Spz, sporozoite; LS, liver stage; AS, asexual blood stage; S, sexual stage.

which led to increased death after challenge (108). In the sexual stage, anti-gamete antibodies were shown to enhance transmission to the mosquito (109).

Antibodies against proteins expressed in one stage of the parasite's development might mediate an enhancement effect in another stage of the parasite's development. This was observed for antibodies against two antigens, the CS protein and heat-shock protein 1 (HSP-1) (110). The CS protein is expressed during sporogony in the mosquito and in the subsequent sporozoite stage. Do Rosario et al. showed that sporozoites generated during sporogony in the presence of anti-CS protein antibodies cannot be inhibited by human polyclonal anti-CS (sporozoite CS) protein (111). HSP70-1 is expressed by the liver stage and the blood stages (43, 110). HSP70-1 antibodies have no effect against the blood-stage parasites but they can partially mediate inhibition of liver-stage development through ADCI (43). However, vaccination with a recombinant protein against the C-terminal fragment of HSP70-1 increased the number of gametocytes generated and the subsequent transmission to mosquito (112). Thus, it was proposed that antibodies against the liver-stage parasites can drive merozoites to differentiate into gametocytes and facilitate transmission (112). Immune cross-talk between the different stages of the infection has not been extensively studied beyond the two reports mentioned above. This cross-talk has wider implications. Indeed vaccination against one stage may influence the subsequent stages positively or negatively. For any vaccine against the pre-erythrocytic or blood stages, there will be a need for follow-up studies on the effect on transmission and development of the parasite in the mosquitoes.

ANTIGENIC POLYMORPHISM

Similar to all other organisms, the parasite is prone to mutation since the replicative machinery is not error free. In its mammalian host, the parasite is haploid and the mutation rate is ~$1–0.7 \times 10^{-9}$ mutations per base per generation (113). Since the *Plasmodium* parasites have a 24- to 72-h blood cycle, there is a high probability that mutation can occur and generate different parasite clones. In the mosquito host, the parasite undergoes sexual reproduction where two haploid gametocytes will generate four haploid sporozoite progenies. Recombination does occur in the mosquito stage, and, thus, it will increase the occurrence of gene polymorphism. For some antigens, hundreds of haplotypes have been observed (114). Polymorphism in the coding sequence can be due to point mutations, insertions, or deletions. Interestingly, many *Plasmodium* antigens possess regions of repeats which can vary in size and number of repetitive units (115). Such diversity is, in most cases, the result of immune pressure since mutations often occur in regions which can be recognized by antibodies or T cells. Mutations in B epitopes abolish the recognition of

parasites by antibodies and may lead to the selection of parasites with a different haplotype. This is important for vaccine development since antibody-based vaccine formulation targeting polymorphic epitopes may either have limited efficacy or select for vaccine-resistant parasite. One such example is the vaccine based on the AMA-1 protein, a highly polymorphic antigen (116–118). However, it is possible to envisage that immunogen(s) inducing broadly inhibitory antibodies recognizing multiple variants may circumvent polymorphism. Studies to identify structural conserved constraints in different variants may pave the way for new vaccines against polymorphic antigens (56).

Polymorphism in T cell epitopes may have profound consequences in T cell responses and have shown to limit the efficacy of the RTS,S vaccine (119). T cell epitopes are, in general, 8–11 amino acid (aa) and 11–25 aa in length for CD8 T cell and CD4 T cells, respectively (120). After the digestion of parasite protein in the cytoplasm, CD8 antigenic peptides are generated and transported to endoplasmic reticulum by the transporter antigenic peptide protein in the endoplasmic reticulum where they are loaded onto MHC class I molecules and beta 2-microglobulin. CD4 epitopes are generated in endosomes after phagocytosis and protease digestion where they meet MHC class II. Peptide–MHC complexes are then expressed in the surface. Exogenous antigens can also be presented by cross-presentation by MHC class I (121). These complexes can be recognized by the T cell receptor (122). Mutations in the amino acids that anchor the epitope to the MHC grove can prevent binding of the peptides to the MHC or recognition by the TCR and thus abrogate T cell activity (123, 124). This was demonstrated for the CS protein (125). Altered peptide ligand (APL) can still bind to the MHC molecules; however, they prevent T cell proliferation, no matter if it was used singly or concurrently with a wild-type peptide. They can also prevent cytokine production (126) or change their production pattern, i.e., from IFN-γ to the immunosuppressive cytokine interleukin-10 (127). These APL can also interfere with induction of memory T cells from naïve T cells (128). This potent mechanism of immune escape is a major hurdle for vaccine development. For any T cell-based vaccine to succeed, a thorough analysis of T cell polymorphism and their effect should be performed.

ANTIGENIC VARIATION

Antigenic variation was first described by Neil Brown during *P. knowlesi* chronic relapsing infection in Rhesus monkeys induced by sub-curative drug treatment (129). In an agglutination assay, antibodies produced against different relapse parasite broods agglutinated only schizonts of the immunizing brood but not the other broods. It was further shown that antibodies (130) and spleen could induce antigenic variation in Rhesus monkey infected with a cloned line of *P. knowlesi* (67) or monkey infected with cloned lines of *P. falciparum* (131). The latter experiments suggested that, contrary to antigenic polymorphism where parasites have different alleles and can be categorized as genomic clones, antigenic variation is a phenotypic variation occurring with the same genomic clone of the parasite. The molecular basis of this phenomenon was elucidated with the sequencing of large segments of the *P. falciparum* and *P. knowlesi* genomes and the subsequent complete sequencing of the genome of many *Plasmodium* species. Antigens prone to antigenic variation are often expressed on the surface of iRBC. Examples includes the multigene family, such the *var* genes (62), the *stevor* gene family, the *rifin* gene family (63), the *surfin* gene family (132), the *sicavar* gene family (133), and the *Plasmodium* interspersed repeats (*pir*) genes. The *var* gene family is the most studied and has been shown to be the target of protective anti-blood-stage antibodies (134, 135). It is also involved in many of malaria pathologies due to parasite sequestration resulting from iRBC cytoadherence (136). PfEMP1 proteins, encoded by the *var* gene family, are highly polymorphic and have different variable domains, called Duffy binding-like domain, which determine their binding specificities to various ligands on endothelial cells such as thrombospondin (137), CD36 (138), ICAM-1/CD54 (139), chondroitin sulfate A (140–142), VCAM-1, E-selectin (143), αvβ3 integrin (144), hyaluronic acid (145), PECAM-1/CD31 (146), gC1qR/HABP1/p32 (147), or endothelial protein C receptor (148). PfEMP-1 proteins bind also to RBC through complement receptor 1 to form rosettes (149). *P. falciparum* parasites possess ~60 var genes distributed over the 14 chromosomes of the parasite (37). *Var* genes expression is extremely regulated and only one PfEMP1 type is produced and displayed on the surface of iRBC (150). At each cycle, the parasite switches the expression of the *var* gene at a rate of 2% *in vitro*, generating new clones with new antigenic and adhesive phenotype (151, 152). Vir proteins, members of a superfamily of the *pir* multigene superfamily, also mediate cytoadherence of *P. vivax* iRBC to endothelial cells (153), resulting to sequestration of mature iRBC (154). There are ~350 *vir* genes, and they are also highly polymorphic (155, 156). Stevor and Rifin proteins are involved in cytoadherence processes and are also highly polymorphic (70, 71). In the recent years, it has become clear that some members of these multigene family has a particular role and may be involved in certain pathology. As example, *var2*CSA mediated the specific cytoadherence of iRBC to placenta (142) and is associated with placental pathology, such as still birth and fetus growth retardation Vaccination using immunogens based on *var2*CSA will thus induce inhibitory antibodies, preventing cytoadherence (157, 158) and placental sequestration. Thus defining the role of members of these multigene families may lead to tailored immune intervention.

ANTIGENIC DIVERSION

Antigenic diversion occurs when non-inhibitory anti-parasite antibodies prevent the action of inhibitory antibodies. Antigenic diversion has been observed with the merozoite surface protein (MSP)-1. MSP-1 is a surface protein which binds to glycophorin A, a molecule present on the surface of RBC, and thus is essential for merozoite invasion (159, 160). MSP-1 is cleaved at the time of invasion. Neutralizing antibodies, which block the proteolytic cleavage in the C-terminal part ($MSP\text{-}1_{19}$) of the protein (antibodies), can prevent invasion (152, 161). However, antibodies against the adjacent or overlapping region can block the effect of $MSP1_{19}$-inhibitory antibodies (blocking antibodies) and thus allowing invasion to occur (162). One possibility to overcome this

phenomenon is to design immunogen(s) that either induce(s) neutralizing but not blocking antibodies in naïve individuals or tip the balance to greater production of neutralizing antibodies over blocking antibodies in naturally exposed individuals. So far, there has been limited success with MSP-1 (159, 163).

EPITOPE MASKING

Epitope masking is the capacity of non-parasite-specific antibodies to prevent parasite-specific inhibitory antibodies to react with their epitopes. During the establishment of an antibody response, IgM precedes IgG production and thus are the first line of humoral response either alone or in combination with complement (164). Malaria-specific IgM have been shown to have efficient inhibitory activity against sporozoite and iRBC (165). However, IgM with different specificities can also bind to PfEMP-1 molecules expressed at the surface of iRBC through their Fc portion (Fc) (166). Non-parasite-specific IgM (NpsIgM) promote rosetting and thus may facilitate sequestration, in order to avoid splenic elimination. NpsIgM binding to the PfEMP-1 VARCSA2, which is involved in the binding of iRBC to chondroitin sulfate in the placenta, appears to protect iRBC from phagocytosis mediated by IgG (167). NpsIgM also binds to the MSP DBLMSP and DBLMSP2 and prevents IgG binding to these molecules by masking epitopes (168). The role of these two molecules in merozoite biology is still unclear but non-specific IgM epitope masking may be important for protecting the parasite against a specific IgG inhibitory response.

SMOKE-SCREEN STRATEGY BY CROSS-REACTIVITY

Smoke-screen strategy is often used by the parasite to divert the antibodies specific for one antigen (antigen A, e.g., an epitope of inhibitory antibodies) to react against another antigen (antigen B, e.g., an epitope of non-inhibitory antibodies) which possess regions homologous to antigen A. Antibody reactivity to segments shared by the two proteins will decrease the amount of antibodies reacting to antigen A and thus reducing efficacy of inhibitory antibodies that can inhibit parasite invasion or development. Many *Plasmodium* antigens contain repeats, and it was shown that cross-reactivity can occur between different blocks of repeats (169). Cross-reactivity has been observed: (i) between different epitopes from the same block of repeats [i.e., within the same block of repeats in the CS protein, or Ring-erythrocyte surface (RESA), or S-antigen or falciparum interspersed repeat antigen (FIRA)]; (ii) between epitopes present in different blocks of the same antigen (i.e., RESA or FIRA), between repeats of different antigens [between the CS protein and the cross-reactive antigen (CRA), between the histidine-rich proteins]. So far, the importance of this mechanism in the immune response evasion has remained uncertain.

DYSREGULATION OF B CELLS

Atypical memory B cells are a population of hypo-responsive memory cells which have been first described in chronic HIV infections (170). They increase in numbers during chronic exposure to malaria and are poor responders to antigen restimulation (171). However, they are able to produce neutralizing antibodies (172). These cells might be important during repeated stimulation due to constant exposure and help to control parasite density. However, in regions with lower exposure or in the absence of reinfection, immunity might wane rapidly.

As mentioned in the previous section, many *Plasmodium* antigens contain repeats. Similar to many viral antigens (173) and haptenated polymers (174), repeats-containing *Plasmodium* antigens can stimulate B cell independently of T cell help. However, this often leads to the production of mainly IgM and limited B cell memory (175). In addition, exposure to antigens containing repeated motifs can result in the suppression of an ongoing T cell response (176, 177).

HOMOLOGIES WITH HOST PROTEINS

Many *Plasmodium* antigens involved in the invasion of host cells have regions which strong homologies with host proteins involved in protein–protein interactions. Thrombospondin type-1 repeat (TSR) domains and von Willebrand factor (vWF)-like A domains are present in the CS protein, thrombospondin-related anonymous protein (TRAP) (178, 179), CS protein TRAP-related protein (CTRP) sporozoite surface protein (180), secreted protein with an altered thrombospondin (SPATR) (181), and thrombospondin-related apical merozoite protein (TRAMP) (182). These molecules are involved in different stages of parasite development, in sporozoite and merozoite motility, and in invasion of mosquito midguts, salivary glands, hepatocytes, and RBC. Many merozoite proteins such as MSP-1, MSP-4, MSP-5, MSP-8, and MSP-10 (183), PfRipr (184) and sexual-stage protein, such as P25 or P28 (185, 186), have been found to contain an epidermal growth factor domain that is involved in its binding to their receptor. Due to the homology to the host protein, the induction of antibodies to epitopes contained in these homologous regions is difficult since the host is tolerized against its own proteins. Immunization with immunogens containing these motifs with strong adjuvants could possibly escape immunological tolerance but may have the risk of inducing auto-immunity.

IMMUNOSUPPRESSION

Myeloid cells are essential mediators of an efficient immune response against the malaria parasites. Monocytes, macrophages, and neutrophils phagocytose iRBC, which eventually leads to the elimination of the parasites (187). Phagocytosis by monocytes/macrophages can be mediated through the interactions of PfEMP-1 and CD36 without inducing or increasing a protective pro-inflammatory response (188). However, during infection, phagocyte functions can be diminished after the ingestion of the malaria pigment or hemozoin (the digested product of hemoglobin by the parasites) (189, 190). Pigment-loaded macrophages cannot phagocytose more iRBC, and their capacity to generate radical oxygen intermediates is also reduced (191).

Dendritic cells (DCs) are essential to induce adaptive immune responses (192). Engagement of CD36 and CD51 (the αv integrin

chain) on DCs by PfEMP-1-expressing parasites impair DC maturation and its capacity to prime T cell responses, leading to reduced production of pro-inflammatory cytokines such as IL-12 and increased production of immunosuppressive cytokines such as IL-10 (193).

Acute blood-stage infection induces a strong activation of mononuclear cells. This can result in apoptosis in monocytes, B cells, T cells, and DCs (194–199). Acute infection can also lead to thymus apoptosis and depletion, thereby diminishing the output of new naïve T cells (200). On the other hand, chronically activated T cells (both αβ and γδ) during lingering blood infection or multiple reinfections can also enter a phase of anergy (201–203) and/or exhaustion (204). *Plasmodium* infection activates check-point inhibitors molecules such as program cell death protein 1 (PD-1), program death ligand 1 (PD-L1), PD-L2, lymphocyte-activation gene 3 (LAG-3), and cytotoxic T lymphocyte antigen-4 (CTLA-4) in CD4 and CD8 T cells. In rodent models, blockade of these molecules facilitates the elimination of blood parasites (91, 205, 206) and the establishment of T cell memory (207). Regulatory T cells often expand during infection (208). Their principal role is to control the excessive pro-inflammatory response which depending on the parasite species may be beneficial or detrimental (209–211). Taken together, these mechanisms leading to immunosuppression are likely to favor survival of the parasite in the host and prevent the establishment of an efficient memory response. In addition, while it might also be beneficial for the host to prevent immunopathogenesis (212), ensuring host survival is advantageous to parasite survival.

REDUNDANT PATHWAYS OF RED BLOOD CELL INVASION

Invasion of RBC by merozoites is a complex process. It involves a precise and coordinated expression of different sets of proteins which are either expressed on the surface of the merozoite or in the organelles such as rhoptries, micronemes, and dense granules located at the apical end of the merozoite (183). The invasion process has been amply studied for *P. falciparum*. It was shown that the parasites can use multiple pathways involving at least nine ligand/receptor combinations to invade a RBC. There are two parasite protein families involved in the host-cell selection and invasion process; the reticulocyte-binding protein homologs (Rh) and the erythrocyte binding-like proteins (EBL) (213). These proteins are polymorphic, and their expression varies depending on parasite clones. Polymorphisms in a *P. falciparum* EBL have been shown to result in a change in their receptor specificity (214) or in a switch to Rh-dependent invasion, a different invasion pathway for RBC invasion (215). Variation in the use of erythrocyte invasion pathways results in evasion of human inhibitory antibodies (216). Thus, this suggests that vaccines targeting only one pathway will select parasite using other pathways. However, so far, the pathway involving Rh5 and its ligand Basigin/CD147 has been shown to be required for all parasites clones and isolates tested so far and has shown limited polymorphism (217). It thus represents a promising vaccine candidate (218, 219).

Contrary to *P. falciparum*, *P. vivax* was thought to use only one pathway to invade RBC (220). *P. vivax* merozoites only invade reticulocytes, the immature RBC. Since people in West Africa or with West African origins naturally lack the Duffy antigen, they are resistant to *P. vivax* blood infection (221, 222). This serves as a strong argument for the existence of only one invasion pathway for *P. vivax*. However, in the recent years, many studies have reported *P. vivax* infections in Duffy-negative patients (220, 222–228), suggesting that *P. vivax* merozoites may use another pathway to invade reticulocytes (229). This may limit the efficacy of vaccine based on the Duffy-binding protein, the ligand of the Duffy antigen (230), by selecting for parasites that are able to invade *via* this alternative pathway.

SHELTERS

Infection with sporozoites from some human/primate parasites, such as *P. vivax*, *P. ovale*, *P. cynomolgi*, *Plasmodium simiovale*, and *Plasmodium fieldi*, may lead to relapse (231). After a patent blood infection is completely cleared by the immune system or by drug treatment, blood parasites may reappear and induce new clinical attacks (232). This new infection originates from long-lasting liver forms, called hypnozoites (233). These non-dividing and metabolically active forms (234) can persist in the liver of their infected hosts for long period of time. Histological analysis on liver from infected rhesus monkeys did not show any signs of cellular immune responses against hypnozoites or late-developing schizonts originating from these forms (235, 236). This suggests that hypnozoites do not trigger any immune response, and do not lead to the expression of MHC molecules containing parasites-derived peptides. Due to the difficulty in studying these forms *in vitro* and *in vivo* (237), the mechanisms by which the parasite subverts immune recognition is unknown. Vaccine development against the pre-erythrocytic of *P. vivax* has been limited, and formulations targeting the liver forms may not work against the hypnozoites.

As mentioned above, during primary sporozoite-induced infection in natural hosts, no cellular immune responses are observed (8, 238–240). The liver is rich in macrophages and Kupffer cells, which can phagocytose emerging hepatic merozoites. To avoid being recognized by phagocytes, merozoites are released inside vesicles called merosomes in to the sinusoid lumen. Merosomes do not express macrophage-recognition signal such as phosphatidyl-serine and thus escape being phagocytosed (8). Merosomes are carried to the lung vasculature where blood circulation is low (241) before releasing their merozoite cargo (242).

Vesicles containing merozoites have been observed inside other cell types other than RBC, such as platelets (243), in macrophages (244), and in DCs (245, 246). It has been shown that merozoites can also divide in the DCs expressing CD317/tetherin, and eventually initiate new infections (245). It is not yet known how the phagocytosed parasites evade digestion or if a population of merozoites can infect and multiply in these cells. Free merozoite-containing vesicles, called merophores, have also been observed in the lymph circulation during rodent malaria infection (247, 248). This could explain recrudescence or latency of infection. However, as for the merosomes, these vesicles may be devoid of recognition signals for phagocyte uptake and may not express parasite antigens on their surface.

CONCLUSION

Malaria parasites have been interacting with its mammalian hosts for more than 150 million years (249) and have efficiently evolved to survive under the pressure of the host immune system. The parasite has developed numerous immune evasion mechanisms. Since new host immune mechanisms against the parasite are constantly being discovered, it will be of no surprise that new immune escape mechanisms by the parasite will be uncovered. For example, there is still little known on how innate immunity are induced during infection and on how protective epitopes are generated. The ultimate goal of understanding the immune responses to the malaria parasites is the development of vaccines. The selection of antigens and delivery system is governed by the target. Historically, before the whole genome was sequenced, the first malarial antigens, that were cloned and sequenced, have been assumed to be good vaccine candidates (250). However, all these antigens are immunodominant and are involved in immune escape. For vaccines to develop in a timely manner, the selection criteria should involve a more stringent GO-NO Go selection based on the analysis of the potential of the vaccine candidate to avoid immune escape. This also calls for an immunological approach to define correlates of protection to guide vaccine development. The development of the experimental human malarial challenge model, where complete sterile protection can be obtained (34, 35), is strong evidence that a vaccine against malaria can be obtained. Together with parasite genetics, the development of the rodent models and the experimental human challenge model would greatly assist in making the critical GO-NO Go decisions and facilitate the development of an efficacious vaccine against malaria in the foreseeable future.

AUTHOR CONTRIBUTIONS

Both the authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Millar SB, Cox-Singh J. Human infections with *Plasmodium knowlesi* – zoonotic malaria. *Clin Microbiol Infect* (2015) 21:640–8. doi:10.1016/j.cmi.2015.03.017
2. Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JM. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. *Malar J* (2014) 13:68. doi:10.1186/1475-2875-13-68
3. World Health Organisation. *World Malaria Report*. Geneva: World Health Organisation (2015).
4. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, et al. Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med* (2006) 12:220–4. doi:10.1038/nm1350
5. Amino R, Giovannini D, Thiberge S, Gueirard P, Boisson B, Dubremetz JF, et al. Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell Host Microbe* (2008) 3:88–96. doi:10.1016/j.chom.2007.12.007
6. Mota MM, Pradel G, Vanderberg JP, Hafalla JCR, Frevert U, Nussenzweig RS, et al. Migration of *Plasmodium* sporozoites through cells before infection. *Science* (2001) 291:141–4. doi:10.1126/science.291.5501.141
7. Tavares J, Formaglio P, Thiberge S, Mordelet E, van Rooijen N, Medvinsky A, et al. Role of host cell traversal by the malaria sporozoite during liver infection. *J Exp Med* (2013) 210:905–15. doi:10.1084/jem.20121130
8. Sturm A, Amino R, Van De Sand C, Regen T, Retzlaff S, Rennenberg A, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* (2006) 313:1287–90. doi:10.1126/science.1115257
9. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. *Lancet* (2013) 383:7223. doi:10.1016/S0140-6736(13)60024-0
10. Pathak VA, Ghosh K. Erythropoiesis in malaria infections and factors modifying the erythropoietic response. *Anemia* (2016) 2016:9310905. doi:10.1155/2016/9310905
11. Storm J, Craig AG. Pathogenesis of cerebral malaria-inflammation and cytoadherence. *Front Cell Infect Microbiol* (2014) 4:100. doi:10.3389/fcimb
12. Haldar K, Murphy SC, Milner DA, Taylor TE. Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease. *Annu Rev Pathol* (2007) 2:217–49. doi:10.1146/annurev.pathol.2.010506.091913
13. Milner DA Jr, Whitten RO, Kamiza S, Carr R, Liomba G, Dzamalala C, et al. The systemic pathology of cerebral malaria in African children. *Front Cell Infect Microbiol* (2014) 4:104. doi:10.3389/fcimb.2014.00104
14. Kanyuka M, Ndawala J, Mleme T, Chisesa L, Makwemba M, Amouzou A, et al. Malawi and millennium development goal 4: a countdown to 2015 country case study. *Lancet Glob Health* (2016) 4:e201–2124. doi:10.1016/S2214-109X(15)00294-6
15. Dondorp AM, Yeung S, White L, Nguon C, Day NP, Socheat D, et al. Artemisinin resistance: current status and scenarios for containment. *Nat Rev Microbiol* (2010) 8:272–80. doi:10.1038/nrmicro2331
16. White LJ, Flegg JA, Phyo AP, Wiladpai-Ngern JH, Bethell D, Plowe CV, et al. Defining the *in vivo* phenotype of artemisinin-resistant falciparum malaria: a modelling approach. *PLoS Med* (2015) 12:e1001823. doi:10.1371/journal.pmed.1001823
17. Canavati SE, Lawford HL, Fatunmbi BS, Lek D, Top-Samphor N, Leang R, et al. Establishing research priorities for malaria elimination in the context of the emergency response to artemisinin resistance framework-the Cambodian approach. *Malar J* (2016) 15:120. doi:10.1186/s12936-016-1117-9
18. von Seidlein LV, Dondorp AM. Fighting fire with fire: mass antimalarial drug administrations in an era of antimalarial resistance. *Expert Rev Anti Infect Ther* (2015) 13:715–30. doi:10.1586/14787210.2015.1031744
19. White NJ, Pukrittayakamee S, Phyo AP, Rueangweerayut R, Nosten F, Jittamala P, et al. Spiroindolone KAE609 for falciparum and vivax malaria. *N Engl J Med* (2014) 371:403–10. doi:10.1056/NEJMoa1315860
20. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell BM, et al. Spiroindolones, a potent compound class for the treatment of malaria. *Science* (2010) 329:1175–80. doi:10.1126/science.1193225
21. White NJ, Duong TT, Uthaisin C, Nosten F, Phyo AP, Hanboonkunupakarn B, et al. Antimalarial activity of KAF156 in falciparum and vivax malaria. *N Engl J Med* (2016) 375:1152–60. doi:10.1056/NEJMoa1602250
22. McNamara CW, Lee MC, Lim CS, Lim SH, Roland J, Nagle A, et al. Targeting *Plasmodium* PI(4)K to eliminate malaria. *Nature* (2013) 504:248–53. doi:10.1038/nature1278
23. Celli A, Santori FS. Intorno alla siero-profilassi della malaria. 1a comunicazione. *Annali d'Igiene Sperimentale* (1897) 7:183–90.
24. McGregor IA. Malarial immunity: current trends and prospects. *Ann Trop Med Parasitol* (1987) 81:647–56.
25. Sergent E, Parrot L. L'immunite, la premunition et la resistance. *Ann Inst Pasteur Algers* (1935) 13:279–315.
26. Sergent E. Sur l'immunite dans le paludisme des oiseaux. Conservation *in vitro* des sporozoites de *Plasmodium relictum*. Immunite relative obtenue par inoculation de ces sporozoites. *C R Acad Sci* (1910) 151:407–9.
27. Heidelberger M, Mayer MM, Alving AS, Craige BJ, Jones R Jr, Pullman TN, et al. Studies in human malaria. IV. An attempt at vaccination of volunteers against mosquito-borne infection with *Plasmodium vivax*. *J Immunol* (1946) 53:113–8.

28. Heidelberger M, Mayer MM, Demarest CR. Studies in human malaria. I. The preparation of vaccines and suspensions containing plasmodia. *J Immunol* (1946) 52:325–30.
29. Freund J, Sommer HE, Walter AW. Immunization against malaria: vaccination of ducks with killed parasites incorporated with adjuvants. *Science* (1945) 102:200–2. doi:10.1126/science.102.2643.200
30. Freund J, Thomson KJ, Sommer HE, Walter AW, Schenkein EL. Immunization of rhesus monkeys against malarial infection (*P. knowlesi*) with killed parasites and adjuvants. *Science* (1945) 102:202–4. doi:10.1126/science.102.2643.202
31. Halbroth BR, Draper SJ. Recent developments in malaria vaccinology. *Adv Parasitol* (2015) 88:1–49. doi:10.1016/bs.apar.2015.03.001
32. Kaslow DC, Biernaux S. RTS,S: toward a first landmark on the malaria vaccine technology roadmap. *Vaccine* (2015) 33:7425–32. doi:10.1016/j
33. Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, et al. Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. *N Engl J Med* (2013) 368:1111–20. doi:10.1056/NEJMoa1207564
34. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJF, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* (2009) 361:468–77. doi:10.1056/NEJMoa0805832
35. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science* (2013) 341:1359–65. doi:10.1126/science.1241800
36. Ishizuka AS, Lyke KE, DeZure A, Berry AA, Richie TL, Mendoza FH, et al. Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nat Med* (2016) 22:614–23. doi:10.1038/nm.4110
37. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* (2002) 419:498–511. doi:10.1038/nature01097
38. Stevenson MM, Riley EM. Innate immunity to malaria. *Nat Rev Immunol* (2004) 4:169–80. doi:10.1038/nri1311
39. Langhorne J, Ndungu FM, Sponaas AM, Marsh K. Immunity to malaria: more questions than answers. *Nat Immunol* (2008) 9:725–32. doi:10.1038/ni.f.205
40. Vanderberg JP, Frevert U. Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. *Int J Parasitol* (2004) 34:991–6. doi:10.1016/j.ijpara.2004.05.005
41. Schwenk R, Lumsden JM, Rein LE, Juompan L, Kester KE, Heppner DG Jr, et al. Immunization with the RTS,S/AS malaria vaccine induces IFN-gamma+ CD4 T cells that recognize only discrete regions of the circumsporozoite protein and these specificities are maintained following booster immunizations and challenge. *Vaccine* (2011) 29:8846–54. doi:10.1016/j.vaccine.2011.09.098
42. Nudelman S, Renia L, Charoenvit Y, Yuan L, Miltgen F, Beaudoin RL, et al. Dual action of anti-sporozoite antibodies in vitro. *J Immunol* (1989) 143:996–1000.
43. Renia L, Mattei DM, Goma J, Pied S, Dubois P, Miltgen F, et al. A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by nonparenchymal liver cells. *Eur J Immunol* (1990) 20:1445–9. doi:10.1002/eji.1830200706
44. Renia L, Marussig MS, Grillot D, Pied S, Corradin G, Miltgen F, et al. *In vitro* activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide. *Proc Natl Acad Sci U S A* (1991) 88:7963–7. doi:10.1073/pnas.88.18.7963
45. Renia L, Grillot DA, Marussig M, Corradin G, Miltgen F, Lambert PH, et al. Effector functions of circumsporozoite peptide-primed $CD4^+$ T cell clones against *Plasmodium yoelii* liver stages. *J Immunol* (1993) 150:1471–8.
46. Bongfen SE, Torgler R, Romero JF, Renia L, Corradin G. *Plasmodium berghei*-infected primary hepatocytes process and present the circumsporozoite protein to specific CD8+ T cells *in vitro*. *J Immunol* (2007) 178:7054–63. doi:10.4049/jimmunol.178.11.7054
47. Pichugin A, Steers N, De la Vega P, Zarling S, Chalom I, Krzych U. TAP-mediated processing of exoerythrocytic antigens is essential for protection induced with radiation-attenuated *Plasmodium* sporozoites. *Eur J Immunol* (2016) 46:885–96. doi:10.1002/eji.201545748
48. Mellouk S, Hoffman SL, Liu ZZ, de la Vega P, Billiar TR, Nussler AK. Nitric oxide-mediated antiplasmodial activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. *Infect Immun* (1994) 62:4043–6.
49. Nussler AK, Renia L, Pasquetto V, Miltgen F, Matile H, Mazier D. *In vivo* induction of the nitric oxide pathway in hepatocytes after injection with irradiated malaria sporozoites, malaria blood parasites or adjuvants. *Eur J Immunol* (1993) 23:882–7. doi:10.1002/eji.1830230417
50. Seguin MC, Klotz FW, Schneider I, Weir JP, Goodbary M, Slayter M, et al. Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated *Plasmodium berghei* infected mosquitoes: involvement of interferon-gamma and CD8+ T cells. *J Exp Med* (1994) 180:353–8. doi:10.1084/jem.180.1.353
51. Cockburn IA, Amino R, Kelemen RK, Kuo SC, Tse SW, Radtke A, et al. *In vivo* imaging of CD8+ T cell-mediated elimination of malaria liver stages. *Proc Natl Acad Sci U S A* (2013) 110:9090–5. doi:10.1073/pnas.1303858110
52. Liehl P, Meireles P, Albuquerque IS, Pinkevych M, Baptista F, Mota MM, et al. Innate immunity induced by *Plasmodium* liver infection inhibits malaria reinfections. *Infect Immun* (2015) 83:1172–80. doi:10.1128/IAI.02796-14
53. Liehl P, Zuzarte-Luis V, Chan J, Zillinger T, Baptista F, Carapau D, et al. Host-cell sensors for *Plasmodium* activate innate immunity against liver-stage infection. *Nat Med* (2013) 20:47–53. doi:10.1038/nm.3424
54. Miller JL, Sack BK, Baldwin M, Vaughan AM, Kappe SH. Interferon-mediated innate immune responses against malaria parasite liver stages. *Cell Rep* (2014) 7:436–47. doi:10.1016/j.celrep.2014.03.018
55. Reiling L, Richards JS, Fowkes FJ, Barry AE, Triglia T, Chokejindachai W, et al. Evidence that the erythrocyte invasion ligand PfRh2 is a target of protective immunity against *Plasmodium falciparum* malaria. *J Immunol* (2010) 185:6157–67. doi:10.4049/jimmunol.1001555
56. Dutta S, Lee SY, Batchelor AH, Lanar DE. Structural basis of antigenic escape of a malaria vaccine candidate. *Proc Natl Acad Sci U S A* (2007) 104:12488–93. doi:10.1073/pnas.0701464104
57. Jiang L, Gaur D, Mu J, Zhou H, Long CA, Miller LH. Evidence for erythrocyte-binding antigen 175 as a component of a ligand-blocking blood-stage malaria vaccine. *Proc Natl Acad Sci USA* (2011) 108:7553–8. doi:10.1073/pnas.1104050108
58. Marsh K, Kinyanjui SM. Immune effector mechanisms in malaria. *Parasite Immunol* (2006) 28:51–60. doi:10.1111/j.1365-3024.2006.00808.x
59. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes. *J Exp Med* (1990) 172:1633–41. doi:10.1084/jem.172.6.1633
60. Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. *J Exp Med* (1995) 182:409–18. doi:10.1084/jem.182.2.409
61. Frech C, Chen N. Variant surface antigens of malaria parasites: functional and evolutionary insights from comparative gene family classification and analysis. *BMC Genomics* (2013) 14:427. doi:10.1186/1471-2164-14-427
62. Su XZ, Heatwole VM, Wertheimer SP, Guinet F, Herrfeldt JA, Peterson DS, et al. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* (1995) 82:89–100. doi:10.1016/0092-8674(95)90055-1
63. Cheng Q, Cloonan N, Fischer K, Thompson J, Waine GJ, Lanzer MP, et al. Stevor and rif are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. *Mol Biochem Parasitol* (1998) 97:161–76. doi:10.1016/S0166-6851(98)00144-3
64. Wang CW, Hviid L. Rifins, rosetting, and red blood cells. *Trends Parasitol* (2015) 31:285–6. doi:10.1016/j.pt.2015.04.009
65. Kyes SA, Rowe JA, Kriek N, Newbold CI. Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* (1999) 96:9333–8. doi:10.1073/pnas.96.16.9333
66. Janssen CS, Phillips RS, Turner CM, Barrett MP. *Plasmodium* interspersed repeats: the major multigene superfamily of malaria parasites. *Nucleic Acids Res* (2004) 32:5712–20. doi:10.1093/nar/gkh907

67. Barnwell JW, Howard RJ, Coon HG, Miller LH. Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. *Infect Immun* (1983) 40:985–94.
68. David PH, Hommel M, Miller LH, Udeinya IJ, Oligino LD. Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc Natl Acad Sci U S A* (1983) 80:5075–9. doi:10.1073/pnas.80.16.5075
69. Udomsangpetch R, Wahlin B, Carlson J, Berzins K, Torii M, Aikawa M, et al. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocytes rosettes. *J Exp Med* (1987) 169:1835–40. doi:10.1084/jem.169.5.1835
70. Niang M, Yan YX, Preiser PR. The *Plasmodium falciparum* STEVOR multigene family mediates antigenic variation of the infected erythrocyte. *PLoS Pathog* (2009) 5:e1000307. doi:10.1371/annotation/c58250db-8cce-40c5-b7ac-42204050069a
71. Goel S, Palmkvist M, Moll K, Joannin N, Lara P, Akhouri R, et al. RIFINs are adhesins implicated in severe *Plasmodium falciparum* malaria. *Nat Med* (2015) 21:314–7. doi:10.1038/nm.3812
72. Lee WC, Malleret B, Lau YL, Mauduit M, Fong MY, Cho JS, et al. Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes. *Blood* (2014) 123:e100–9. doi:10.1182/blood-2013-12-541
73. Pain A, Ferguson DJP, Kai O, Urban BC, Lowe B, Marsh K, et al. Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc Natl Acad Sci U S A* (2001) 98:1805–10. doi:10.1073/pnas.98.4.1805
74. Tan J, Pieper K, Piccoli L, Abdi A, Foglierini M, Geiger R, et al. A LAIR1 insertion generates broadly reactive antibodies against malaria variant antigens. *Nature* (2016) 529:105–9. doi:10.1038/nature16450
75. Chan JA, Fowkes FJ, Beeson JG. Surface antigens of *Plasmodium falciparum*-infected erythrocytes as immune targets and malaria vaccine candidates. *Cell Mol Life Sci* (2014) 71:3633–57. doi:10.1007/s00018-014-1614-3
76. Olivier M, Van Den Ham KM, Shio MT, Kassa FA, Fougeray S. Malarial pigment hemozoin and the innate inflammatory response. *Front Immunol* (2014) 5:25. doi:10.3389/fimmu.2014.00025
77. Corbett Y, Parapini S, D'Alessandro S, Scaccabarozzi D, Rocha BC, Egan TJ, et al. Involvement of Nod2 in the innate immune response elicited by malarial pigment hemozoin. *Microbes Infect* (2015) 17:184–94. doi:10.1016/j.micinf.2014.11.001
78. Boura M, Frita R, Gois A, Carvalho T, Hanscheid T. The hemozoin conundrum: is malaria pigment immune-activating, inhibiting, or simply a bystander? *Trends Parasitol* (2013) 29:469–76. doi:10.1016/j.pt.2013.07.005
79. Chang Z, Jiang N, Zhang Y, Lu H, Yin J, Wahlgren M, et al. The TatD-like DNase of *Plasmodium* is a virulence factor and a potential malaria vaccine candidate. *Nat Commun* (2016) 7:11537. doi:10.1038/ncomms11537
80. Bhatt TK, Khan S, Dwivedi VP, Banday MM, Sharma A, Chandele A, et al. Malaria parasite tyrosyl-tRNA synthetase secretion triggers pro-inflammatory responses. *Nat Commun* (2011) 2:530. doi:10.1038/ncomms1522
81. Wijesekera SK, Carter RL, Rathnayaka L, Mendis KN. A malaria parasite toxin associated with *Plasmodium vivax* paroxysms. *Clin Exp Immunol* (1996) 104:221–7. doi:10.1046/j.1365-2249.1996.07699.x
82. Schofield L, Hewitt MC, Evans K, Siomos MA, Seeberger PH. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* (2002) 418:785–9. doi:10.1038/nature00937
83. von der Weid T, Kopf MA, Kohler G, Langhorne J. The immune response to *Plasmodium chabaudi* malaria in interleukin-4-deficient mice. *Eur J Immunol* (1994) 24:2285–93. doi:10.1002/eji.1830241004
84. Seixas EM, Fonseca L, Langhorne J. The influence of gammadelta T cells on the CD4+ T cell and antibody response during a primary *Plasmodium chabaudi chabaudi* infection in mice. *Parasite Immunol* (2002) 24:131–40. doi:10.1046/j.1365-3024.2002.00446.x
85. Bouharoun-Tayoun H, Druilhe P. *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infect Immun* (1992) 60:1473–81.
86. Chitnis CE, Mukherjee P, Mehta S, Yazdani SS, Dhawan S, Shakri AR, et al. Phase I clinical trial of a recombinant blood stage vaccine candidate for *Plasmodium falciparum* malaria based on MSP1 and EBA175. *PLoS One* (2015) 10:e0117820. doi:10.1371/journal.pone.0137816
87. Suss G, Eichmann K, Kury E, Linke A, Langhorne J. Roles of CD4 and CD8 bearing T lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi*. *Infect Immun* (1988) 56:3081–8.
88. Podoba JE, Stevenson MM. CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. *Infect Immun* (1991) 59:51–8.
89. Imai T, Ishida H, Suzue K, Taniguchi T, Okada H, Shimokawa C, et al. Cytotoxic activities of CD8+ T cells collaborate with macrophages to protect against blood-stage murine malaria. *Elife* (2015) 4:e04232. doi:10.7554/eLife.04232
90. Imai T, Shen J, Chou B, Duan X, Tu L, Tetsutani K, et al. Involvement of CD8+ T cells in protective immunity against murine blood-stage infection with *Plasmodium yoelii* 17XL strain. *Eur J Immunol* (2010) 40:1053–61. doi:10.1002/eji.200939525
91. Horne-Debets JM, Faleiro R, Karunarathne DS, Liu XQ, Lineburg KE, Poh CM, et al. PD-1 dependent exhaustion of CD8 T cells drives chronic malaria. *Cell Rep* (2013) 5:1204–13. doi:10.1038/srep26210
92. Read D, Lensen AHW, Begarnie S, Haley S, Raza A, Carter RL. Transmission-blocking antibodies against multiple, non-variant target epitopes of the *Plasmodium falciparum* gamete surface antigen Pfs230 are all complement-fixing. *Parasite Immunol* (1994) 16:511–9. doi:10.1111/j.1365-3024.1994.tb00305.x
93. Margos G, Navarette S, Butcher GA, Davies A, Willers C, Sinden RE, et al. Interaction between host complement and mosquito-midgut-stage *Plasmodium berghei*. *Infect Immun* (2001) 69:5064–71. doi:10.1128/IAI.69.8.5064-5071.2001
94. Blagborough AM, Sinden RE. *Plasmodium berghei* HAP2 induces strong malaria transmission-blocking immunity *in vivo* and *in vitro*. *Vaccine* (2009) 27:5187–94. doi:10.1016/j.vaccine.2009.06.069
95. Volohonsky G, Steinert S, Levashina EA. Focusing on complement in the antiparasitic defense of mosquitoes. *Trends Parasitol* (2010) 26:1–3. doi:10.1016/j.pt.2009.10.003
96. Sieber KP, Huber M, Kaslow DC, Banks SM, Torii M, Aikawa M, et al. The peritrophic membrane as a barrier: its penetration by *Plasmodium gallinaceum* and the effect of a monoclonal antibody to ookinetes. *Exp Parasitol* (1991) 72:145–56. doi:10.1016/0014-4894(91)90132-G
97. Duffy PE, Pimenta P, Kaslow DC. Pgs28 belongs to a family of epidermal growth factor-like antigens that are targets of malaria transmission-blocking antibodies. *J Exp Med* (1993) 177:505–10. doi:10.1084/jem.177.2.505
98. Ranawaka GR, Fleck SL, Blanco AR, Sinden RE. Characterization of the modes of action of anti-Pbs21 malaria transmission-blocking immunity: ookinete to oocyst differentiation in vivo. *Parasitology* (1994) 109:403–11. doi:10.1017/S0031182000080653
99. Baton LA, Ranford-Cartwright LC. Do malaria ookinete surface proteins P25 and P28 mediate parasite entry into mosquito midgut epithelial cells? *Malar J* (2005) 4:15. doi:10.1186/1475-2875-4-15
100. Naotunne TS, Karunaweera ND, Mendis KN, Carter RL. Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates. *Immunology* (1993) 78:555–62.
101. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res* (2010) 20:34–50. doi:10.1038/cr.2009.139
102. Simon N, Lasonder E, Scheuermayer M, Kuehn A, Tews S, Fischer R, et al. Malaria parasites co-opt human factor H to prevent complement-mediated lysis in the mosquito midgut. *Cell Host Microbe* (2013) 13:29–41. doi:10.1016/j.chom.2012.11.013
103. Ramphul UN, Garver LS, Molina-Cruz A, Canepa GE, Barillas-Mury C. *Plasmodium falciparum* evades mosquito immunity by disrupting JNK-mediated apoptosis of invaded midgut cells. *Proc Natl Acad Sci U S A* (2015) 112:1273–80. doi:10.1073/pnas.1423586112
104. Morens DM. Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clin Infect Dis* (1994) 19:500–12. doi:10.1093/clinids/19.3.500
105. Hollingdale MR, Appiah A, Leland P, Do Rosario VE, Mazier D, Pied S, et al. Activity of human volunteer sera to candidate *Plasmodium falciparum* circumsporozoite vaccines in the inhibition of sporozoite invasion (ISI) of human hepatoma cells and hepatocytes assays. *Trans R Soc Trop Med Hyg* (1990) 84:325–9. doi:10.1016/0035-9203(90)90303-V

106. Franzen L, Wahlin B, Wahlgren M, Aslund L, Perlmann P, Wigzell H, et al. Enhancement or inhibition of *Plasmodium falciparum* erythrocyte reinvasion *in vitro* by antibodies to an asparagine rich protein. *Mol Biochem Parasitol* (1989) 32:201–11. doi:10.1016/0166-6851(89)90071-6
107. Biryukov S, Angov E, Landmesser ME, Spring MD, Ockenhouse CF, Stoute JA. Complement and antibody-mediated enhancement of red blood cell invasion and growth of malaria parasites. *EBioMedicine* (2016) 9:207–16. doi:10.1016/j.ebiom.2016.05.015
108. Jerusalem CR, Weiss ML, Poels LG. Immunologic enhancement in malaria infection (*Plasmodium berghei*). *J Immunol* (1971) 107:260–8.
109. Mendis KN, Peiris JSM, Premawansa S, Udagama PV, Munesinghe YD, Ranawaka MB, et al. Immune modulation of parasite transmission in *Plasmodium vivax* malaria. Anti-gamete antibodies can both block and enhance transmission. In: Agabian N, Goodman H, Nogueira N, editors. *Molecular Strategies of Parasite invasion.UCLA symposium on Molecular and Cellular Biology*. New York: Alan R. Liss, Inc. (1987) 42:417–26.
110. Mattei DM, Sherf A, Bensaude O, Pereira da Silva LH. A heat shock-like protein from the human malaria parasite *Plasmodium falciparum* induces autoantibodies. *Eur J Immunol* (1989) 19:1823–8. doi:10.1002/eji.1830191010
111. Do Rosario VE, Appiah A, Vaughan JA, Hollingdale MR. *Plasmodium falciparum*: administration of anti-sporozoite antibodies during sporogony results in production of sporozoites which are not neutralized by human anti-circumsporozoite protein sera. *Trans R Soc Trop Med Hyg* (1989) 83:305–7. doi:10.1016/0035-9203(89)90481-1
112. Motard A, Marussig M, Renia L, Baccam D, Landau I, Mattei DM, et al. Immunization with the malaria heat shock like protein hsp70-1 enhances transmission to the mosquito. *Int Immunol* (1995) 7:147–50. doi:10.1093/intimm/7.1.147
113. Bopp SE, Manary MJ, Bright AT, Johnston GL, Dharia NV, Luna FL, et al. Mitotic evolution of *Plasmodium falciparum* shows a stable core genome but recombination in antigen families. *PLoS Genet* (2013) 9:e1003293. doi:10.1371/journal.pgen.1003293
114. Anders RF, Smythe JA. Polymorphic antigens in *Plasmodium falciparum*. *Blood* (1989) 74:1865–75.
115. Anders RF, Brown GV, Coppel RL, Kemp DJ. Repeat structures in malaria antigens. *PNG Med J* (1986) 29:87–93.
116. Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, et al. A field trial to assess a blood-stage malaria vaccine. *N Engl J Med* (2011) 365:1004–13. doi:10.1056/NEJMoa1008115
117. Takala SL, Coulibaly D, Thera MA, Batchelor AH, Cummings MP, Escalante AA, et al. Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. *Sci Transl Med* (2009) 1:2ra5. doi:10.1126/scitranslmed.3000257
118. Drew DR, Wilson DW, Elliott SR, Cross N, Terheggen U, Hodder AN, et al. A novel approach to identifying patterns of human invasion-inhibitory antibodies guides the design of malaria vaccines incorporating polymorphic antigens. *BMC Med* (2016) 14:144. doi:10.1186/s12916-016-0691-6
119. Neafsey DE, Juraska M, Bedford T, Benkeser D, Valim C, Griggs A, et al. Genetic diversity and protective efficacy of the RTS,S/AS01 malaria vaccine. *N Engl J Med* (2015) 373:2025–37. doi:10.1056/NEJMoa1505819
120. Yaneva R, Schneeweiss C, Zacharias M, Springer S. Peptide binding to MHC class I and II proteins: new avenues from new methods. *Mol Immunol* (2010) 47:649–57. doi:10.1016/j.molimm.2009.10.008
121. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. *Annu Rev Immunol* (2013) 31:443–73. doi:10.1016/j.vaccine.2009.06.069
122. Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. *Cell* (2001) 104:1–4. doi:10.1016/S0092-8674(01)00185-4
123. De la Cruz VF, Maloy WL, Miller LH, Lal AA, Good MF, McCutchan TF. Lack of cross-reactivity between variant T cell determinants from malaria circumsporozoite protein. *J Immunol* (1988) 141:2456–60.
124. Zevering Y, Khamboonruang C, Good MF. Natural amino acid polymorphisms of the circumsprozoite protein of *Plasmodium falciparum* abrogate specific human CD4+ T cell responsiveness. *Eur J Immunol* (1994) 24:1418–25. doi:10.1002/eji.1830240627
125. Plebanski M, Lee EA, Hill AVS. Immune evasion in malaria: altered peptide ligands of the circumsporozoite protein. *Parasitology* (1997) 115:S55–66. doi:10.1017/S0031182097002035
126. Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo MA, et al. Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* (1998) 279:1173–7. doi:10.1126/science.279.5354.1173
127. Plebanski M, Flanagan KL, Lee EA, Reece WH, Hart K, Gelder C, et al. Interleukin 10-mediated immunosuppression by a variant CD4 T cell epitope of *Plasmodium falciparum*. *Immunity* (1999) 10:651–60. doi:10.1016/S1074-7613(00)80064-3
128. Plebanski M, Lee AM, Hanan CM, Flanagan KL, Gilbert SC, Gravenor MB, et al. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. *Nat Med* (1999) 5:565–71. doi:10.1038/8444
129. Brown KN, Brown IN. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature* (1965) 208:1286–8. doi:10.1038/2081286a0
130. Brown KN. Antibody induced variation in malaria parasites. *Nature* (1973) 242:49–50. doi:10.1038/242049a0
131. Hommel M, David PH, Oligino LD. Surface alterations of erythrocytes in *Plasmodium falciparum* malaria antigenic variation, antigenic diversity, and the role of the spleen. *J Exp Med* (1983) 157:1137–48. doi:10.1084/jem.157.4.1137
132. Winter G, Kawai S, Haeggstrom M, Kaneko O, von Eule A, Kawazu S, et al. SURFIN is a polymorphic antigen expressed on *Plasmodium falciparum* merozoites and infected erythrocytes. *J Exp Med* (2005) 201:1853–63. doi:10.1084/jem.20041392
133. al-Khedery B, Barnwell JW, Galinski MR. Antigenic variation in malaria: a 3' genomic alteration associated with the expression of a *Plasmodium knowlesi* variant antigen. *Mol Cell* (1999) 3:131–41. doi:10.1016/S1097-2765(00)80304-4
134. Chan JA, Howell KB, Reiling L, Ataide R, Mackintosh CL, Fowkes FJ, et al. Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria immunity. *J Clin Invest* (2012) 122:3227–38. doi:10.1172/JCI62182
135. Beeson JG, Chan JA, Fowkes FJ. PfEMP1 as a target of human immunity and a vaccine candidate against malaria. *Expert Rev Vaccines* (2013) 12:105–8. doi:10.1038/71582
136. Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Peterson DS, et al. Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* (1995) 82:101–10. doi:10.1016/0092-8674(95)90056-X
137. Roberts DD, Sherwood JA, Spitalnik SL, Panton LJ, Howard RJ, Dixit VM, et al. Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence. *Nature* (1985) 318:64–6. doi:10.1038/318064a0
138. Oquendo P, Hundt E, Lawler JW, Seed B. CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitized erythrocytes. *Cell* (1989) 58:95–101. doi:10.1016/0092-8674(89)90406-6
139. Berendt AR, Simmons DL, Tansey J, Newbold C, Marsh K. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. *Nature* (1989) 341:57–9. doi:10.1038/341057a0
140. Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* (1996) 272:1502–4. doi:10.1126/science.272.5267.1502
141. Reeder JC, Cowman AF, Davern KM, Beeson JG, Thompson JK, Rogerson SJ, et al. The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. *Proc Natl Acad Sci U S A* (1999) 96:5198–202. doi:10.1073/pnas.96.9.5198
142. Buffet PA, Gamain B, Scheidig C, Baruch DI, Smith JD, Hernandez-Rivas R, et al. *Plasmodium falciparum* domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc Natl Acad Sci U S A* (1999) 96:12743–8. doi:10.1073/pnas.96.22.12743
143. Ockenhouse CF, Tegoshi T, Maeno Y, Benjamin C, Ho M, Kan KE, et al. Human vascular endothelial cell adhesion receptors for *Plasmodium falciparum*-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1. *J Exp Med* (1992) 176:1183–9. doi:10.1084/jem.176.4.118
144. Siano JP, Grady KK, Millet P, Wick TM. *Plasmodium falciparum*: cytoadherence to alpha v beta3 on human microvascular endothelial cells. *Am J Trop Med Hyg* (1998) 59:77–9.

145. Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, et al. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* (2000) 6:86–90. doi:10.1038/71582
146. Treutiger CJ, Heddini A, Fernandez V, Muller WA, Wahlgren M. PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes. *Nat Med* (1997) 3:1405–8. doi:10.1038/nm1297-1405
147. Biswas AK, Hafiz A, Banerjee B, Kim KS, Datta K, Chitnis CE. *Plasmodium falciparum* uses gC1qR/HABP1/p32 as a receptor to bind to vascular endothelium and for platelet-mediated clumping. *PLoS Pathog* (2007) 3:1271–80. doi:10.1371/journal.ppat.0030130
148. Turner L, Lavstsen T, Berger SS, Wang CW, Petersen JE, Avril M, et al. Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature* (2013) 499:223–7. doi:10.1038/nature12361
149. Rowe JA, Moulds JM, Newbold CI, Miller LH. *Plasmodium falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* (1997) 388:292–5. doi:10.1038/40888
150. Chen Q, Fernandez V, Sundstrom A, Schlichtherle M, Datta S, Hagblom P, et al. Developmental selection of *var* gene expression in *Plasmodium falciparum*. *Nature* (1998) 394:392–5. doi:10.1038/28660
151. Roberts DJ, Craig AG, Berendt AR, Pinches RA, Nash GB, Marsh K, et al. Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* (1992) 357:689–92. doi:10.1038/357689a0
152. Blackman MJ, Scott-Finnigan TJ, Shai S, Holder AA. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. *J Exp Med* (1994) 180:389–93. doi:10.1084/jem.180.1.389
153. Carvalho BO, Lopes SC, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, et al. On the cytoadhesion of *Plasmodium vivax*-infected erythrocytes. *J Infect Dis* (2010) 202:638–47. doi:10.1086/654815
154. Lopes SC, Albrecht L, Carvalho BO, Siqueira AM, Thomson-Luque R, Nogueira PA, et al. Paucity of *Plasmodium vivax* mature schizonts in peripheral blood is associated with their increased cytoadhesive potential. *J Infect Dis* (2014) 209:1403–7. doi:10.1093/infdis/jiu018
155. Carlton JMR, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* (2008) 455:757–63. doi:10.1038/nature07327
156. Neafsey DE, Galinsky K, Jiang RH, Young L, Sykes SM, Saif S, et al. The malaria parasite *Plasmodium vivax* exhibits greater genetic diversity than *Plasmodium falciparum*. *Nat Genet* (2012) 44:1046–50. doi:10.1038/ng.2373
157. Srivastava A, Gangnard S, Round A, Dechavanne S, Juillerat A, Raynal B, et al. Full-length extracellular region of the var2CSA variant of PfEMP1 is required for specific, high-affinity binding to CSA. *Proc Natl Acad Sci U S A* (2010) 107:4884–9. doi:10.1073/pnas.1000951107
158. Gangnard S, Lewit-Bentley A, Dechavanne S, Srivastava A, Amirat F, Bentley GA, et al. Structure of the DBL3X-DBL4epsilon region of the VAR2CSA placental malaria vaccine candidate: insight into DBL domain interactions. *Sci Rep* (2015) 5:14868. doi:10.1038/srep14868
159. Holder AA. The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. *Parasitology* (2009) 136:1445–56. doi:10.1017/S0031182009990515
160. Ayi K, Turrini F, Piga A, Arese P. Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait. *Blood* (2004) 104:3364–71. doi:10.1182/blood-2003-11-3820
161. Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J Exp Med* (1990) 172:379–82. doi:10.1084/jem.172.1.379
162. Guevara-Patino JA, Holder AA, McBride JS, Blackman MJ. Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. *J Exp Med* (1997) 186:1689–99. doi:10.1084/jem.186.10.1689
163. Okafor CM, Anumudu CI, Omosun YO, Uthaipibull C, Ayede I, Awobode HO, et al. Cellular response to modified *Plasmodium falciparum* MSP119 antigens in individuals previously exposed to natural malaria infection. *Malar J* (2009) 8:263. doi:10.1186/1475-2875-8-263
164. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* (2010) 10:778–86. doi:10.1038/nri2849
165. Pleass RJ, Moore SC, Stevenson L, Hviid L. Immunoglobulin M: restrainer of inflammation and mediator of immune evasion by *Plasmodium falciparum* malaria. *Trends Parasitol* (2016) 32:108–19. doi:10.1016/j.pt.2015.09.007
166. Ghumra A, Semblat JP, McIntosh RS, Raza A, Rasmussen IB, Braathen R, et al. Identification of residues in the Cmu4 domain of polymeric IgM essential for interaction with *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). *J Immunol* (2008) 181:1988–2000. doi:10.4049/jimmunol.181.3.1988
167. Barfod LK, Dalgaard MB, Pleman ST, Ofori MF, Pleass RJ, Hviid L. Evasion of immunity to *Plasmodium falciparum* malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. *Proc Natl Acad Sci U S A* (2011) 108:12485–90. doi:10.1073/pnas.1103708108
168. Crosnier C, Iqbal Z, Knuepfer E, Maciuca S, Perrin AJ, Kamuyu G, et al. Binding of *Plasmodium falciparum* merozoite surface proteins DBLMSP and DBLMSP2 to human immunoglobulin M is conserved amongst broadly diverged sequence variants. *J Biol Chem* (2016) 291:14285–99. doi:10.1074/jbc.M116.722074
169. Anders RF. Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective immunity against malaria. *Parasite Immunol* (1986) 8:529–35. doi:10.1111/j.1365-3024.1986.tb00867.x
170. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med* (2008) 205:1797–805. doi:10.1084/jem.20072683
171. Portugal S, Tipton CM, Sohn H, Kone Y, Wang J, Li S, et al. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. *Elife* (2015) 4:e07218. doi:10.7554/eLife.07218
172. Muellenbeck MF, Ueberheide B, Amulic B, Epp A, Fenyo D, Busse CE, et al. Atypical and classical memory B cells produce *Plasmodium falciparum* neutralizing antibodies. *J Exp Med* (2013) 210:389–99. doi:10.1084/jem.20121970
173. Fehr T, Bachmann MF, Bucher E, Kalinke U, Padova FED, Lang AB, et al. Role of repetitive antigen patterns for induction of antibodies against antibodies. *J Exp Med* (1997) 185:1785–92. doi:10.1084/jem.185.10.1785
174. Dintzis RZ, Okajima M, Middleton MH, Greene G, Dintzis HM. The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence. *J Immunol* (1989) 143:1239–44.
175. Zubler RH. Naive and memory B cells in T-cell-dependent and T-independent responses. *Springer Semin Immunopathol* (2001) 23:405–19. doi:10.1007/s281-001-8167-7
176. Symer DE, Reim J, Dintzis RZ, Voss EW Jr, Dintzis HM. Durable elimination of high affinity, T cell-dependent antibodies by low molecular weight antigen arrays in vivo. *J Immunol* (1995) 155:5608–16.
177. Puentes F, Dickhaut K, Hofstatter M, Pfeil J, Lauer U, Hamann A, et al. Immune modulation and prevention of autoimmune disease by repeated sequences from parasites linked to self antigens. *J Neuroimmune Pharmacol* (2016). doi:10.1007/s11481-016-9701-x
178. Robson KJH, Hall JR, Davies LC, Crisanti A, Hill AVS, Wellems TE. Polymorphism of the TRAP gene of *Plasmodium falciparum*. *Proc Biol Sci* (1990) 242:205–16. doi:10.1098/rspb.1990.0126
179. Muller HM, Scarselli E, Crisanti A. Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* in parasite-host cell interactions. *Parassitologia* (1993) 35:69–72.
180. Trottein F, Triglia T, Cowman AF. Molecular cloning of a gene from *Plasmodium falciparum* that codes for a protein sharing motifs found in adhesive molecules from mammals and plasmodia. *Mol Biochem Parasitol* (1995) 74:129–41. doi:10.1016/0166-6851(95)02489-1
181. Chattopadhyay R, Rathore D, Fujioka H, Kumar S, De la Vega P, Haynes D, et al. PfSPATR – a *Plasmodium falciparum* protein containing an altered thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is the target of inhibitory antibodies. *J Biol Chem* (2003) 278:25977–81. doi:10.1074/jbc.M300865200
182. Siddiqui FA, Dhawan S, Singh S, Singh B, Gupta P, Pandey A, et al. A thrombospondin structural repeat containing rhoptry protein from *Plasmodium falciparum* mediates erythrocyte invasion. *Cell Microbiol* (2013) 15:1341–56. doi:10.1111/cmi.12118

183. Cowman AF, Berry D, Baum J. The cell biology of disease: the cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J Cell Biol* (2012) 198:961–71. doi:10.1083/jcb.201206112
184. Chen L, Lopaticki S, Riglar DT, Dekiwadia C, Uboldi AD, Tham WH, et al. An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by *Plasmodium falciparum*. *PLoS Pathog* (2011) 7:e1002199. doi:10.1371/journal.ppat.1002199
185. Kaslow DC, Quakyi IA, Syin C, Raum MG, Keister DB, Coligan JE, et al. A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. *Nature* (1988) 333:74–6. doi:10.1038/333074a0
186. Duffy PE, Kaslow DC. A novel malaria protein, Pfs28, and Pfs25 are genetically linked and synergistic as falciparum malaria transmission-blocking vaccines. *Infect Immun* (1997) 65:1109–13.
187. Urban BC, Roberts DJ. Malaria, monocytes, macrophages and myeloid dendritic cells: sticking of infected erythrocytes switches off host cells. *Curr Opin Immunol* (2002) 14:458–65. doi:10.1016/S0952-7915(02)00368-0
188. McGilvray ID, Serghides L, Kapus A, Rotstein OD, Kain KC. Nonopsonic monocyte/macrophage phagocytosis of *Plasmodium falciparum*-parasitized erythrocytes: a role for CD36 in malarial clearance. *Blood* (2000) 96:3231–40.
189. Schwarzer E, Turrini F, Ulliers D, Giribaldi G, Ginsburg HD, Arese P. Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. *J Exp Med* (1992) 176:1033–41. doi:10.1084/jem.176.4.1033
190. Cunnington AJ, Njie M, Correa S, Takem EN, Riley EM, Walther M. Prolonged neutrophil dysfunction after *Plasmodium falciparum* malaria is related to hemolysis and heme oxygenase-1 induction. *J Immunol* (2012) 189:5336–46. doi:10.4049/jimmunol.1201028
191. Schwarzer E, Arese P. Phagocytosis of malarial pigment hemozoin inhibits NADPH-oxidase activity in human monocyte-derived macrophages. *Biochim Biophys Acta* (1996) 1316:169–75. doi:10.1016/0925-4439(96)00021-X
192. Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* (2012) 30:1–22. doi:10.1146/annurev-immunol-100311-102839
193. Urban BC, Ferguson DJP, Pain A, Willcox N, Plebanski M, Austyn JM, et al. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* (1999) 400:73–7. doi:10.1038/21900
194. Toure Balde A, Sarthou JL, Aribot G, Michel P, Trape JF, Rogier C, et al. *Plasmodium falciparum* induces apoptosis in human mononuclear cells. *Infect Immun* (1996) 64:744–50.
195. Helmby H, Jonsson G, Troye-Blomberg M. Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage *Plasmodium chabaudi chabaudi* AS. *Infect Immun* (2000) 68:1485–90. doi:10.1128/IAI.68.3.1485-1490.2000
196. Wykes MN, Zhou YH, Liu XQ, Good MF. *Plasmodium yoelii* can ablate vaccine-induced long-term protection in mice. *J Immunol* (2005) 175:2510–6. doi:10.4049/jimmunol.175.4.2510
197. Woodberry T, Minigo G, Piera KA, Amante FH, Pinzon-Charry A, Good MF, et al. Low-level *Plasmodium falciparum* blood-stage infection causes dendritic cell apoptosis and dysfunction in healthy volunteers. *J Infect Dis* (2012) 206:333–40. doi:10.1093/infdis/jis366
198. Matsumoto J, Kawai S, Terao K, Kirinoki M, Yasutomi Y, Aikawa M, et al. Malaria infection induces rapid elevation of the soluble Fas ligand level in serum and subsequent T lymphocytopenia: possible factors responsible for the differences in susceptibility of two species of *Macaca* monkeys to *Plasmodium coatneyi* infection. *Infect Immun* (2000) 68:1183–8. doi:10.1128/IAI.68.3.1183-1188.2000
199. Pinzon-Charry A, Woodberry T, Kienzle V, McPhun V, Minigo G, Lampah DA, et al. Apoptosis and dysfunction of blood dendritic cells in patients with falciparum and vivax malaria. *J Exp Med* (2013) 210:1635–46. doi:10.1084/jem.20121972
200. Carvalho LJ, Ferreira-da-Cruz MF, Daniel-Ribeiro CT, Pelajo-Machado M, Lenzi HL. *Plasmodium berghei* ANKA infection induces thymocyte apoptosis and thymocyte depletion in CBA mice. *Mem Inst Oswaldo Cruz* (2006) 101:523–8. doi:10.1590/S0074-02762006000500007
201. Theander TG, Bygbjerg IC, Jacobsen L, Jepsen S, Larsen PB, Kharazmi A. Low parasite specific T cell response in clinically immune individuals with low grade *Plasmodium falciparum* parasitaemia. *Trans R Soc Trop Med Hyg* (1986) 80:1000–1. doi:10.1016/0035-9203(86)90295-6
202. Martini F, Paglia MG, Montesano C, Enders PJ, Gentile M, Pauza CD, et al. Vgamma9Vdelta2 T-cell anergy and complementarity-determining region 3-specific depletion during paroxysm of nonendemic malaria infection. *Infect Immun* (2003) 71:2945–9. doi:10.1128/IAI.71.5.2945-2949.2003
203. Alves FA, Pelajo-Machado M, Totino PR, Souza MT, Goncalves EC, Schneider MP, et al. Splenic architecture disruption and parasite-induced splenocyte activation and anergy in *Plasmodium falciparum*-infected *Saimiri sciureus* monkeys. *Malar J* (2015) 14:128. doi:10.1186/s12936-015-0641-3
204. Illingworth J, Butler NS, Roetynck S, Mwacharo J, Pierce SK, Bejon P, et al. Chronic exposure to *Plasmodium falciparum* is associated with phenotypic evidence of B and T cell exhaustion. *J Immunol* (2012) 190:1038–47. doi:10.4049/jimmunol.1202438
205. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nat Immunol* (2012) 13:188–95. doi:10.1038/ni.2180
206. Karunarathne DS, Horne-Debets JM, Huang JX, Faleiro R, Leow CY, Amante FH, et al. Programmed death-1 ligand 2-mediated regulation of the PD-L1 to PD-1 axis is essential for establishing CD4+ T cell immunity. *Immunity* (2016) 45:333–45. doi:10.1016/j.immuni.2016.07.017
207. Horne-Debets JM, Karunarathne DS, Faleiro RJ, Poh CM, Renia L, Wykes MN. Mice lacking programmed cell death-1 show a role for CD8+ T cells in long-term immunity against blood-stage malaria. *Sci Rep* (2016) 6:26210. doi:10.1038/srep26210
208. Kho S, Marfurt J, Handayuni I, Pava Z, Noviyanti R, Kusuma A, et al. Characterization of blood dendritic and regulatory T cells in asymptomatic adults with sub-microscopic *Plasmodium falciparum* or *Plasmodium vivax* infection. *Malar J* (2016) 15:328. doi:10.1186/s12936-016-1382-7
209. Cambos M, Belanger B, Jacques A, Roulet A, Scorza T. Natural regulatory CD4+CD25+FOXP+ T cells control the production of pro-inflammatory cytokines during *Plasmodium chabaudi adami* infection and do not contribute to immune evasion. *Int J Parasitol* (2008) 38:229–38. doi:10.1016/j.ijpara.2007.07.006
210. Hisaeda H, Tetsutani K, Imai T, Moriya C, Tu L, Hamano S, et al. Malaria parasites require TLR9 signaling for immune evasion by activating regulatory T cells. *J Immunol* (2008) 180:2496–503. doi:10.4049/jimmunol.180.4.2496
211. Kho S, Marfurt J, Noviyanti R, Kusuma A, Piera KA, Burdam FH, et al. Preserved dendritic cell HLA-DR expression and reduced regulatory T cell activation in asymptomatic *Plasmodium falciparum* and *P. vivax* Infection. *Infect Immun* (2015) 83:3224–32. doi:10.1128/IAI.00226-15
212. Hafalla JCR, Claser C, Couper KN, Grau GE, Renia L, De Souza JB, et al. The CTLA-4 and PD-1/PD-L1 inhibitory pathways independently regulate host resistance to *Plasmodium*-induced acute immune pathology. *PLoS Pathog* (2012) 8:e1002504. doi:10.1371/journal.ppat.1002504
213. Iyer JK, Gruner AC, Renia L, Snounou G, Preiser PR. Invasion of host cells by malaria parasites: a tale of two protein families. *Mol Microbiol* (2007) 65:231–49. doi:10.1111/j.1365-2958.2007.05791.x
214. Mayer DCG, Mu JB, Feng X, Su XZ, Miller LH. Polymorphism in a *Plasmodium falciparum* erythrocyte-binding ligand changes its receptor specificity. *J Exp Med* (2002) 196:1523–8. doi:10.1084/jem.20020750
215. Stubbs J, Simpson KM, Triglia T, Plouffe D, Tonkin CJ, Duraisingh MT, et al. Molecular mechanism for switching of *Plasmodium falciparum* invasion pathways into human erythrocytes. *Science* (2005) 309:1384–7. doi:10.1126/science.1115257
216. Persson KE, McCallum FJ, Reiling L, Lister NA, Stubbs J, Cowman AF, et al. Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. *J Clin Invest* (2008) 118:342–51. doi:10.1172/JCI32138
217. Crosnier C, Bustamante LY, Bartholdson SJ, Bei AK, Theron M, Uchikawa M, et al. Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* (2011) 480:534–7. doi:10.1038/nature10606
218. Douglas AD, Baldeviano GC, Lucas CM, Lugo-Roman LA, Crosnier C, Bartholdson SJ, et al. A PfRH5-based vaccine is efficacious against heterologous strain blood-stage infection in *Aotus* monkeys. *Cell Host Microbe* (2015) 17:130–9. doi:10.1016/j.chom.2014.11.017
219. Douglas AD, Williams AR, Illingworth JJ, Kamuyu G, Biswas S, Goodman AL, et al. The blood-stage malaria antigen PfRH5 is susceptible to vaccine-

inducible cross-strain neutralizing antibody. *Nat Commun* (2011) 2:601. doi:10.1038/ncomms1615
220. Zimmerman PA, Ferreira MU, Howes RE, Mercereau-Puijalon O. Red blood cell polymorphism and susceptibility to *Plasmodium vivax*. *Adv Parasitol* (2013) 81:27–76. doi:10.1016/B978-0-12-407826-0.0000
221. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med* (1976) 295:302–4. doi:10.1056/NEJM197608052950602
222. Livingstone FB. The Duffy blood groups, vivax malaria, and malaria selection in human populations: a review. *Hum Biol* (1984) 56:413–25.
223. Menard D, Barnadas C, Bouchier C, Henry-Halldin CN, Gray LR, Ratsimbasoa A, et al. *Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people. *Proc Natl Acad Sci U S A* (2010) 107:5967–71. doi:10.1073/pnas.0912496107
224. Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, et al. Duffy negative antigen is no longer a barrier to *Plasmodium vivax*-molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis* (2011) 5:e1192. doi:10.1371/journal.pntd.0001192
225. Abdelraheem MH, Albsheer MM, Mohamed HS, Amin M, Abdel Hamid MM. Transmission of *Plasmodium vivax* in Duffy-negative individuals in central Sudan. *Trans R Soc Trop Med Hyg* (2016) 110:258–60. doi:10.1093/trstmh/trw014
226. Lo E, Yewhalaw D, Zhong D, Zemene E, Degefa T, Tushune K, et al. Molecular epidemiology of *Plasmodium vivax* and *Plasmodium falciparum* malaria among Duffy-positive and Duffy-negative populations in Ethiopia. *Malar J* (2015) 14:84. doi:10.1186/s12936-015-0596-4
227. Ngassa Mbenda HG, Das A. Molecular evidence of *Plasmodium vivax* mono and mixed malaria parasite infections in Duffy-negative native Cameroonians. *PLoS One* (2014) 9:e103262. doi:10.1371/journal.pone.0103262
228. Rosenberg RL. *Plasmodium vivax* in Africa: hidden in plain sight? *Trends Parasitol* (2007) 23:193–6. doi:10.1016/j.pt.2007.02.009
229. Ntumngia FB, Thomson-Luque R, de Menezes Torres L, Gunalan K, Carvalho LH, Adams JH. A novel erythrocyte binding protein of *Plasmodium vivax* suggests an alternate invasion pathway into Duffy-Positive reticulocytes. *MBio* (2016) 7:e1261. doi:10.1128/mBio.01261-16
230. Fang XD, Kaslow DC, Adams JH, Miller LH. Cloning of the *Plasmodium vivax* Duffy receptor. *Mol Biochem Parasitol* (1991) 44:125–32. doi:10.1016/0166-6851(91)90228-X
231. Cogswell FB. The hypnozoite and relapse in primate malaria. *Clin Microbiol Rev* (1992) 5:26–35. doi:10.1128/CMR.5.1.26
232. Fairley NH. Chemotherapeutic suppression and prophylaxis in malaria. An experimental investigation undertaken by medical research teams in Australia. *Trans R Soc Trop Med Hyg* (1945) 38:311–65. doi:10.1016/0035-9203(45)90038-1
233. Krotoski WA, Garnham PCC, Bray RS, Krotoski DM, Killick-Kendrick R, Draper CC, et al. Observations on early and late post-sporozoite tissue stages in primate malaria. I. The discovery of a new latent form of *Plasmodium cynomolgi* (the hypnozoite), and failure to detect forms within the first 24 hours after infection. *Am J Trop Med Hyg* (1982) 31:24–35.
234. Mikolajczak SA, Vaughan AM, Kangwanrangsan N, Roobsoong W, Fishbaugher M, Yimamnuaychok N, et al. *Plasmodium vivax* liver stage development and hypnozoite persistence in human liver-chimeric mice. *Cell Host Microbe* (2015) 16:526–35. doi:10.1016/j.chom.2015.02.011
235. Krotoski WA, Bray RS, Garnham PCC, Gwadz RW, Killick-Kendrick R, Draper CC, et al. Observations on early and late post-sporozoite tissue stages in primate malaria. II. The hypnozoite of *Plasmodium cynomolgi bastianellii* from 3 to 105 days after infection, and detection of 36- to 40-hour pre-erythrocytic forms. *Am J Trop Med Hyg* (1982) 31:211–25.
236. Krotoski WA, Krotoski DM, Garnham PCC, Bray RS, Killick-Kendrick R, Draper CC, et al. Relapses in primate malaria: discovery of two populations of exoerythrocytic stages. Preliminary note. *Br Med J* (1980) 280:153–4. doi:10.1136/bmj.280.6208.153-a
237. Mazier D, Renia L, Snounou G. A pre-emptive strike against malaria's stealthy hepatic forms. *Nat Rev Drug Discov* (2009) 8:854–64. doi:10.1038/nrd29
238. Garnham PCC, Bray RS. The influence of immunity upon the stages (including late exo-erythrocytic schizonts) of mammalian malaria parasites. *Rev Bras Malariol Doencas Trop* (1956) 8:151–60.
239. Shortt HE, Garnham PCC. The pre-erythrocytic stage of human malaria, *Plasmodium vivax*. *Br Med J* (1948) 1:547. doi:10.1136/bmj.1.4550.547
240. Shortt HE, Fairley NH. The pre-erythrocytic stage of *Plasmodium falciparum*; a preliminary note. *Br Med J* (1949) 2:1006–8. doi:10.1136/bmj.2.4635.1006
241. Coquelin F, Boulard Y, Mora-Silvera E, Richard F, Chabaud AG, Landau I. Final stage of maturation of the erythrocytic schizonts of rodent *Plasmodium* in the lungs. *C R Acad Sci* (1999) 322:55–62. doi:10.1016/S0764-4469(99)80017-1
242. Baer K, Klotz C, Kappe SH, Schnieder T, Frevert U. Release of hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. *PLoS Pathog* (2007) 3:e171. doi:10.1371/journal.ppat.0030171
243. Fajardo LF. The role of platelets in infections. I. Observations in human and murine malaria. *Arch Pathol Lab Med* (1979) 103:131–4.
244. Mora-Silvera E, Coquelin F, Vuong P, Deharo E, Gautret P, Renia L, et al. Role of macrophages as possible transporters of *Plasmodium yoelii nigeriensis* merozoites through the lymphatic system. Preliminary note. *Parasite* (1997) 4:83–5. doi:10.1051/parasite/1997041083
245. Wykes MN, Kay JG, Manderson A, Liu XQ, Brown DL, Richard DJ, et al. Rodent blood-stage *Plasmodium* survive in dendritic cells that infect naive mice. *Proc Natl Acad Sci U S A* (2011) 108:11205–10. doi:10.1073/pnas.1108579108
246. Boulard Y, Coquelin F, Mora-Silvera E, Renia L, Gautret P, Deharo E, et al. Dendritic leucocytes as possible carriers of murine *Plasmodium* merozoites. *Parasite* (1996) 3:383–6. doi:10.1051/parasite/1996034383
247. Landau I, Chabaud AG, Vuong PN, Deharo E, Gautret P. Circulation in the lymphatic system and latency of *Plasmodium* merozoites. Preliminary note. *Parasite* (1995) 2:185–6. doi:10.1051/parasite/1995022185
248. Landau I, Chabaud AG, Mora-Silvera E, Coquelin F, Boulard Y, Renia L, et al. Survival of rodent malaria merozoites in the lymphatic network: potential role in chronicity of the infection. *Parasite* (1999) 6:311–22. doi:10.1051/parasite/1999064311
249. Carter RL, Mendis KN. Evolutionary and historical aspects of the burden of malaria. *Clin Microbiol Rev* (2002) 15:564–94. doi:10.1128/CMR.15.4.564-594.2002
250. Cox FEG. Malaria. Cloning genes for antigens of *Plasmodium falciparum*. *Nature* (1983) 304:13–4. doi:10.1038/304013a0

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IL-6 Improves the Nitric Oxide-Induced Cytotoxic CD8+ T Cell Dysfunction in Human Chagas Disease

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Reactive oxygen and nitrogen species are important microbicidal agents and are also involved in lymphocyte unresponsiveness during experimental infections. Many of the biological effects attributed to nitric oxide are mediated by peroxynitrites, which induce the nitration of immune cells, among others. Our group has demonstrated that nitric oxide is involved in the suppressive activity of myeloid-derived suppressor cells in *Trypanosoma cruzi*-infected mice, with a higher number of CD8+ T cells suffering surface-nitration compared to uninfected controls. Studying the functional and phenotypic features of peripheral CD8+ T cells from chagasic patients and human cells experimentally infected with *T. cruzi*, we found that different regulatory mechanisms impaired the effector functions of T cytotoxic population from seropositive patients. Peripheral leukocytes from chagasic patients showed increased nitric oxide production concomitant with increased tyrosine nitration of CD8+ T cells. Additionally, this cytotoxic population exhibited increased apoptotic rate, loss of the TCRζ-chain, and lower levels of CD107a, a marker of degranulation. Strikingly, IL-6 stimulation of *in vitro*-infected peripheral blood mononuclear cells obtained from healthy donors, blunted *T. cruzi*-induced nitration of CD3+CD8+ cells, and increased their survival. Furthermore, the treatment of these cultures with an IL-6 neutralizing antibody increased the percentage of *T. cruzi*-induced CD8+ T cell nitration and raised the release of nitric oxide. The results suggest that the under-responsiveness of cytotoxic T cell population observed in the setting of long-term constant activation of the immune system could be reverted by the pleiotropic actions of IL-6, since this cytokine improves its survival and effector functions.

Keywords: tyrosine nitration, peroxynitrite, CD39, CD73, oxidative stress, *Trypanosoma cruzi* infection

INTRODUCTION

CD8+ T cells play a critical role in the immunity against intracellular pathogens, including the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas cardiomyopathy (1). Chagas disease is characterized by two distinct phases: the acute phase, which lasts several weeks and is characterized by non-specific symptoms; and the chronic phase, lasting lifelong. The host's ability to control *T. cruzi* infection is substantial, but not fully effective, since most infected individuals tightly

limit parasite numbers but fail to completely clear the infection due to diverse and fascinating immune evasion processes (2). Indeed, parasite persistence at low levels into target cells is the hallmark of the indeterminate or asymptomatic chronic phase (3). Up to 40% of infected individuals develop progressive heart disease leading to an end-stage dilated cardiomyopathy.

One key defense mechanism against *T. cruzi* is exerted by nitric oxide (NO), which is produced by inducible NO synthase, among other enzymes, present in monocytes/macrophages and cardiomyocytes (4, 5). The parasite triggers activation of macrophage NADPH oxidase, resulting in a continuous production of superoxide anion $\left(O_2^-\right)$ and also stimulates infected macrophages to produce different amounts of NO. Despite its importance as a microbicidal agent at high levels, persistent low levels of NO have been involved in the establishment and maintenance of lymphocyte unresponsiveness in different experimental models of parasite infections (6–8). These mechanisms illustrate a clear parasite evasion strategy. Moreover, NO induces apoptosis of many different cell types *in vitro* and *in vivo* (9, 10). We found that IL-6 regulates inflammasome activation and, consequently, IL-1β-induced NO production in a murine model of *T. cruzi* infection. The anti-inflammatory action of IL-6 seems to be central for controlling local and systemic oxidative stress, promoting cellular rescue of apoptosis, and protecting infected IL-6-deficient mice against death (unpublished observation). This could be a novel mechanism that regulates NO release in the setting of *T. cruzi* infection.

The presence of low levels of NO rapidly initiates a reaction with the superoxide anion generating peroxynitrites that induce the nitration of surface proteins on T cells (11, 12). It is widely accepted that reactive nitrogen species (NO and peroxynitrites) contribute to the immunosuppressive attitude of myeloid-derived suppressor cells (MDSCs), a heterogeneous cell population associated with tumors, infectious, and inflammatory diseases. Our group has demonstrated that MDSCs during acute *T. cruzi* experimental infection were able to inhibit T cell proliferation *in vitro* (13). Furthermore, we also observed a higher number of splenic CD8+ T cells suffering surface nitration in infected mice compared to uninfected controls.

Chronic exposure to antigens may cause functional defects in pathogen-specific T cells. It has been reported that individuals with more severe clinical disease have a significantly lower frequency of *T. cruzi*-specific CD8+IFNγ+ T cells than subjects in the asymptomatic stage of the infection (14). This impairment in *T. cruzi*-specific CD8+ T cell responses was associated with an increased frequency of fully differentiated memory cells and an increased rate of apoptosis in the total peripheral CD8+ T cell population. The results reveal a progressive exhaustion in the parasite-specific cytotoxic T cell compartment in patients with long-term *T. cruzi* infection. However, during persistent infection, the chronic exposure to an inflammatory microenvironment may also contribute to the impairment of CD8+ T cell responses, resulting in a less efficient control of the pathogen and promoting its persistence.

These findings prompted us to investigate the frequency and functionality of circulating CD8+ T cells from patients with chronic Chagas disease, with particular focus on the molecular mechanisms triggered by IL-6 associated with cytotoxicity and NO-induced cell death. In agreement with previous reports in seropositive patients with Chagas disease, we found a substantial reduction in the total peripheral T cell compartment at the expense of CD8+ T cells (14). Additionally, we observed increased NO-producing leukocytes concomitant with increased nitration of CD8+ T cells and impaired cytotoxic functionality of T cells. Strikingly, IL-6 prevented the nitration of CD8+ T cells and increased their survival when healthy donor peripheral blood mononuclear cells (PBMCs) were infected *in vitro*. In this context, IL-6 led to a decrease in IL-1β levels. The results suggest that the under-responsiveness of the whole cytotoxic T cell population from chagasic patients could be reverted by the pleiotropic actions of IL-6, since this cytokine functions as a survival factor for CD8+ cells and improves the cytotoxic T cell functionality in the setting of long-term constant activation of the immune system.

MATERIALS AND METHODS

Ethics Statement

Subjects were recruited at the "Hospital Nuestra Señora de la Misericordia" (HNSM) from Córdoba-Argentina. All studies were conducted according to the principles expressed in the Declaration of Helsinki. Signed informed consent was obtained from all donors before inclusion in the study. Venous blood was drawn from 46 non-chagasic and chagasic subjects (age 25–60 years) using a protocol that was reviewed and approved by the Comité Institucional de Ética de la Investigación en Salud del Adulto, Ministerio de Salud de la Provincia de Córdoba (Acta number 194/2014, Argentina). *T. cruzi* infection was determined by a combination of indirect hemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) performed in the laboratory of HNSM. Subject positive on these two tests were considered infected. Chronic chagasic patients ($n = 22$) were evaluated clinically and by electrocardiogram and chest X-ray. The uninfected control group ($n = 24$) consisted of age-matched individuals were serologically negative for *T. cruzi*. All donors with chronic or inflammatory pathology or erythrocyte sedimentation rate >30 mm or white blood cells count <4,000 or >10,000/mm^3 were excluded from the study.

Blood Collection

Approximately 15 mL of blood was drawn from each individual by venipuncture and placed into heparinized tubes (Vacutainer, BD Bioscience). An aliquot of 50 µL of total peripheral blood was stained for FACS analysis and subjected to ACK lysing buffer to remove erythrocytes. PBMCs were isolated through density gradient centrifugation using Ficoll-Hypaque PLUS (GE Healthcare Bioscience) and resuspended in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS (Natocor).

Ex Vivo Flow Cytometry

Peripheral blood was lysed with ACK lysing buffer to remove erythrocytes, and 01×10^6 cells were blocked with Fc block and stained with anti-human CD3 Alexa 488 or PerCP, anti-human

CD4 Alexa 647, anti-human CD8 PECy7, anti-human CD39 biotin and streptavidin APC, anti-human CD73 PE (eBioscience and Biolegend), anti-nitrotyrosine (Sigma-Aldrich), and anti-rabbit Alexa 647 or CD20 PECy7 (Biolegend). The intracellular expression of ζ-chain was analyzed with anti-human TCRζ PE antibody (Beckman Coulter). Stained cells were analyzed by flow cytometry (FACS Canto II, Becton Dickinson), with cellular debris being excluded from the analysis. Data were analyzed using the FlowJo software.

Cell Viability Measurement

Leukocytes from peripheral blood were stained with anti-human CD3 Alexa 488, anti-human CD4 Alexa 647, anti-human CD8 PECy7 or APCCy7, anti-human CCR7 Alexa 488, anti-human CD45RA PECy7, and labeled with 5 μL of Annexin VPE (BD Pharmingen) for 15 min on ice. Before acquisition, the cells were stained with 7-AAD (BD Bioscience). A minimum of 300,000 events for each condition were analyzed by flow cytometry. Staining of peripheral blood T cells with antibodies to CD45RA and CCR7 reveals four cells subsets: naïve T cells are CD45RA+CCR7+, central memory cells are CD45RA−CCR7+, effector memory (EM) cells are CD45RA−CCR7−, and terminally differentiated effector memory (EMRA) are CD45RA+CCR7− (15).

To determine Bcl-2 expression, PBMCs stained for CD3 PerCP, CD8, CD4, CCR7, and CD45RA surface expression were permeabilized with FOXP3 staining buffer set (eBioscience) and labeled with anti-Bcl-2 rabbit (Cell Signaling) and then with anti-rabbit Alexa 488 or anti-rabbit Alexa 647. Stained cells were analyzed by flow cytometry.

CD8+ T Cell Functionality

Peripheral blood mononuclear cells were cultured with anti-CD107a PE (Biolegend), monensin, brefeldin A, phorbol 12-myristate 13-acetate (PMA), and ionomycin (Sigma) for 4 h, then stained with anti-IFNγ FITC, anti-TNF APC, and anti-IL-2 APC-Cy7 (Biolegend or eBioscience), and analyzed by flow cytometry.

To evaluate the effect of TCR-dependent activation, PBMCs were cultured with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 mg/mL). After 72 h, PBMCs were cultured with monensin, brefeldin A, and anti-CD107a PE for 6 h and then stained with anti-human CD8 APCCy7, anti-NT rabbit and anti-rabbit Alexa 647, anti-IL-2 PECy7, anti-TNF PerCPCy5.5, or anti-IFNγ PerCPCy5.5.

CD8+ T Cell Exhaustion

Peripheral blood mononuclear cells were cultured with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 mg/mL). After 72 h, the cells were stained with anti-human CD8 APCCy7, anti-Tim3 PerCPCy5.5, anti-PD1 PECy7, anti-CTLA4 PE, anti-CCR7 Alexa 488, and anti-NT rabbit and anti-rabbit Alexa 647.

Measurement of Reactive Oxygen and Nitrogen Species

The nitrite/nitrate content, indicative of NO production, was monitored by the Griess reagent assay (16). In 96-well plates, plasma samples were mixed with 50 μL of Griess reagent, consisting of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-napthyl) ethylenediamine dihydrochloride [1:1 ratio (vol/vol)], and incubated for 10 min. The change in absorbance was monitored at 545 nm (standard curve, 0–200 μmol sodium nitrite).

The production of ROS and NO was evaluated using the molecular probes: H_2DCF-DA (10 μM, Invitrogen Inc.) and DAF-FM DA (10 μM, Molecular Probes, Inc.), respectively. All samples were acquired on a FACS Canto II cytometer and then analyzed using the Flow Jo software.

Quantification of Cytokines

Plasma samples were analyzed for TNF, IL-6, IL-10, IFNγ, and IL-4 by using bead-based immunoassays and flow cytometry, according to the manufacturer's instructions (LegendPlex-Biolegend). In cultured supernatant, IL-1β levels were measured by Ready-SET-Go ELISA kit from eBioscience.

Culture of PBMCs and Parasites

Vero cell monolayers were infected with trypomastigote forms of *T. cruzi* Tulahuen strain for 3 h and then washed and maintained in RPMI (Gibco Invitrogen Corporation) at 37°C in a 5% CO_2 atmosphere. After 7 days, the parasites were collected from the supernatant of infected cells and harvested by centrifugation at 4,400 rpm for 5 min.

Peripheral blood mononuclear cells from non-chagasic donors were cultured with *T. cruzi* Tulahuen trypomastigotes (1:1 rate) for 3 h, then the cells were washed and cultured with recombinant bioactive IL-6 (20 ng/mL) or with anti-IL-6 (2 μg/mL) (Biolegend) or anti-IL-6 plus anti-IL-1β (2 μg/mL), or maintained in medium alone for 48 h. Then, culture supernatants were evaluated for IL-1β/NO levels, and the cells were analyzed for NT staining or NO production by flow cytometry. In addition, peripheral blood from chagasic patients and control donors (250 μL) were cultured with 7,500 trypomastigotes with or without IL-6 (20 ng/mL) or maintained in medium for 24 h and then cells were harvested to evaluate NO and ROS production and NT by flow cytometry.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5.0 software by using parametric or non-parametric paired *t* test according to data distribution. p Values <0.05 were considered significant.

RESULTS

Chagasic Patients Showed a Lower Number of Peripheral Blood CD8+ T Lymphocytes

A total of 46 peripheral blood samples from chagasic and non-chagasic donors of both sexes, collected from people living in Cordoba (Argentina) were analyzed for *T. cruzi*-specific antibodies by ELISA and IHA (**Table 1**). The median value for anti-*T. cruzi* antibody titers detected by IHA was 1/256 (local

cutoff titer 1/32). Only one patient showed a complete right-bundle branch block, left anterior hemiblock, and left atrial enlargement, clinical features of chagasic heart disease. Cordoba is considered by the Argentine Ministry of Health to be at high risk for vector transmission, since there is a reemergence of this infection route by an increase in house infestation and a high seroprevalence in vulnerable groups (17).

TABLE 1 | Description of subject groups.

	Non-chagasic donors ($n = 24$)	Chagasic patients ($n = 22$)
Age (years old)		
Range	25–60	25–48
Median	30	32
Gender		
Female	$n = 16$	$n = 17$
Male	$n = 8$	$n = 5$
Clinical evaluation		
Electrocardiographic changes	NE	$n = 1$[a]
Echocardiographic changes	NE	$n = 1$[b]
Chest X-rays abnormalities	NE	$n = 0$

[a]*Complete right-bundle branch block and left anterior hemiblock.*
[b]*Left atrial enlargement.*
NE, not evaluated.

TABLE 2 | Peripheral blood lymphocyte subpopulations.

	Non-chagasic donors ($n = 15$)	Chagasic patients ($n = 8$)	p-Value
Lymphocyte subsets in peripheral blood			
CD3+ (%)	58.6 ± 3.9	43.2 ± 5.3	$p = 0.03$*
CD3+/μL (absolute number)	1,139 ± 102	754 ± 135	$p = 0.04$*
CD3+CD4+ (%)	58.7 ± 3.7	57.6 ± 2.0	$p = 0.84$
CD3+CD4+/μL (absolute number)	600 ± 72	535 ± 65	$p = 0.59$
CD3+CD8+ (%)	39.9 ± 3.5	30.74 ± 2.2	$p = 0.04$*
CD3+CD8+/μL (absolute number)	381 ± 56	193 ± 32	$p = 0.049$*
CD20+ (%)	14.1 ± 1.5	12.3 ± 1.6	$p = 0.44$
CD20+/μL (absolute number)	305 ± 40	218 ± 32	$p = 0.16$

Values are expressed as mean value ± SE.
**$p < 0.05$, significant.*

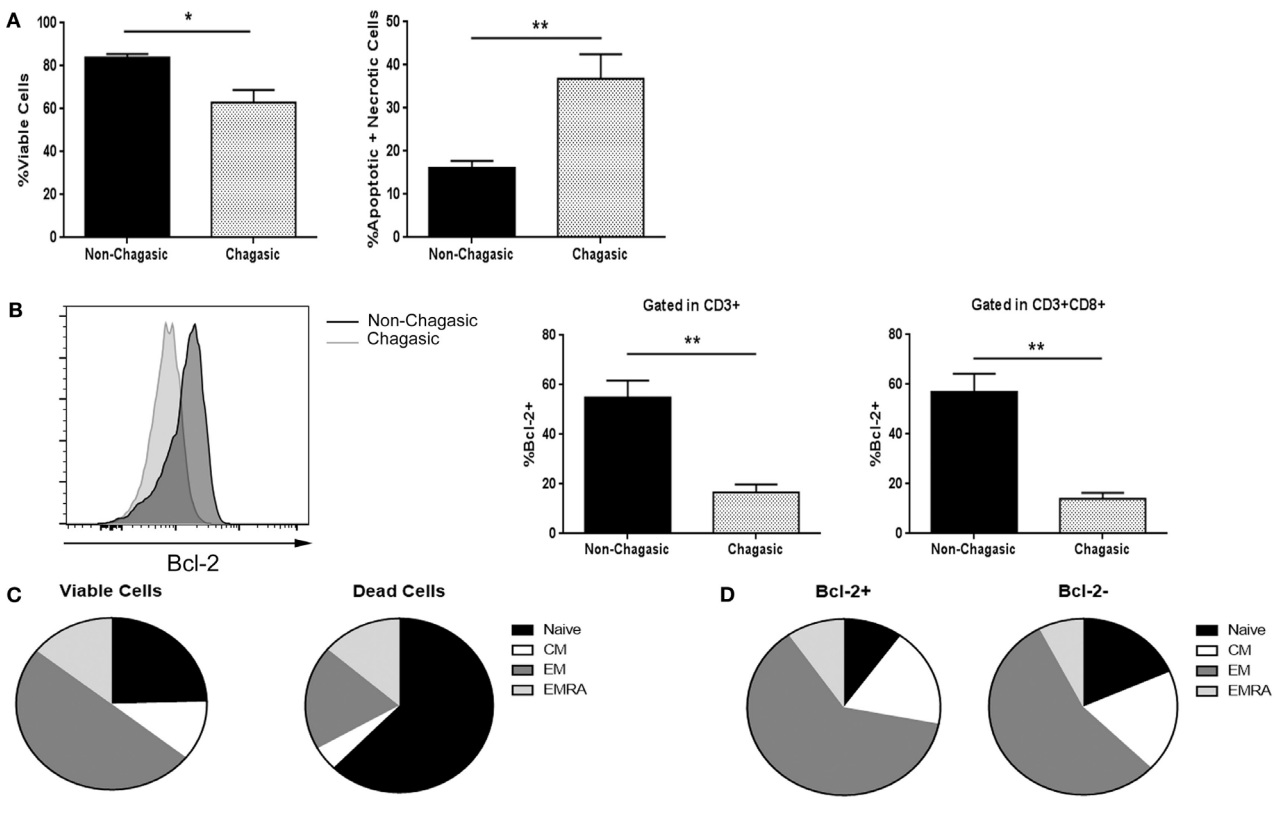

FIGURE 1 | Cytotoxic T lymphocytes from chagasic patients show higher death rate and less Bcl-2 expression. **(A)** Percentage of viable and apoptotic + necrotic CD3+CD8+ from chagasic patients ($n = 5$) and non-chagasic donors ($n = 5$) analyzed by Annexin-V and 7AAD staining. **(B)** Representative histogram of Bcl-2 expression in cytotoxic T cells and percentage of T lymphocytes and CD8+ T cells expressing Bcl-2 in chagasic patients ($n = 5$) and in non-chagasic donors ($n = 5$) (*$p < 0.05$ and **$p < 0.01$). **(C)** Frequency of naïve, central memory (CM), effector memory (EM), and effector memory RA (EMRA) gated in viable cells (7AAD−) or dead cells (7AAD+) in CD8+ lymphocytes from seropositive patients ($n = 4$) (viable naïve vs. dead naïve $p = 0.002$; viable EM vs. dead EM, $p = 0.0002$). **(D)** Frequency of naïve, CM, EM, and EMRA gated in Bcl-2+ or Bcl-2− in CD8+ cells from chagasic patients ($n = 4$) (naïve Bcl-2+ vs. naïve Bcl-2−, $p = 0.0123$).

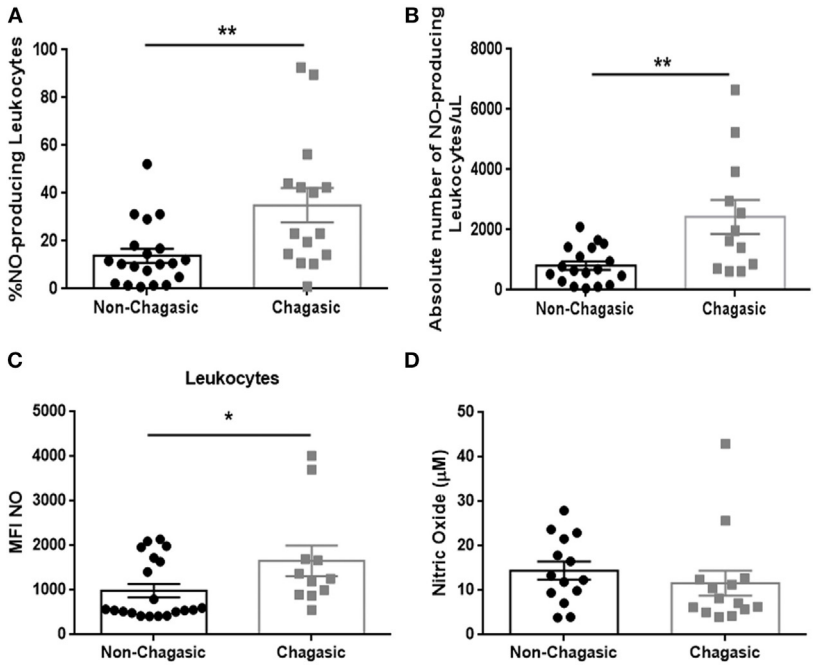

FIGURE 2 | Chagasic patients exhibit a higher number and frequency of nitric oxide-producing leukocytes. **(A)** Percentage and **(B)** absolute number of nitric oxide (NO)-producing leukocytes from chagasic patients ($n = 15$) and non-chagasic donors ($n = 19$). **(C)** Mean fluorescence intensity of NO in leukocytes from chagasic patients ($n = 11$) and non-chagasic donors ($n = 20$). **(D)** Serum nitric oxide levels in seropositive patients ($n = 14$) and seronegative donors ($n = 14$) ($^*p < 0.05$ and $^{**}p < 0.01$).

Seropositive patients with Chagas disease showed a diminished percentage and absolute number of total peripheral T lymphocytes (CD3+) compared to seronegative donors. Although no differences were observed between non-chagasic and chagasic patients in the percentage and absolute number of T helper cells (CD3+CD4+), the percentage and absolute number of cytotoxic T cells (CD3+CD8+) were significantly lower in seropositive than in seronegative patients (**Table 2**).

No differences were observed in the percentage and absolute number of B lymphocytes (CD20+) between the analyzed groups (**Table 2**).

Cytotoxic T Lymphocytes from Chagasic Patients Showed Higher Cell Death and Less Bcl-2 Expression

Through Annexin V and 7-AAD staining, we found that fresh explanted CD8+ T lymphocytes from chagasic patients showed a diminished percentage of viable cells, and concomitantly, a higher rate of apoptotic and necrotic cells compared to non-chagasic donors (**Figure 1A**). In agreement, the expression of the anti-apoptotic protein Bcl-2 was significantly higher in cytotoxic lymphocytes from non-chagasic than from chagasic patients (**Figure 1B**). Among cytotoxic T cells from Chagas patients, naïve CD8 T cells were the phenotype most susceptible to die (viable cells vs. dead cells: 24.53 ± 6.11 vs. $62.48 \pm 3.95\%$). In contrast, EM cytotoxic T cells showed significantly increased survival rate (viable cells vs. dead cells: 49.75 ± 3.06 vs. $19.48 \pm 2.45\%$) (**Figure 1C**). In accordance, the frequency of naïve cells negative for intracellular Bcl-2 expression was significantly lower than naïve cells positive for the expression of this anti-apoptotic protein (Bcl-2+ vs. Bcl-2−: 9.95 ± 0.87 vs. 17.67 ± 2.07) (**Figure 1D**). Furthermore, the percentage of EM CD8 T cells was higher in Chagas subjects in comparison with seronegative donors (Figure S1 in Supplementary Material).

Nitric Oxide-Producing Leukocytes Were Increased in Chagasic Patients

Seropositive patients showed an increased percentage and absolute number of NO-producing leukocytes (**Figures 2A,B**), with higher production of NO per leukocyte (**Figure 2C**), but no significant differences were detected in levels of plasma NO in comparison with seronegative subjects (**Figure 2D**). The infection of peripheral blood from seronegative and seropositive patients with trypomastigotes (Tulahuen strain) significantly increased the percentage of NO- and ROS-producing leukocytes (Figure S2 in Supplementary Material).

CD8+ T Lymphocytes from Chagasic Patients Exhibited High Levels of Nitrated Tyrosine Residues

Non-chagasic individuals exhibited significantly lower tyrosine nitration (NT) of leukocytes, lymphocytes, and T cells than chagasic patients (**Figure 3A**). In particular, chagasic patients showed

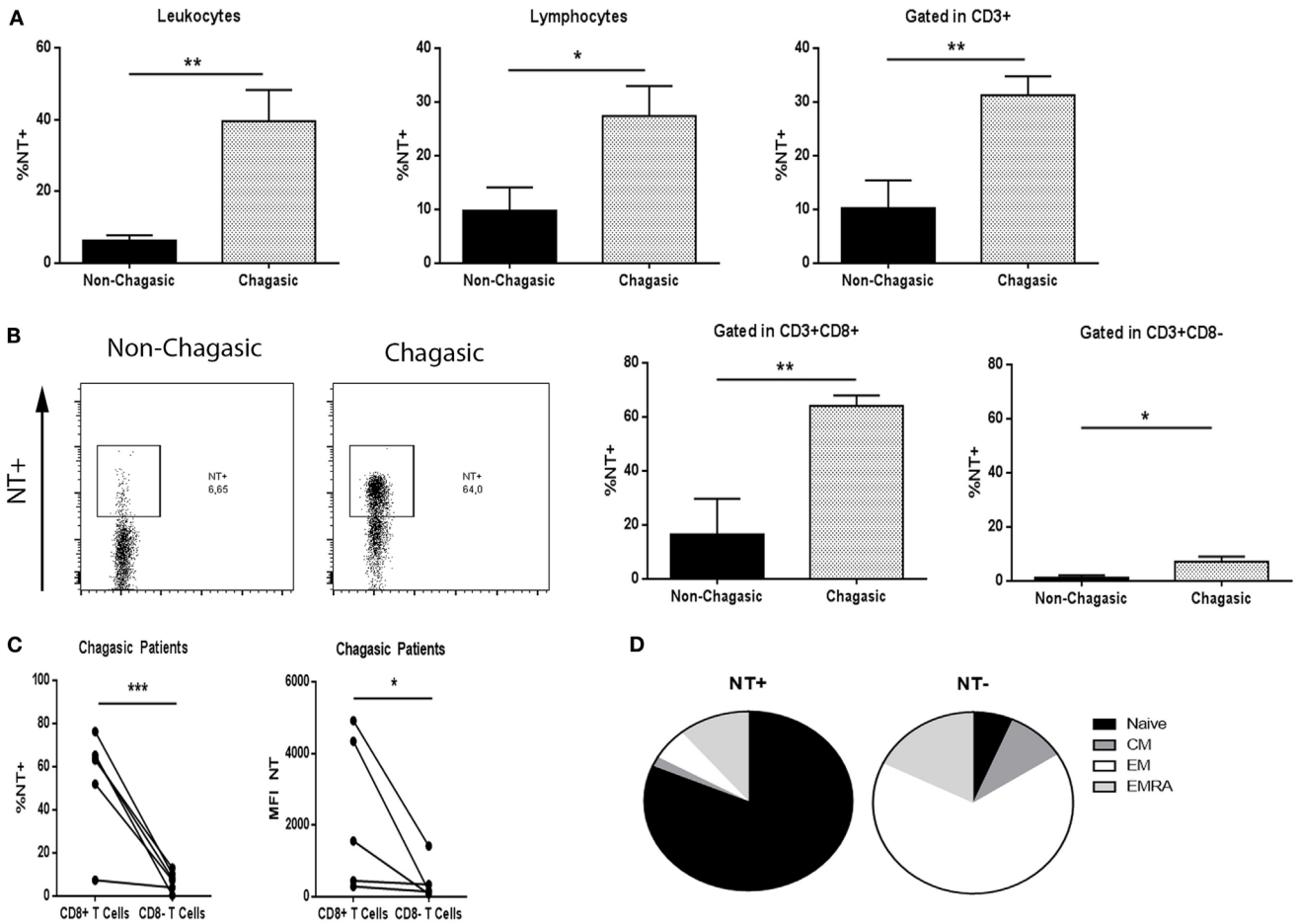

FIGURE 3 | CD8+ T lymphocytes from chagasic patients present high tyrosine nitration. **(A)** Percentage of nitrated (NT) leukocytes, lymphocytes, and T cells from seropositive patients ($n = 6$) and seronegative donors ($n = 6$). **(B)** Representative dot plot of tyrosine nitration on cytotoxic T-cell surface and percentage of NT+CD3+CD8+ and NT+CD3+CD8− lymphocytes from chagasic patients ($n = 6$) and non-chagasic donors ($n = 6$). **(C)** Percentage of NT+CD3+CD8+ and NT+CD3+CD8− and mean fluorescence intensity of NT in CD3+CD8+ and CD3+CD8− cells from chagasic patients ($n = 5$) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). **(D)** Frequency of naïve, central memory, effector memory (EM), and effector memory RA gated in NT+ or NT− in CD8+ cells from chagasic patients ($n = 4$) (naïve NT+ vs. naïve NT−, $p < 0.0001$; EM NT+ vs. EM NT−, $p < 0.0001$).

more NT+CD8+ T lymphocytes compared to non-chagasic donors (**Figure 3B**). Although nitration also increased in CD8− T cell population from seropositive patients, a main effect was observed in CD8+ T lymphocytes compared to CD8− T cells in this group of patients (**Figure 3C**). Among NT+ cytotoxic cells from Chagas patients, we have identified naïve cells as the main subpopulation that undergoes tyrosine nitration (NT+ vs. NT− 81.45 ± 3.00 vs. 6.50 ± 1.61%). On the contrary, the majority of NT− cells were EM CD8+ T cells (NT+ vs. NT− 5.73 ± 1.22 vs. 66.33 ± 4.46%) (**Figure 3D**).

Cytotoxic T Cells from Chagas Patients Were Less Functional

Even though CD4+ T lymphocytes from seropositive and seronegative individuals showed similar levels of expression of the TCRζ chain, the cytotoxic T cells exhibited a diminished amount of TCRζ in chagasic patients, as compared to non-chagasic donors (**Figure 4A**). In line with these observations, CD8 T cell population from seropositive patients showed lower frequency of CD107a+ cells, as well as IFNγ, TNF, and IL-2-producing cells after stimulation with PMA/Ionomycin (unspecific stimuli) in comparison with the same population from seronegative donors (**Figure 4B**). Furthermore, after anti-CD3/anti-CD28 stimulation (*via* TCR), the percentage of CD8+ T cells from Chagas patients positive for CD107a, IFNγ, TNF, and IL-2 was significantly diminished in NT+ cells compared to NT− cells (**Figure 4C**). The same behavior was observed when we compared the functionality of nitrated vs. no nitrated CD8+ T cells within the EM–EMRA populations (Figure S3 in Supplementary Material). These results indicated that nitrated cytotoxic T cells from chagasic patients were less functional than non-nitrated population. Moreover, chagasic patients showed a decreased percentage of CD39+ and CD73+ lymphocytes compared to control donors (**Figure 4D**). However,

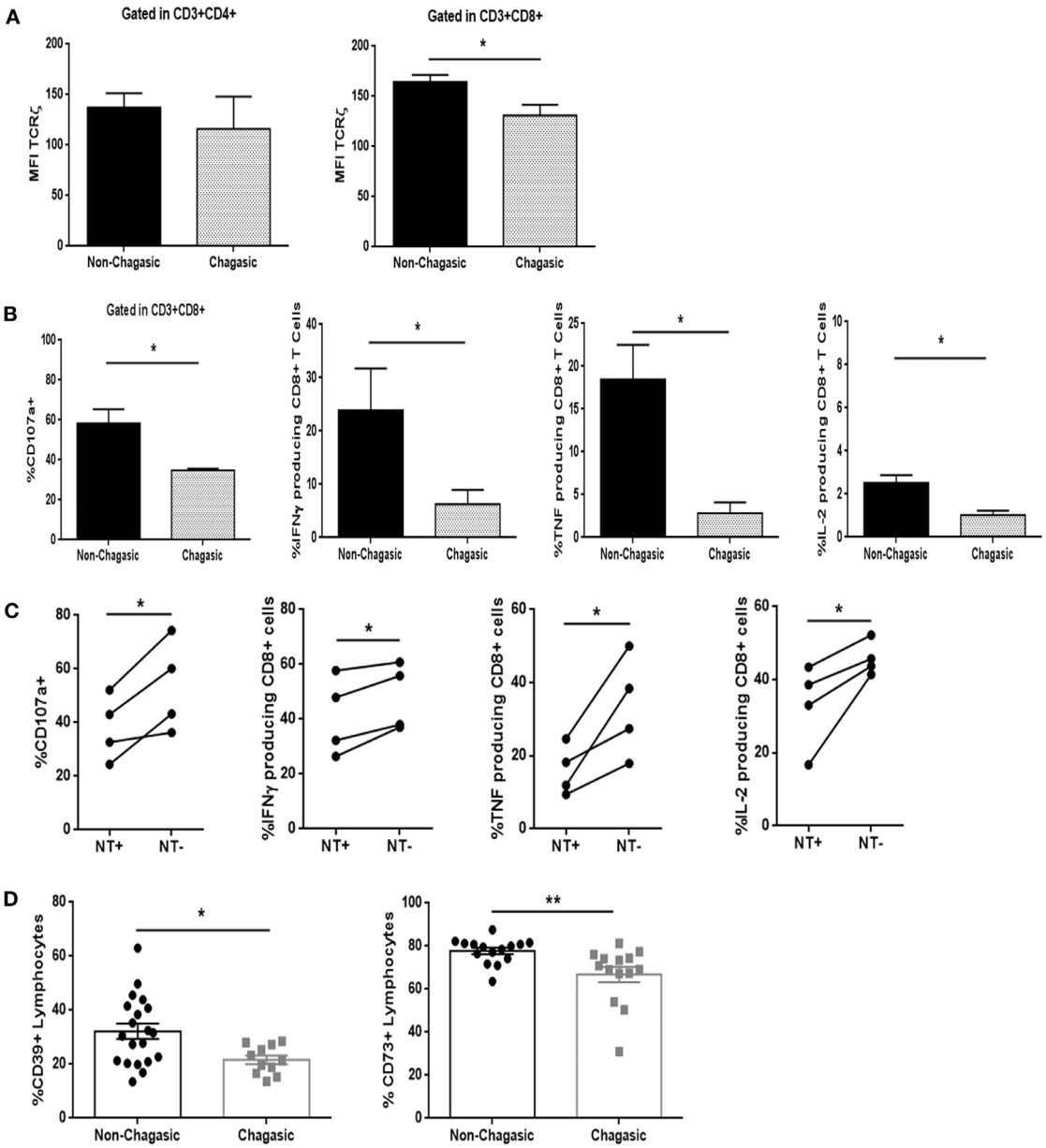

FIGURE 4 | **Cytotoxic T cells from chagasic patients are less functional than those from healthy individuals**. **(A)** Mean fluorescence intensity of TCRζ-chain in CD3+CD4+ and CD3+CD8+ cells; **(B)** frequency of CD107a+ cells, IFNγ, TNF, and IL-2-producing CD8+ T lymphocytes from chagasic patients ($n = 5$) and seronegative donors ($n = 5$) after PMA/ionomycin stimulation. **(C)** Frequency of CD107a+ cells, IFNγ, TNF, and IL-2-producing NT+ and NT− gated in CD8+ T lymphocytes from chagasic patients ($n = 4$) after anti-CD3+ anti-CD28 stimulation. **(D)** Percentage of CD39+ and CD73+ leukocytes from seropositive ($n = 20$) and seronegative ($n = 14$) individuals (*$p < 0.05$ and **$p < 0.01$).

frequency of CD8+ T cells expressing Tim-3, PD-1, or CTLA-4 was no different between chagasic patients and seronegative donors (Figure S4 in Supplementary Material). The results suggest that although cytotoxic T cells are less functional in seropositive subjects, they appear not to be a classically exhausted population since the expression of inhibitory receptors was not upregulated compared to CD8+ T cells from seronegative subjects.

IL-1β Plasma Levels Were Increased in Seropositive Patients

The cytokine plasma levels were assayed using a panel of capture beads. In seronegative and seropositive individuals, the levels of IL-6, IFNγ, TNF, and IL-4 were similar. However, IL-1β levels were higher, and the amount of IL-10 was lower in plasma from chagasic people compared with control donors (**Figure 5**).

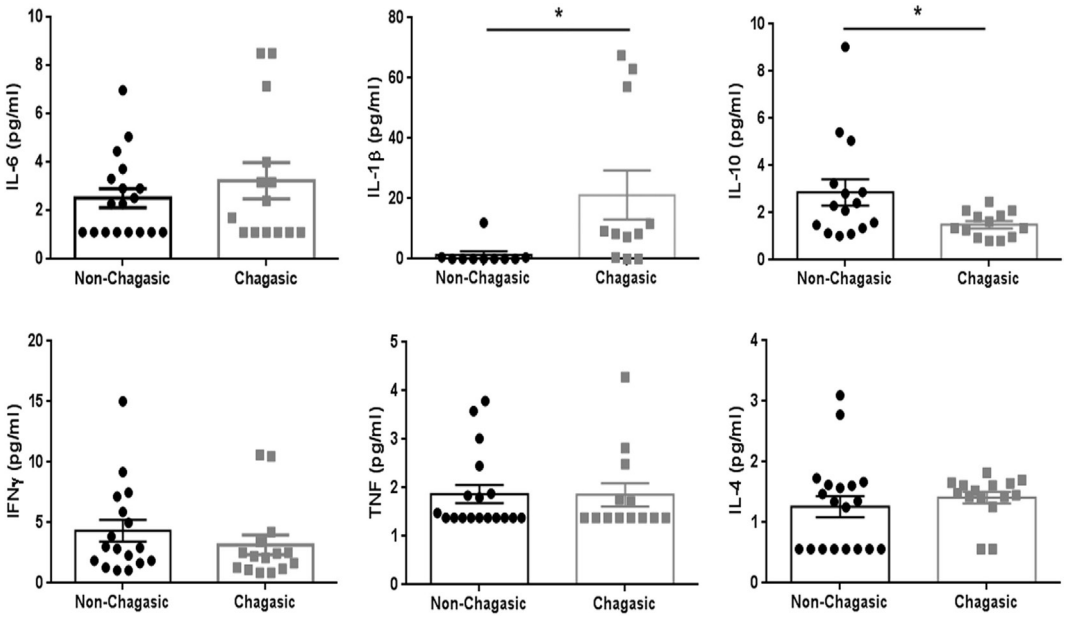

FIGURE 5 | IL-1β plasma levels are higher in seropositive patients than in seronegative donors. Plasma levels of IL-6, IL-10, IFNγ, TNF, and IL-4 (assayed by a panel of capture beads) and IL-1β (evaluated by enzyme-linked immunosorbent assay) in chagasic patients ($n = 18$) and non-chagasic donors ($n = 15$) (*$p < 0.05$).

IL-6 Prevented CD8+ T Cell Nitration and Apoptosis Susceptibility and Decreased IL-1β Production by Infected PBMCs

The IL-6 stimulation of *in vitro*-infected PBMCs obtained from healthy donors blunted *T. cruzi*-induced NT in CD3+CD8+ cells. Conversely, the treatment of these cultures with a neutralizing antibody specific for IL-6 (αIL-6) increased the percentage of *T. cruzi*-induced NT in this subpopulation (**Figure 6A**). Moreover, IL-6 treatment rescued infected CD8+ T cells from apoptosis (**Figure 6B**). Concomitantly, while IL-6 stimulation diminished the levels of IL-1β in culture supernatants from *T-cruzi*-infected PBMCs, αIL-6 treatment increased the release of IL-1β (**Figure 6C**). Furthermore, although IL-6 stimulation did not affect NO production by *T. cruzi*-infected PBMCs, the blockage of IL-6 significantly increased *T. cruzi*-induced NO production (**Figure 6D**). Additionally, IL-6 stimulation diminished NT in infected CD3+CD8+ cells from peripheral blood of chagasic patients (Figure S5 in Supplementary Material). In accordance, we found that blocking IL-6 and IL-1β significantly diminished the percentage of NT+CD8+ T cells from *in vitro*-infected PBMCs in comparison with cultures incubated with anti-IL-6 alone (**Figure 7A**). Moreover, the percentage of NO-producing monocytes significantly diminished when both cytokines were blocked compared with the inhibition of IL-6 alone (**Figure 7B**).

DISCUSSION

Multiple immunological effector mechanisms are critical for resolving *T. cruzi* infection, but considering that this parasite invades and replicates in essentially all types of mammalian cells, T cells and monocytes/macrophages are particularly important for controlling the infection. As with other intracellular parasites, *T. cruzi* antigens are processed and presented on MHC-class I molecules, leading to the recognition of parasite components by CD8+ T cells. In this regard, the study of the induction of CD8+ T cell-mediated protective immunity has become a center of intense research efforts to find control measures and prophylactic tools that could be used to produce effective therapeutic vaccines. This study shows that chronic *T. cruzi* infection leads to a significant nitration of T lymphocytes, mainly of the CD8+ T cell subset. The increased tyrosine nitration was associated with impaired effector functions and a significant fall in the number and percentage of circulating CD8+ T cells in chronic Chagas patients.

Among the reactive oxygen and nitrogen species mediating T-cell suppression is peroxynitrite, one of the most potent oxidants in the body. The hyperproduction of peroxynitrite is associated with nitration of the surface proteins in T cells (11, 12). Regarding CD8+ T cells, nitration of tyrosines within the TCR/CD8 complex disrupts the binding of the specific peptide-MHC dimers to CD8 molecules, which results in the inability of this T cell subset to bind MHC (18, 19). Furthermore, peroxynitrites also inhibit TCR signaling by preventing the association of CD3-ζ with the TCR. Our group has reported that *T. cruzi* induces an increase in splenic NT+CD8+ and NT+CD4+ T cells from infected mice by performing confocal and flow cytometric analysis of immunofluorescence staining (13). In this work, we found that the increased nitration of CD8+ T cells was associated with a lower capacity for activation (TCRζ expression), a

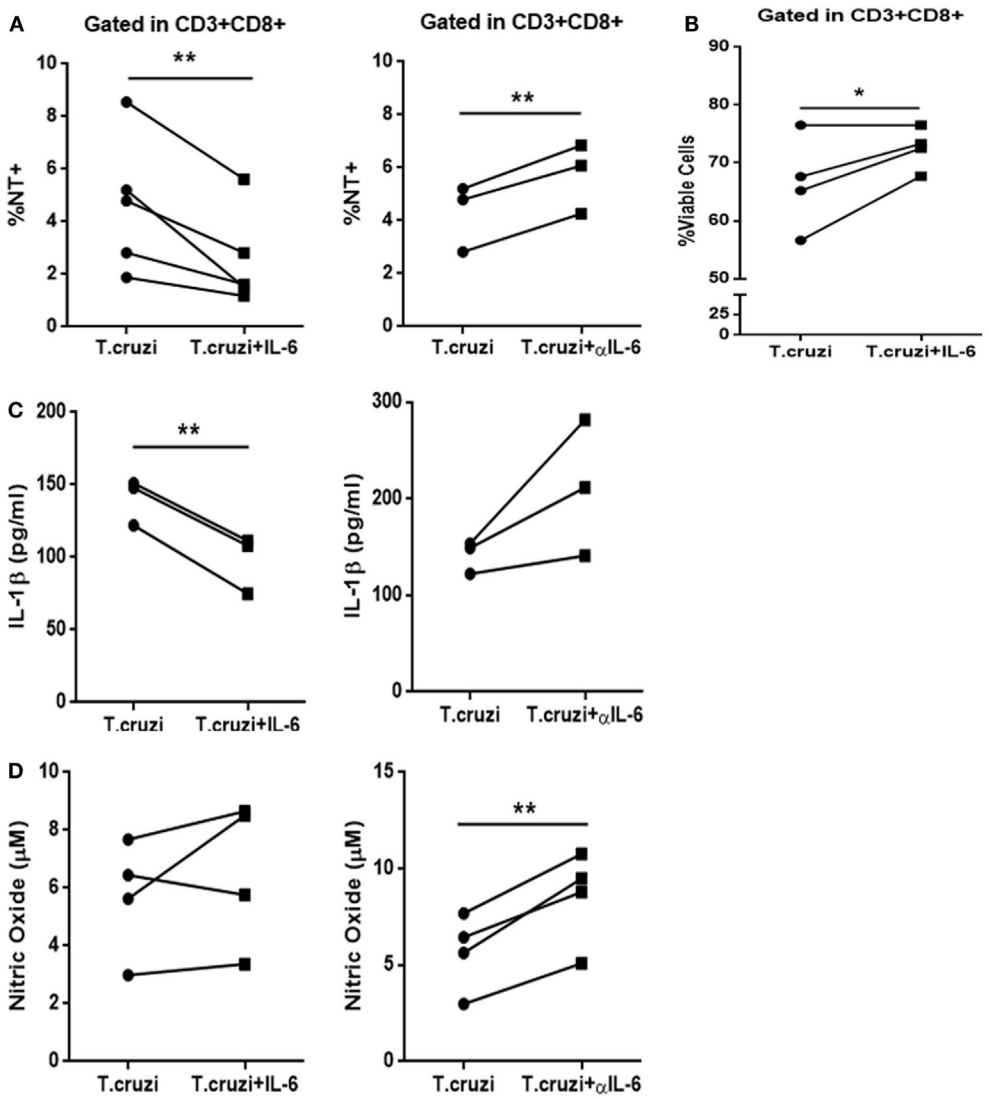

FIGURE 6 | IL-6 prevents nitration, increases survival of CD3+CD8+ cells, and decreases IL-1β levels after infection. **(A)** Percentage of NT+CD3+CD8+ cells in infected peripheral blood mononuclear cells (PBMCs) from non-chagasic ($n = 5$) donors after 48 h of stimulation with IL-6 (*Trypanosoma cruzi* + IL-6) or αIL-6 (*T. cruzi* + αIL-6) or medium alone (*T. cruzi*). **(B)** Percentage of viable CD3+CD8+ cells infected with *T. cruzi* (*T. cruzi*) or infected and stimulated with IL-6 (*T. cruzi* + IL-6). **(C)** IL-1β levels and **(D)** nitric oxide levels in culture supernatants of infected PBMCs after 48 h of stimulation with IL-6 (*T. cruzi* + IL-6) or αIL-6 (*T. cruzi* + αIL-6) or medium alone (*T. cruzi*) (*$p < 0.05$ and **$p < 0.01$).

diminished production of cytokines (IFNγ, TNF, and IL-2) and deactivation of cytotoxic functions (CD107a expression) in this cell population. Strikingly, cellular nitration seems to be reversible, since the incubation of infected leukocytes from seropositive patients with IL-6 significantly diminished the percentage of NT+ lymphocytes.

Leukocytes from chagasic patients significantly increased the production of NO, which correlated with increased susceptibility of CD8+ T cells to undergo spontaneous apoptosis. NO-mediated suppression of T-cell activation does not seem to be mediated by events triggered by TCR recognition but, instead, with the signaling cascade that is downstream of IL-2 (20). In human T cells, NO affects the stability of IL-2 mRNA and the release of IL-2. In this sense, NO negatively regulates intracellular signaling proteins either directly, by S-nitrosylation of crucial cysteine residues, or indirectly, by activation of cyclic-GMP-dependent protein kinase (21). Furthermore, while NO also sensitizes cells to Fas-L-mediated apoptosis (22), the anti-apoptotic molecule Bcl-2 diminished the susceptibility to NO-induced apoptosis (23). In line with these reports, we found that CD8+ T cells from seropositive individuals showed a significantly decreased expression of Bcl-2 concomitant with the significant diminution in their viability. The increased susceptibility to apoptosis was in accordance with the diminution in the percentage as well as

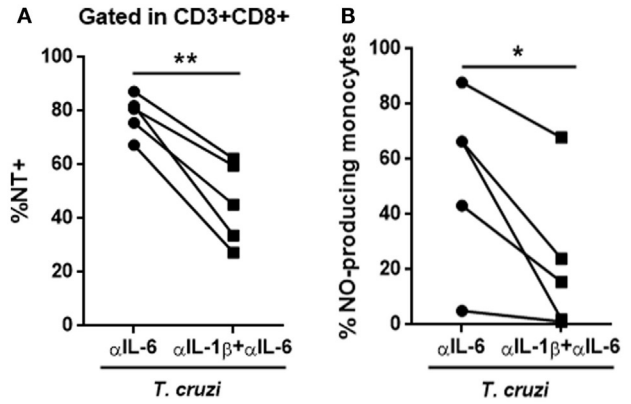

FIGURE 7 | The blockage of IL-6 and IL-1β reverts the phenotype induced by anti-IL-6. **(A)** Percentage of NT+CD8+ T cells and **(B)** NO-producing monocytes (CD14+) in *in vitro*-infected peripheral blood mononuclear cells from seronegative ($n = 5$) subjects after 48 h of stimulation with anti-IL-6 or anti-IL-6 + anti-IL-1β (*$p < 0.05$ and **$p < 0.01$).

absolute number of total T cells at the expense of diminution of CD8+ T cells but not of T helper cells or B cells.

It was reported that NO levels, as measured by Griess reagent assay, were not significantly increased in the plasma of seropositive subjects compared with those in seronegative samples. However, the plasma level of 3-nitrotyrosine (NT), was increased in seropositive subjects (24). The results indicated that seropositive subjects with Chagas disease are exposed to increased nitrosative stress. In total agreement with this report, we observed no significant increase in plasma levels of nitrate/nitrite of seropositive subjects compared with seronegative donors. Nevertheless, the leukocytes from chagasic patients significantly increased the production of NO, suggesting that NO produced by leukocytes, rather than soluble NO, may contribute to increased nitrosative stress in patients with Chagas disease, evidencing the importance of cell-to-cell contact for NO inhibitory effector functions (25, 26). Although tyrosine nitration constitutes one of the mechanisms employed by MDSC to suppress T cell response through cell-to-cell contact, it may also be dependent on the activity of myeloperoxidase, secreted by monocytes and polymorphonuclear neutrophils (27).

In accordance with our recent observations, while IL-1β plasma levels were significantly increased in chagasic patients, the stimulation of *in vitro*-infected PBMCs with IL-6 significantly diminished the production of IL-1β. Moreover, IL-6 diminished the nitration rate and improved the survival of CD8+ T cells. In agreement with this result, we have previously shown that IL-6 acts as a survival factor for infected target cells (28, 29). We have observed that IL-6 regulates IL-1β-induced NO production, and that excessive oxidative stress accounts for the increased mortality of *T. cruzi*-infected IL6KO mice (unpublished results). The anti-inflammatory action of IL-6 appears to be central to controlling cardiac and systemic oxidative stress, promoting cellular rescue against apoptosis, and protecting infected IL-6-deficient mice against death. In this model, this was clearly illustrated by the fact that the percentage of nitrated CD8+ T cells observed after blocking IL-6 was significantly diminished when anti-IL-6 was combined with anti-IL-1β. In accordance, IL-6 stimulation of peripheral blood cells from chagasic patients induced a significant diminution of nitration of CD8+ T cells. Our results strongly suggest that IL-6 could represent a key factor for regulating the nitration of proteins on human cytotoxic T cells.

One potential regulatory system that could have a role in the IL-6-induced anti-inflammatory effects is the extracellular levels of adenosine. *T. cruzi* infection of cardiac tissue induces an influx of immune cells that consume large quantities of oxygen. Ischemic cells rapidly respond to the hypoxic and inflammatory environment by releasing ATP (normally present within cardiomyocytes in millimolar concentrations). Once in the extracellular environment, ATP is converted to AMP and then to adenosine by the CD39 and CD73 ectoenzymes, respectively (30). In pathological conditions, high levels of ATP act as a pro-inflammatory danger signal, activating the inflammasome that processes pro-IL-1β into mature IL-1β (31, 32). In this sense, it has been suggested that CD39 expression may contribute to dampening the ongoing inflammatory processes and/or rescue the cells from ATP-induced apoptosis/necrosis (33). Ultimately, adenosine exerts potent anti-inflammatory effects on different immune cell types. Recently, we reported that purinergic signaling is clue in regulating the immune response to experimental *T. cruzi* infection. The temporal pharmacological inhibition of CD73 during the early acute phase of the infection induces microbicidal mechanisms, with the concomitant reduction in cardiac parasite load, improving the outcome of chronic cardiomyopathy (34). In agreement with a previous report that indicated CD73 expression on T cells is downregulated in chronic HIV infection (35), in this work, we observed lower expression of CD39 and CD73 on fresh explanted lymphocytes from chagasic patients compared to seronegative donor. Thus, it is plausible that the downregulation of ATP catabolic enzymes could be involved in the increased plasma IL-1β levels in chagasic patients.

In summary, the results of this study show that chronic *T. cruzi* infection leads to a decrease in the number and percentage of total circulating CD8+ T cells and that NO produced by leukocytes may contribute to lowering the effector function of these cells, which ultimately may results in an inefficient control of parasite replication. The findings also suggest that IL-6 could be a key factor to improve CD8+ T cell activation and survival.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LS, MR, and MA. Performed the experiments: LS, LV, NE, MR, and NP. Analyzed the data: LS, LV, NE, MR, NP, NS, MV, GB, SG, AM, and MA. Patients handling and human samples: LV, NS, MV, GB, and AM. Wrote the paper: LS and MA.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Tarleton RL. CD8+ T cells in *Trypanosoma cruzi* infection. *Semin Immunopathol* (2015) 37(3):233–8. doi:10.1007/s00281-015-0481-9
2. Savino W, Villa-Verde DM, Mendes-da-Cruz DA, Silva-Monteiro E, Perez AR, Aoki Mdel P, et al. Cytokines and cell adhesion receptors in the regulation of immunity to *Trypanosoma cruzi*. *Cytokine Growth Factor Rev* (2007) 18(1–2):107–24. doi:10.1016/j.cytogfr.2007.01.010
3. Burgos JM, Diez M, Vigliano C, Bisio M, Risso M, Duffy T, et al. Molecular identification of *Trypanosoma cruzi* discrete typing units in end-stage chronic Chagas heart disease and reactivation after heart transplantation. *Clin Infect Dis* (2010) 51(5):485–95. doi:10.1086/655680
4. Aoki MP, Guinazu NL, Pellegrini AV, Gotoh T, Masih DT, Gea S. Cruzipain, a major *Trypanosoma cruzi* antigen, promotes arginase-2 expression and survival of neonatal mouse cardiomyocytes. *Am J Physiol Cell Physiol* (2004) 286(2):C206–12. doi:10.1152/ajpcell.00282.2003
5. Aoki MP, Carrera-Silva EA, Cuervo H, Fresno M, Girones N, Gea S. Nonimmune cells contribute to crosstalk between immune cells and inflammatory mediators in the innate response to *Trypanosoma cruzi* infection. *J Parasitol Res* (2012) 2012:13. doi:10.1155/2012/737324
6. Mabbott NA, Sutherland IA, Sternberg JM. Suppressor macrophages in *Trypanosoma brucei* infection: nitric oxide is related to both suppressive activity and lifespan in vivo. *Parasite Immunol* (1995) 17(3):143–50. doi:10.1111/j.1365-3024.1995.tb01016.x
7. Jenson JS, O'Connor R, Osborne J, Devaney E. Infection with *Brugia microfilariae* induces apoptosis of CD4(+) T lymphocytes: a mechanism of immune unresponsiveness in filariasis. *Eur J Immunol* (2002) 32(3):858–67. doi:10.1002/1521-4141(200203)32:3<858::AID-IMMU858>3.0.CO;2-E
8. Rockett KA, Awburn MM, Rockett EJ, Cowden WB, Clark IA. Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunol* (1994) 16(5):243–9. doi:10.1111/j.1365-3024.1994.tb00346.x
9. Saio M, Radoja S, Marino M, Frey AB. Tumor-infiltrating macrophages induce apoptosis in activated CD8(+) T cells by a mechanism requiring cell contact and mediated by both the cell-associated form of TNF and nitric oxide. *J Immunol* (2001) 167(10):5583–93. doi:10.4049/jimmunol.167.10.5583
10. Hirst DG, Robson T. Nitrosative stress as a mediator of apoptosis: implications for cancer therapy. *Curr Pharm Des* (2010) 16(1):45–55. doi:10.2174/138161210789941838
11. Sawa T, Akaike T, Maeda H. Tyrosine nitration by peroxynitrite formed from nitric oxide and superoxide generated by xanthine oxidase. *J Biol Chem* (2000) 275(42):32467–74. doi:10.1074/jbc.M910169199
12. Gunaydin H, Houk KN. Mechanisms of peroxynitrite-mediated nitration of tyrosine. *Chem Res Toxicol* (2009) 22(5):894–8. doi:10.1021/tx800463y
13. Arocena AR, Onofrio LI, Pellegrini AV, Carrera-Silva AE, Paroli A, Cano RC, et al. Myeloid-derived suppressor cells are key players in the resolution of inflammation during a model of acute infection. *Eur J Immunol* (2014) 44(1):184–94. doi:10.1002/eji.201343606
14. Albareda MC, Laucella SA, Alvarez MG, Armenti AH, Bertochi G, Tarleton RL, et al. *Trypanosoma cruzi* modulates the profile of memory CD8+ T cells in chronic Chagas' disease patients. *Int Immunol* (2006) 18(3):465–71. doi:10.1093/intimm/dxh387
15. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* (2004) 22:745–63. doi:10.1146/annurev.immunol.22.012703.104702
16. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol* (1988) 141:2407–12.
17. Plan.Nacional.de.Chagas. *Plan Nacional de Chagas 2011–2015*. Boletín Oficial Junio – Res 867/2012. Argentina: Official Bulletin of National Ministry of Health (2012).
18. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* (2007) 13(7):828–35. doi:10.1038/nm1609
19. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J Immunol* (2010) 184(6):3106–16. doi:10.4049/jimmunol.0902661
20. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol* (2002) 168(2):689–95. doi:10.4049/jimmunol.168.2.689
21. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* (2005) 5(8):641–54. doi:10.1038/nri1668
22. Bonavida B, Garban H. Nitric oxide-mediated sensitization of resistant tumor cells to apoptosis by chemo-immunotherapeutics. *Redox Biol* (2015) 6:486–94. doi:10.1016/j.redox.2015.08.013
23. Messmer UK, Reed UK, Brune B. Bcl-2 protects macrophages from nitric oxide-induced apoptosis. *J Biol Chem* (1996) 271(33):20192–7. doi:10.1074/jbc.271.33.20192
24. Dhiman M, Estrada-Franco JG, Pando JM, Ramirez-Aguilar FJ, Spratt H, Vazquez-Corzo S, et al. Increased myeloperoxidase activity and protein nitration are indicators of inflammation in patients with Chagas' disease. *Clin Vaccine Immunol* (2009) 16(5):660–6. doi:10.1128/CVI.00019-09
25. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* (2009) 9(3):162–74. doi:10.1038/nri2506
26. Lu T, Gabrilovich DI. Molecular pathways: tumor-infiltrating myeloid cells and reactive oxygen species in regulation of tumor microenvironment. *Clin Cancer Res* (2012) 18(18):4877–82. doi:10.1158/1078-0432.CCR-11-2939
27. Predonzani A, Cali B, Agnellini AH, Molon B. Spotlights on immunological effects of reactive nitrogen species: when inflammation says nitric oxide. *World J Exp Med* (2015) 5(2):64–76. doi:10.5493/wjem.v5.i2.64
28. Ponce NE, Cano RC, Carrera-Silva EA, Lima AP, Gea S, Aoki MP. Toll-like receptor-2 and interleukin-6 mediate cardiomyocyte protection from apoptosis during *Trypanosoma cruzi* murine infection. *Med Microbiol Immunol* (2012) 201(2):145–55. doi:10.1007/s00430-011-0216-z
29. Ponce NE, Carrera-Silva EA, Pellegrini AV, Cazorla SI, Malchiodi EL, Lima AP, et al. *Trypanosoma cruzi*, the causative agent of Chagas disease, modulates interleukin-6-induced STAT3 phosphorylation via gp130 cleavage in different host cells. *Biochim Biophys Acta* (2013) 1832(3):485–94. doi:10.1016/j.bbadis.2012.12.003
30. Antonioli L, Pacher P, Vizi ES, Hasko G. CD39 and CD73 in immunity and inflammation. *Trends Mol Med* (2013) 19(6):355–67. doi:10.1016/j.molmed.2013.03.005

31. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. *Cell* (2014) 157(5):1013–22. doi:10.1016/j.cell.2014.04.007
32. Garg NJ. Inflammasomes in cardiovascular diseases. *Am J Cardiovasc Dis* (2011) 1(3):244–54.
33. Zhou Q, Yan J, Putheti P, Wu Y, Sun X, Toxavidis V, et al. Isolated CD39 expression on CD4+ T cells denotes both regulatory and memory populations. *Am J Transplant* (2009) 9(10):2303–11. doi:10.1111/j.1600-6143.2009.02777.x
34. Ponce NE, Sanmarco LM, Eberhardt N, Garcia MC, Rivarola HW, Cano RC, et al. CD73 inhibition shifts cardiac macrophage polarization toward a microbicidal phenotype and ameliorates the outcome of experimental Chagas cardiomyopathy. *J Immunol* (2016) 197(3):814–23. doi:10.4049/jimmunol.1600371
35. Toth I, Le AQ, Hartjen P, Thomssen A, Matzat V, Lehmann C, et al. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *J Leukoc Biol* (2013) 94(4):551–61. doi:10.1189/jlb.0113018

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Vaccination with Altered Peptide Ligands of a *Plasmodium berghei* Circumsporozoite Protein CD8 T-Cell Epitope: A Model to Generate T Cells Resistant to Immune Interference by Polymorphic Epitopes

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Many pathogens, including the malaria parasite *Plasmodium falciparum*, display high levels of polymorphism within T-cell epitope regions of proteins associated with protective immunity. The T-cell epitope variants are often non-cross-reactive. Herein, we show in a murine model, which modifies a protective CD8 T-cell epitope from the circumsporozoite protein (CS) of *Plasmodium berghei* (SYIPSAEKI), that simultaneous or sequential co-stimulation with two of its putative similarly non-cross-reactive altered peptide ligand (APL) epitopes (SYIPSAEDI or SYIPSAEAI) has radically different effects on immunity. Hence, co-immunization or sequential stimulation *in vivo* of SYIPSAEKI with its APL antagonist SYIPSAEDI decreases immunity to both epitopes. By contrast, co-immunization with SYIPSAEAI has no apparent initial effect, but it renders the immune response to SYIPSAEKI resistant to being turned off by subsequent immunization with SYIPSAEDI. These results suggest a novel strategy for vaccines that target polymorphic epitopes potentially capable of mutual immune interference in the field, by initiating an immune response by co-immunization with the desired index epitope, together with a carefully selected "potentiator" APL peptide.

Keywords: cross-reactivity, altered peptide ligand, antagonism, T cell, dendritic cell, vaccine, *Plasmodium*, malaria

INTRODUCTION

Foreign epitopes complexed with host MHC molecules are the target of recognition by cognate antigen-specific T cells. The development of effective preventive vaccines requires the induction of long-lasting immunity in the human population, with the capacity to induce responses to naturally occurring strains of a pathogen bearing different variants of protective epitopes. Pathogens bearing variant epitopes can evade or skew the immune system in a variety of ways leading to loss of protective immunity. A direct form of immune evasion involves the mutation of amino acid residues that are required for peptide binding to MHC. Other more sophisticated forms of polymorphic

immune evasion also exist [reviewed in Ref. (1)]. Altered peptide ligand (APL) antagonism involves the concurrent presentation of selected closely related epitope variants. This can inhibit T cell effector functions such as cytokine production, cytotoxicity, or proliferation. The use of APL ligands for immune evasion has been well documented across human immunodeficiency virus (HIV), hepatitis B and C (HBV and HBC), and malaria parasite infections [reviewed in Ref. (1)]. Another form of immune evasion, known as "immune interference," also involves the concurrent or sequential presentation of related epitope variants to T cells. Immune interference results a failure to induce memory T cells from naive precursors. CD8 T-cell epitope variants causing APL antagonism and immune interference have been identified in *Plasmodium falciparum* and are major contributors to the parasite population structure observed in malaria-endemic regions of the world (2, 3).

Variant-specific immunity has been documented for the lead preerythrocytic malaria vaccine, RTS,S, which contains a single polymorphic variant of the circumsporozoite protein (CS) of *P. falciparum* (4). However, vaccines that target individual variants provide limited or short-lasting benefit in protection from malaria. This occurs because the non-targeted parasite variants take over the vacated niches in the vaccine-treated host population [discussed in Ref. (1)]. Attempts to provide more broadly cross-reactive responses against malaria have included mixing polymorphic variants of a single antigenic protein into one vaccine formulation, for example, including both the 3D7 and FC27 variants of merozoite surface protein 2 from *P. falciparum* into a single recombinant erythrocytic-stage vaccine (5); or mixing multiple target proteins into one formulation, for example, including the two key preerythrocytic-stage antigens CS and thrombospondin-related adhesive protein (TRAP) (6). Both attempts yielded disappointing results, with the combination of antigens showing little increase, and in the case of CS and TRAP, even a decrease, in immunity. As an alternative, recent studies have evaluated whether changing the adjuvant used to deliver a protective preerythrocytic *Plasmodium berghei* CD8 T-cell epitope (SYIPSAEKI) in murine models could broaden the pattern of T cell cross-reactivity (7). SYIPSAEKI (KI) is the immunodominant CD8 T-cell epitope of the *P. berghei* circumsporozoite (CS) protein, and the presence of vaccine-induced IFNγ-producing splenic T cells to this epitope correlates with protective efficacy (8). The contact T cell receptor (TCR) amino acid residue has been identified to be position 8, and in recent studies engineered amino acid changes to this position resulted in the generation of useful variants to model how an amino acid change can lead to loss of T cell cross-reactivity, without a decrease in MHC-binding capacity (8). Disappointingly, although both pro-inflammatory (montanide and poly I:C) and non-inflammatory nanoparticle-based vaccines induced comparable and robust peptide-specific responses to KI, they induced limited cross-reactivity to variants SYISAEDI (DI) or SYIPSAEAI (AI) (8). The limited cross-reactivity was also shown not to be due to holes in the naïve T cell repertoire, since vaccines formulated with each APL individually (including KI, AI, or DI) were capable of inducing robust immune responses to the immunizing index APL (7). While neither DI nor AI are natural variants, inclusion of such APLs in malaria vaccines has the potential to induce a broader spectrum of cross-reactive responses different from immunization with the index epitope alone. Conversely, if any of the pooled variants had antagonistic APL properties, such an approach could restrict the spectrum of responses, or turn off preexisting immunity, thus inducing immune interference, as has been described for naturally occurring variants of CS T-cell epitopes (9). Testing the consequences of immunizing with combinations or mixtures of APL variants has not been explored in such previous studies.

Understanding the mechanism by which immune interference promotes parasite survival and influences parasite population dynamics is central to successful vaccine development. In the present study, a murine model was utilized to dissect the complex patterns of immunity induced by well characterized selected APL variants of the *P. berghei* CD8 T-cell epitope KI, DI, and AI. The findings demonstrate a novel vaccination strategy that broadens T cell immunity and is resistant to being "turned off" by immune interference. This new vaccination strategy is likely to be of utility in tackling diverse pathogens beyond malaria that utilizes APLs for immune evasion, such as HIV and hepatitis C [reviewed in Ref. (1)].

ANIMALS AND METHODS

Animals

Female BALB/c (H-2^d) mice, 6–10 weeks of age were sourced from the Walter and Eliza Hall Institute, Melbourne, VIC, Australia, or bred at the Austin Research Institute Biomedical Animal Research Laboratory. The study was approved by Austin Research Institute Animal Care and Use Committee approved all animal procedures.

Peptides

The immunodominant CD8 epitope of *P. berghei* circumsporozoite protein SYIPSAE**K**I, its variants SYIPSAE**D**I and SYIPSAE**A**I, and the influenza CD8 epitope NPKd (TYQRTRALV) were synthesized by to >99% purity by Auspep (Melbourne, VIC, Australia). Peptides were confirmed to be non-toxic at the doses used by incubation of splenocytes overnight and assessment of impairment of ConA responses by ELISPOT.

Immunizations

Bone marrow-derived dendritic cells (DCs) were generated as previously described 10) by culturing bone marrow cells from female BALB/c mice for 6–8 days in RPMI 1640 (Gibco, USA) supplemented with 10% heat inactivated fetal calf serum (CSL, Australia), 20 mM HEPES buffer (JRH, USA), 4 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin sulfate, 100 mM β-mercaptoethanol (all Sigma, Australia), 1,000 U/mL granulocyte and macrophage colony stimulating factor (GM-CSF, Pharmingen, USA), and 10 ng/mL of interleukin-4 (IL-4, Pharmingen, USA). Female mice were used to avoid rejection of the transferred DCs. DCs were CD11c+MHCII+ and Gr1− with the majority (>80%) co-expressing CD11b+ indicating an inflammatory DC phenotype (data not shown). Harvested DCs were pulsed with 5 μg/mL peptide at 37°C for 1 h. For

co-presentation DCs were pulsed with one peptide for 1 h, followed by the variant peptide for a further 2 h. No phenotypic changes were observed (using markers CD11c, CD11b, Gr1, MHCII, CD40, CD80, or CD86) following the peptide pulse for any of the peptides used, and no changes in inflammatory (IL6, TNF, and IL1) or suppressor cytokines (IL-10) in the supernatant, or changes in DC viability (by trypan blue). 10^6 DCs in 100 µL sterile PBS were injected intradermally into the hind footpads (50 µL per footpad) or into the base of the tail. Experiments involving immunization with one peptide or peptide combination on day 0 followed by booster immunization with another peptide on day 14 included control groups that received the same peptide or peptide combination as single immunization on day 0 only and another group that received a single immunization with the booster peptide on day 14 only. For all sequential immunizations, ELISPOT assays were performed between days 14 and 17 following the last immunization.

Ex Vivo Elispot Assays

ELISPOT assays were carried out in 96 well mixed acetate plates (Millipore, Watford, UK) as previously described (8). MAIPS4510 plates, pretreated with 50 µL/well methanol, were used for IFNγ assays; IL-4 and IL-10 secretion was assessed in MAHAS4510 plates. Plates were coated with 5 µg/mL anti-mouse IFNγ mAb (Mab AN18, Mabtech), 10 µg/mL anti-mouse IL-4 mAb (clone BVD4-1D11, Pharmingen), or 10 µg/mL anti-mouse IL-10 mAb (Pharmingen) and blocked with culture media RPMI 1640 (Gibco, USA) supplemented with 1% heat inactivated mouse serum, 20 mM HEPES buffer (JRH, USA), 4 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin sulfate, and 100 mM β-mercaptoethanol (all Sigma, Australia). Around 500,000 freshly isolated splenocytes were cultured in the presence of 10^{-5}–10^{-11} M peptide in triplicate or quadruplicate wells. Plates were incubated at 37°C, 5% CO_2 for 16 h for IFNγ assays, 24 h for IL-4, and 48 h for IL-10 assays. In preliminary assays, cognate responses from splenic T cells from animals immunized with KI, AI, or DI alone were eliminated when splenocytes were depleted of CD8 T cells (Dynabeads, UK) prior to the assay, confirming responses to these minimal CD8 T cell peptide epitopes come from CD8 T cell as expected.

For *In Vitro* Antagonism Assays

Splenocytes were restimulated with a suboptimal concentration of the index peptide KI for 1 h; the altered variant DI was added at different concentrations and incubated for a further 16 h washed, counted, and plated out as per (9).

Plates were incubated with 1 µg/mL biotinylated anti-mouse IFNγ mAb (Mab R4-6A2-Biotin, Mabtech, Sweden), biotinylated anti-mouse IL-4 mAb (clone BVD6-24G2-biotin, Pharmingen, USA), or biotinylated anti-mouse IL-10 mAb (Pharmingen, USA), followed by 1 µg/mL streptavidin–alkaline phosphatase (AP) (Mabtech, Sweden) for IFNγ assay or 0.1 µg/mL extravidin–AP (Sigma, USA) for IL-4 and IL-10 assays and developed using a colorimetric AP Kit (BioRad, Hercules, CA, USA). The number of spot forming units (SFU) per well was scored using the AID ELISPOT reader (Autoimmun Diagnostika GmbH, Germany) with AID ELISpot software version 2.9 (Autoimmun Diagnostika GmbH, Germany).

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism (version 6.01). ELISPOT data were analyzed using two-way ANOVA followed by Tukey's *post hoc* test for comparison of all groups or Dunnett's *post hoc* test for comparison to the index peptide. The significance level used was $(\alpha) = 0.05$ for all statistical tests.

RESULTS

Co-immunization with Variants KI and DI Impairs the Generation of KI-Specific IFNγ-Secreting CD8 T Cells

To address the question whether co-immunization with epitope variants SYIPSAEKI (KI) and SYIPSAEDI (DI) could broaden the immune response, we immunized BALB/c mice with DCs loaded with both peptides KI and DI. To minimize competition for MHC class I binding clefts, and to ensure KI presentation, DCs were pulsed first with variant KI for 1 h, followed by variant DI for another 2 h at equimolar concentrations (0.5×10^{-6} M). Control mice received DCs pulsed with KI or DI alone or without peptide. No KI-specific IFNγ secreting cells were detected in mice immunized with DI-pulsed DCs, confirming that priming with DI did not induce cross-reactive T cells. DCs pulsed with KI alone induced strong KI-specific IFNγ responses, while DCs co-presenting both KI and DI (KI/DI) induced significantly less KI-specific IFNγ-producing T cells (**Figure 1A**, $p < 0.001$). This significant reduction in KI-specific IFNγ responses was still evident 6 weeks after immunization (**Figure 1B**, $n = 3$, $p < 0.001$). KI-specific IFNγ responses could not be restored to normal level by using more antigen (**Figure 1C**, $n = 3$). To rule out peptide competition for H-2K^d binding, DCs were also pulsed separately with variant KI or DI for 1 h, washed, and then mixed at 1:1 ratio (KI + DI) immediately before injection. Control mice received (i) DCs pulsed with KI + DI, (ii) DCs pulsed with either variant KI or DI alone as positive controls, or (iii) DCs without peptide as a negative control. **Figure 1D** shows that KI-specific IFNγ-secreting cells were reduced irrespective of whether DCs had been loaded with KI and DI together ($p = 0.009$) or separately ($p < 0.001$). No KI-specific IFNγ secretion was observed in DI-immunized mice as observed previously. These data suggest that DI interfered with priming of KI-specific IFNγ secreting T cells *in vivo* when presented together with KI on the same or different APC. Therefore, this interference is unlikely to have been caused by peptide competition for MHC binding. Furthermore, no interference with KI-specific T cell priming was observed with DCs co-presenting KI together with the unrelated H-2K^d-restricted CD8 T-cell epitope NPK^d (TYQRTRALV) from the influenza A virus nucleoprotein (aa 147–155) (11), a peptide with strong binding affinity for H-2k^d (12, 13). The immune interference observed with KI + DI immunization appeared therefore unlikely to be due to the DC-loading protocol but suggests inhibitory effects were mediated specifically by variant DI.

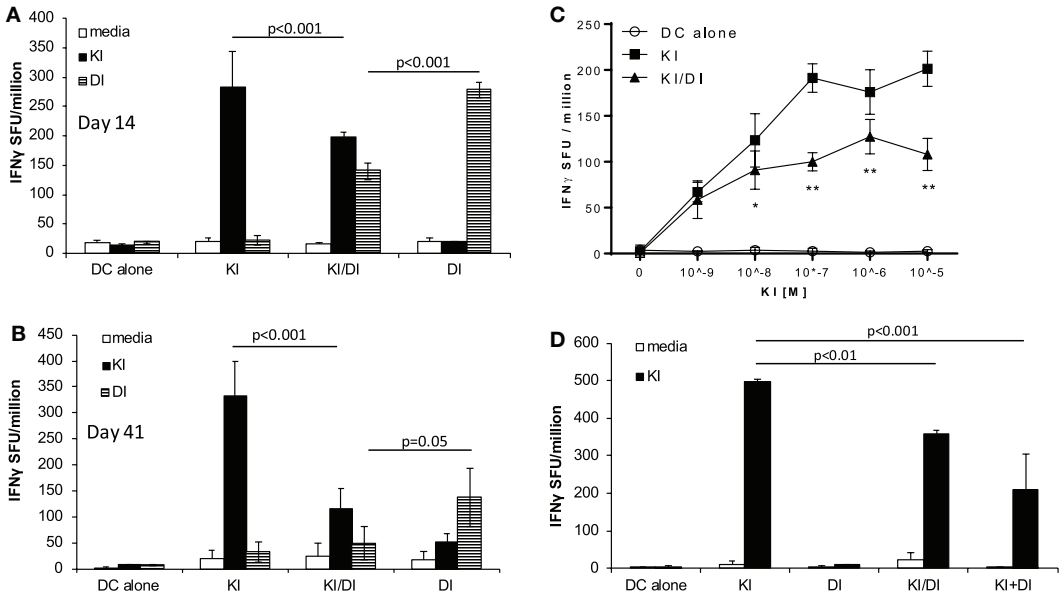

FIGURE 1 | **Impaired IFNγ secretion following co-immunization with peptides KI and DI presented on the same or on different DC**. BALB/c mice were immunized with dendritic cells (DCs) pulsed with either peptide KI or DI alone or with DCs pulsed with KI for 1 h followed by DI for 2 h. Control mice received DCs without peptide. IFNγ secretion was assessed by *ex vivo* ELISpot assay **(A)** 14 days or **(B)** 41 days after immunization. **(C)** IFNγ secretion upon restimulation with peptide KI at concentrations ranging from 10^{-9} to 10^{-5} M was assessed by *ex vivo* ELISpot assay 13 days after immunization. **(D)** BALB/c mice were immunized with DCs pulsed with either peptide KI or DI alone, with DCs pulsed with KI for 1 h then with DI for 2 h (KI/DI), or with a 1:1 mixture of DCs pulsed separately with KI or DI (KI + DI). IFNγ secretion was assessed by *ex vivo* ELISpot 17 days after immunization. Mean spot forming units ± SD are shown ($n = 3$ mice per group). Two-way ANOVA with Tukey's **(A,B,D)** or Dunnett's **(C)** multiple comparison test was used to test for statistical significance when comparing all groups or when compared to the group immunized with the index peptide KI, respectively.

Co-immunization with Variants KI and AI Does Not Interfere with KI-Specific or AI-Specific T Cell Priming

When we tested the effect of co-immunization of KI with another variant SYIPSAEAI (AI), we observed a different effect. Using the same immunization strategy as above, DCs were pulsed with KI for 1 h followed by AI for 2 h at equimolar concentration (5×10^6 M) (KI/AI). Control mice were immunized with DCs pulsed with KI and AI alone or without peptide. KI-specific IFNγ responses were assessed by *ex vivo* ELISpot assay. We observed no significant difference in KI-specific IFNγ responses between mice immunized with KI or KI/AI at 2 (**Figure 2A**) or 5 weeks (**Figure 2B**) post-immunization. Titration of peptide KI from 5×10^{-9} to 5×10^{-6} M for *ex vivo* stimulation showed a similar reactivity pattern for KI-immunized mice and KI/AI-immunized mice at all concentrations tested (**Figure 2C**). Together, these data suggest that epitope variant AI does not interfere with KI-specific T cell priming when presented together with KI *in vivo*, indicating a functional difference between the two altered peptide variants AI and DI. To assess whether KI interfered with AI-specific priming, splenocytes from mice immunized with KI/AI or AI alone were assessed for AI-specific IFNγ responses by *ex vivo* ELISpot assay. Titration of peptide AI showed a similar reactivity pattern for AI- or KI/AI-immunized mice at all concentrations tested (**Figure 2D**). These results show that co-presentation of peptides KI and AI does not interfere with T cell priming.

Across experiments, mice immunized with KI-pulsed DC varied in their KI-specific IFNγ responses. **Figure 3A** shows pooled data from 12 experiments. The mean KI-specific IFNγ response in mice immunized with DC pulsed KI/DI (mean 185 ± 146 SFU/million) was significantly lower compared to those immunized with KI alone (mean 330 ± 164 SFU/million; $p < 0.001$). By contrast, KI-specific IFNγ responses in mice co-immunized with KI/AI (mean 285 ± 197 SFU/million) was comparable to those immunized with KI alone (**Figure 3A**).

Variants KI and DI Show Potential Mutual Inhibitory Effects on T Cell Priming

We next tested the effect of KI/DI co-presentation of the priming on DI-specific IFNγ responses after immunization with DCs co-presenting KI and variant DI (KI/DI) or DI alone. Mice immunized with KI/DI had significantly fewer DI-specific IFNγ secreting cells than mice immunized with DI alone (**Figure 1A**, $p < 0.001$), an effect that was less evident at 6 weeks post-immunization (**Figure 1B**, $p = 0.05$). Across experiments KI/DI immunization elicited significantly lower DI-specific IFNγ responses (mean 98 ± 83 SFU/million) compared to mice immunized with DC pulsed with DI alone (mean 164 ± 107 SFU/million; **Figure 3B**; $p < 0.01$). Although we cannot exclude that this effect might have been due to DC being pulsed with KI first followed by DI, this observation suggests that altered peptide variants KI and DI could be mutually antagonistic/immune interfering during T cell priming *in vivo*. KI- or DI-specific IFNγ responses following

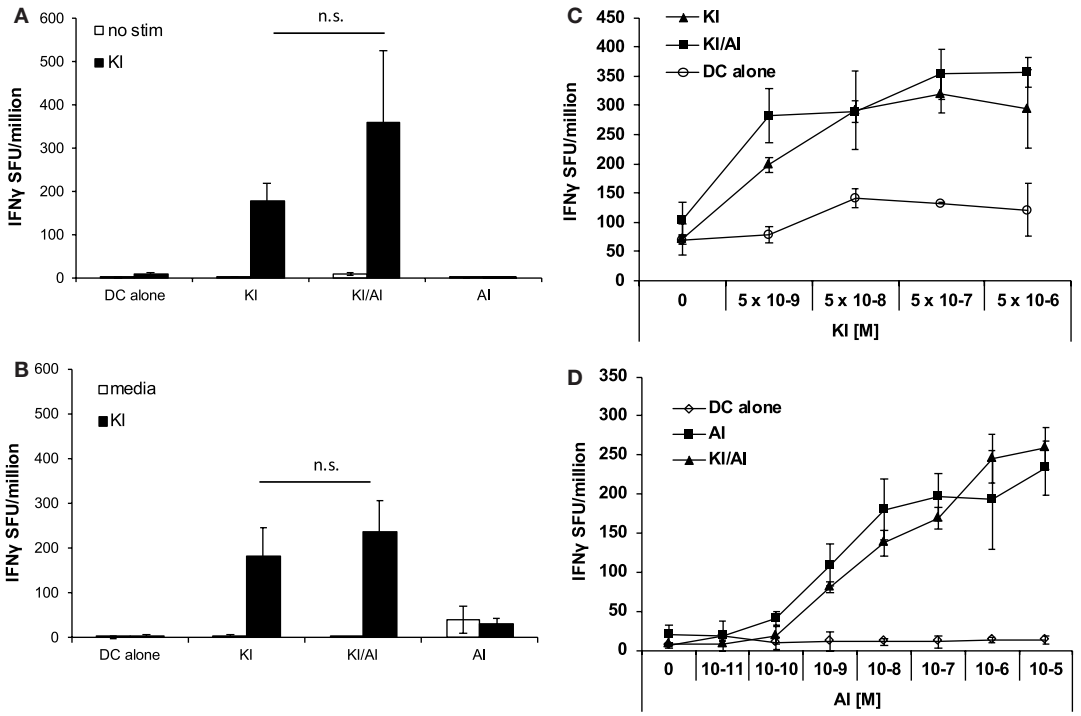

FIGURE 2 | Co-immunization with KI and AI does not interfere with T cell priming. BALB/c mice were immunized with dendritic cells (DCs) pulsed with either peptide KI or AI alone or with DC pulsed with KI for 1 h then with AI for 2 h. Control mice received DCs without peptide. **(A)** 12 days or **(B)** 31 days post-immunization IFNγ secretion was assessed by *ex vivo* ELISpot by restimulation with peptide KI at 0.5 μg/mL. **(C)** BALB/c mice were immunized with DCs pulsed with either peptide KI (triangles) or with DC pulsed with KI for 1 h then with AI for 2 h (squares). Control mice received non-pulsed DCs (open circles). IFNγ secretion was assessed by *ex vivo* ELISpot by restimulation with peptide KI at concentrations ranging from 5×10^{-9} to 5×10^{-6} M 10 days post-immunization. **(D)** BALB/c mice were immunized with DCs pulsed with either peptide AI (triangles) or with DC pulsed with KI for 1 h then with AI for 2 h (squares). Control mice received non-pulsed DCs (open circles). IFNγ secretion was assessed by *ex vivo* ELISpot by restimulation with peptide AI at concentrations ranging from 5×10^{-9} to 5×10^{-6} M 21 days post-immunization. Mean spot forming units ± SD are shown ($n = 3$ mice per group). Two-way ANOVA with Dunnett's multiple comparison test was used to test for statistical significance when compared to the groups immunized with the index peptide KI.

single immunization with the respective peptide displayed a similar response curve with peak responses elicited 2–3 weeks post-immunization (**Figure 3C**). It therefore appears unlikely that the reduced IFNγ responses following co-presentation of both peptides should result from different peptide kinetics. The implication for pathogen immune evasion is that a host exposed to both variants simultaneously might generate impaired IFNγ responses to both epitopes. This potential mutual interference was surprising and led us to investigate what would happen if mice were immunized sequentially with peptides KI and DI.

Variant DI Inhibited KI-Primed Effector T Cells *In Vivo*

To study *in vivo* effects of DI on KI-primed effector T cells, we primed BALB/c mice with the index peptide KI (d0) and 2 weeks later (d14) injected DCs presenting DI. We called this sequential immunization protocol KI–DI. Control mice received KI-pulsed DCs on day 0 (KI) or DI-pulsed DCs on day 14 (DI) or DCs without peptide (DC alone). KI-specific IFNγ responses were measured 14–17 days after the last immunization by *ex vivo* ELISpot assay. Mice primed with the index variant KI that received no further immunizations showed strong KI-specific IFNγ responses detectable 31 days post-immunization (**Figure 4A**). Mice receiving the KI–DI sequential immunizations, however, had significantly reduced numbers of KI-specific IFNγ-secreting cells (**Figure 4A**, black bars; $p < 0.01$). No difference was observed in the numbers of KI-specific IL-4-secreting cells between mice immunized with KI alone or followed by DI 14 days later (**Figure 4B**, black bars), and no KI-specific IL-10 secretion was detected in any of the groups (**Figure 4C**). These results suggest that epitope variant DI has an inhibitory effect on polyclonal KI-primed IFNγ secreting but not IL-4 secreting effector T cells *in vivo*.

DI-specific IFNγ responses were also measured after sequential immunization. Interestingly, there were significantly fewer DI-specific IFNγ-secreting cells detected in mice previously primed with KI compared with mice that were not pre-primed (**Figure 4A**, gray bars, $p < 0.001$). No significant difference in DI-specific IL-4 secretion (**Figure 4B**) and no DI-specific IL-10 secretion (**Figure 4C**) were observed in these mice. These results show that priming with the index peptide KI inhibits subsequent priming with the altered peptide variant DI.

No such interference was observed with AI-specific priming, using the above sequential immunization protocol for KI–AI

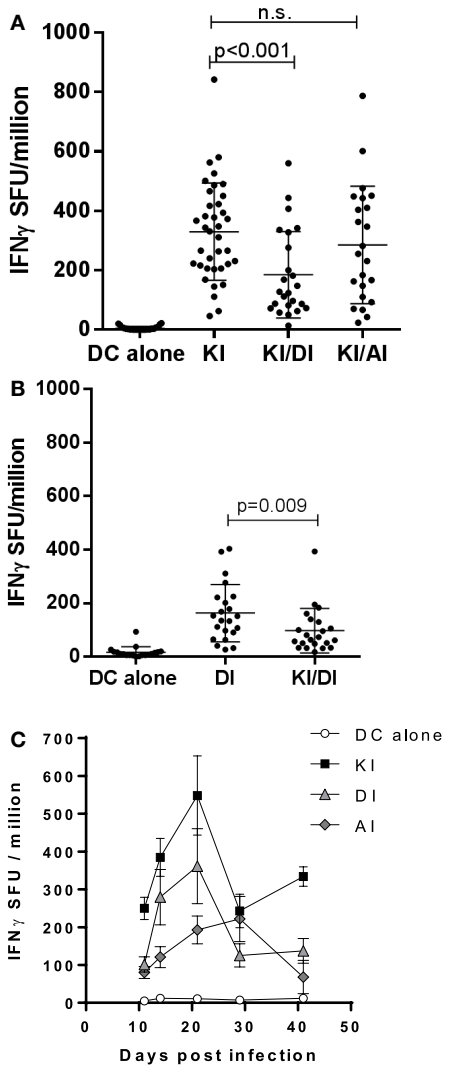

FIGURE 3 | **Pooled data**. **(A)** BALB/c mice immunized with dendritic cells (DCs) pulsed with either peptide KI alone ($n = 37$), with DCs pulsed with KI for 1 h followed by DI for 2 h ($n = 24$) or with DC pulsed with KI for 1 h then with AI for 2 h ($n = 23$). Control mice received DCs without peptide ($n = 30$). KI-specific IFNγ response was assessed by *ex vivo* ELISpot assay. Pooled data from 12 experiments are shown. Dots represent individual mice, and the horizontal line represents the mean ± SD. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test. **(B)** BALB/c mice were immunized with DCs pulsed with either peptide DI alone or with DCs pulsed with KI for 1 h followed by DI for 2 h. Control mice received DCs without peptide. DI-specific IFNγ response was assessed by *ex vivo* ELISpot assay. Pooled data from eight experiments are shown. Dots represent individual mice, and the horizontal line represents the mean ± SD. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test. **(C)** Mice were immunized with DCs pulsed with KI (black squares), DI (gray triangles), or AI (gray diamonds) alone and IFNγ responses to their respective peptides measured by *ex vivo* ELISpot assay at different time points. KI response is shown for control mice that received DCs without peptide. Mean spot forming units ± SD are shown ($n = 3$–9 mice per time point).

immunization. KI-primed mice showed strong KI-specific IFNγ responses irrespective of whether they were later immunized with AI-pulsed DCs or not (**Figure 4D**, black bars) with no statistically significant difference in the frequencies of KI-specific IFNγ-secreting effector T cells between KI- and KI–AI-immunized mice. Variant AI had no inhibitory effect on KI-specific IFNγ effector responses *in vivo*. Similarly, KI-primed mice raised competent AI-specific IFNγ-secreting cells in response KI–AI immunization, similar to AI-immunized mice (**Figure 4D**, gray bars).

KI-Specific IFNγ Responses Were Inhibited in the Presence of Epitope Variant DI *In Vitro*

The effects of antagonistic peptides have been studied on both clonal and polyclonal effector T cell responses (9, 14). Peptide variant DI has previously been shown to partially antagonize KI-specific IFNγ secretion *in vitro* in splenocytes from mice immunized with KI-expressing protein particles derived from a yeast retrotransposon (TyS3) (9). Here, we confirm that peptide DI inhibits KI-specific effector T cell responses *in vitro* using a protocol optimized in previous studies to control for the issue of competition of peptides for binding to MHC (9, 15). For the antagonism assay, splenocytes from KI-immunized mice were incubated with the suboptimal KI concentration of 10^{-8} M (0.01 μg/mL), and after 1 h DI was added at concentrations ranging from 10^{-8} to 10^{-4} M. **Figure 5** shows that addition of the altered variant DI significantly inhibited KI-specific IFNγ secretion from concentrations as low as 10^{-6} M (0.1 μg/mL). The inhibitory effect increased with increasing DI concentration, but inhibition never exceeded 60% of the total IFNγ response to the index peptide KI.

KI/AI-Primed Cells Were Not Susceptible to *In Vivo* Immune Interference by DI

Similar frequencies of KI-specific IFNγ-secreting cells were detected in the spleens from KI-immunized and KI/AI-immunized mice, although mice primed with KI or AI individually showed no cross-reactivity to the other variant (data not shown). In order to determine whether KI-specific CD8 T cells, induced by co-immunization with KI/AI, would have a similar or distinct pattern of cross-reactivity and/or susceptibility to immune interference to those induced with KI alone, mice were immunized with either KI/AI together, or KI alone on DCs (day 0), and 2 weeks later with DI-pulsed DCs (KI/AI–DI and KI–DI, respectively). Control mice received DCs pulsed with KI or without peptide. KI-specific IFNγ responses were measured 2–3 weeks later by *ex vivo* ELISpot assay. Three experiments were performed to assess the effect of variant DI on KI-specific IFNγ responses from KI/AI-primed splenocytes. The responses of mice immunized with KI were as expected susceptible to immune interference by subsequent immunization with DI (**Figure 6**). By contrast, we detected no significant difference in KI-specific IFNγ responses between KI/AI-primed mice that received a subsequent immunization with DI-pulsed DCs on day 14 and those that did not (**Figure 6**). Mice immunized with DCs presenting the variant AI alone on day 0 followed by immunization with DCs pulsed with variant DI on day 14 had no detectable KI-specific IFNγ responses (**Figure 6**), consistent with the previously shown lack

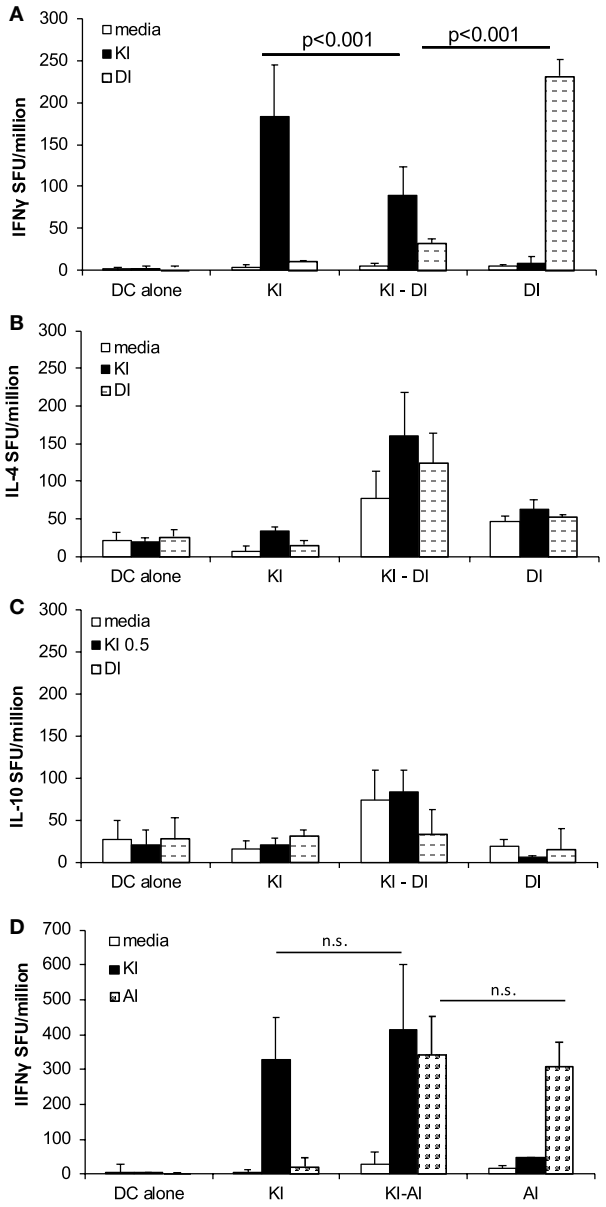

FIGURE 4 | Immunization with variant DI but not variant AI inhibits KI-specific IFNγ-secreting effector T cells *in vivo*. BALB/c mice were immunized with dendritic cells (DCs) pulsed with KI on day 0 (KI), DCs pulsed with DI on day 14 (DI) or with KI-pulsed DCs on day 0 followed by DI-pulsed DCs on day 14 (KI–DI). The negative control group received two immunizations with non-pulsed DCs 14 days apart. Splenocytes were assessed for KI-specific (black bars) and DI-specific (gray bars) **(A)** IFNγ, **(B)** IL-4, and **(C)** IL-10 responses by *ex vivo* ELISpot assay 17 days after the last immunization (day 31). **(D)** BALB/c mice were immunized with DCs pulsed with KI on day 0 (KI), with DCs pulsed with AI on day 14 (AI) or with KI-pulsed DCs on day 0 followed by AI-pulsed DCs on day 14 (KI–AI). The negative control group received two immunizations with non-pulsed DCs 14 days apart. Splenocytes were assessed for KI-specific (black bars) and AI-specific (gray bars) IFNγ responses by *ex vivo* ELISpot assay 14 days after boost immunization (day 28). Mean spot forming units ± SD ($n = 3$ mice per group). Two-way ANOVA with Tukey's multiple comparison test was used to test for statistical significance when comparing all groups **(A,B,D)**. Two-way ANOVA with Dunnett's multiple comparison test was used to test for statistical significance when comparing to the media control (C).

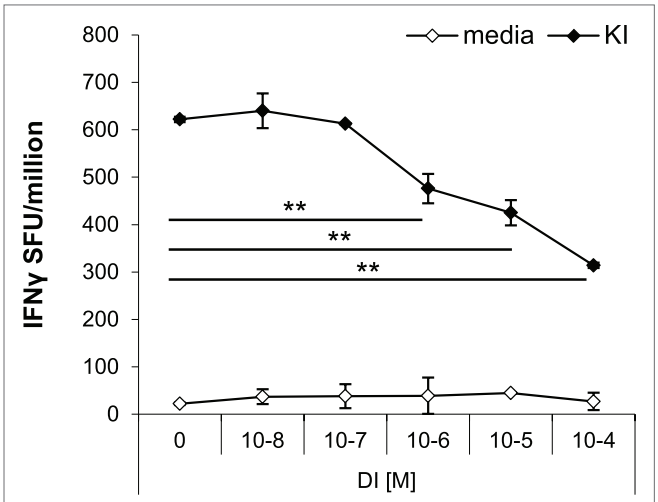

FIGURE 5 | Variant DI antagonizes KI-specific IFNγ secretion *in vitro*. BALB/c mice were immunized with 10^6 dendritic cells (DCs) pulsed with peptide variant KI or with DCs without peptide as negative controls. IFNγ responses were measured 21 days post-immunization by *ex vivo* ELISpot assay. Splenocytes were restimulated with the index peptide KI at 10^{-8} M for 1 h, followed by the altered peptide variant DI at different concentrations (10^{-8}–10^{-4} M). Mean spot forming units ± SD are shown ($n = 3$ mice per group). Two-way ANOVA with Dunnett's multiple comparison test was used to test for statistical significance when compared to the groups immunized with the index peptide KI.

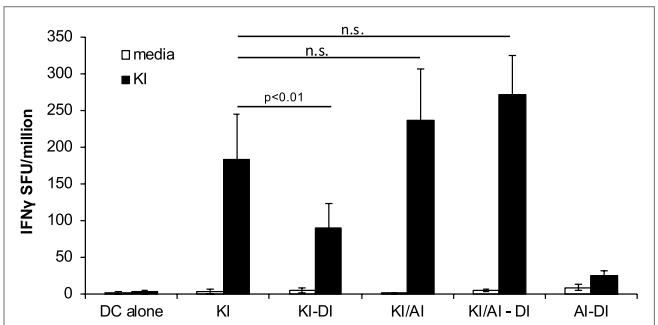

FIGURE 6 | KI/AI-primed cells are resistant to DI-induced immune interference. BALB/c mice were immunized on day 0 with dendritic cells (DCs) pulsed with either KI or AI or with DCs pulsed with KI for 1 h followed by AI for another 2 h. On day 14, three groups received sequential immunization with DI-pulsed DCs (KI–DI, KI/AI–DI, and AI–DI). The negative control group received two immunizations with non-pulsed DCs 14 days apart. Splenocytes were assessed for KI-specific IFNγ responses by *ex vivo* ELISpot assay 17 days after boost immunization (day 31). Mean spot forming units ± SD are shown ($n = 3$ mice per group). Two-way ANOVA with Dunnett's multiple comparison test was used to test for statistical significance when compared to the groups immunized with the index peptide KI.

of cross-reactivity. Our results show that KI/AI immunization, in contrast to immunization with KI or AI alone, induced T cells that recognize KI and AI, while no longer being susceptible to being turned off by immunization with the antagonistic DI APL, rendering the response therefore resistant to this type of immune interference.

DISCUSSION

The present study shows that immunization with combinations of highly related immunogenic variant peptides (KI, DI, and AI), such as those found naturally in pathogen populations with highly polymorphic T-cell epitopes, can have highly variable, and unpredictable, outcomes, with some combinations capable of decreasing immunity to both immunizing peptides (KI and DI). Moreover, immunizing sequentially with such peptides was shown to be capable of turning off responses, again to both immunizing T-cell epitope variants (KI and DI). The existence of "sequential immune interference" has theoretical consequences for pathogen population structures. It may be that polymorphic variants provide a survival advantage to the pathogen population that allows the species to evade the host immune response. By contrast, co-immunization of KI and AI had apparently little effect on the initial induction of immunity to either epitope, but, unexpectedly, the KI-specific responses generated were no longer susceptible to being turned off by subsequent immunization with DI. This finding offers a new practical strategy to elicit robust immune responses against polymorphic variants (by co-immunizing with an index epitope and a carefully selected "potentiator" epitope), which are then further resistant to being turned off by natural polymorphic variants capable of immune interference. *In vivo* challenge experiments with *P. berghei* sporozoites are needed to quantify the extent to which protective immunity is altered in this model.

Each of the three CD8 T-cell epitopes selected for study (KI, DI, and AI) induced high immune responses to themselves, but not to the other two epitopes, thus at face value they represent simple non-cross-reactive T-cell epitopes. However, co-immunization with DC pre-pulsed with KI and DI together, significantly reduced responses to both epitopes, with this mutual immune interference observed even when KI and DI were injected separately on separately peptide pulsed DC. There was no correlation between the magnitude of induced KI response and the level of interference by DI. Furthermore, the DI peptide was also shown to be capable of turning off effector KI-specific responses in a manner consistent with a classical APL antagonist. However, the fact that immune interference was observed even when presenting the epitopes on separate DC shows that the immune interference observed herein is not acting *via* classical mechanisms used by APL antagonism, such as alterations of the TCR:MHC immune-synaptosome structure (16). Further studies will be required to fully explore the extent to which immune interference by KI and DI is potentially fully reciprocal under a range of co-presentation and sequential immunization combinations.

The activity of some APL antagonist peptides has been shown to depend on a high off-rate in relation to MHC binding, but such mechanisms are unlikely to underlie the observed immune interference described in this study, since we have previously shown similarly high affinity binding to MHC for KI, DI, and AI (1). Temporal separation of stimulation by KI and DI by sequential immunization also resulted in reduced immunity demonstrating that immune interference by DI acts by stimulating KI-specific T cells to change their activity profile. Such switching of an activity profile has been observed *in vitro* for CD4 T-cell epitopes from *P. falciparum*. In this case, stimulation with a variant polymorphic epitope of an index peptide converted IFNγ-producing cells into IL-10 producers; a property demonstrated with human T cell clones to Th2R region variants of CS (2). In our study, immunization with DI did not appear to switch KI-specific responses from IFNγ to IL-10, or convert them into IL-4-producing Th2 cells, which have been noted as previous APL mechanisms capable of down modulating Th1 and cytotoxic immunity. These results do not exclude the hypothesis that stimulation with DI promotes other, less obvious, immunosuppressive cell populations, as well as potentially T cell apoptosis, anergy, or partial activation, related in a number of other studies with low TCR affinity interactions or disturbed TCR stimulating signaling micro-domains [reviewed in Ref. (17, 18)].

By using a combination of the index variant KI together with the "potentiator" peptide AI, we have demonstrated the induction of an immune response that is resistant to immune interference by DI stimulation. This finding lays the experimental foundations for a practical immunization strategy that could induce robust and long-lasting immune responses in the malaria setting. Thus, index epitopes could be paired with potentiator epitopes in a single immunization to induce a robust immune response to a polymorphic epitope, which would be predicted to persist even in vaccinees challenged by repeated pathogen stimulation. This is potentially of great significance for those living in malaria-endemic regions and exposed to persistent reinfection by polymorphic parasites capable of turning off immunity using immune interference (1). Moreover, combinations of engineered and natural variants may offer a new path for vaccines against many different pathogens, to create new modalities of cross-reactive responses.

AUTHOR CONTRIBUTIONS

GM and MP designed the study, interpreted the data, and drafted the manuscript. GM performed experiments and analyzed data. KF and RS critically revised the manuscript.

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REFERENCES

1. Flanagan KL, Wilson KL, Plebanski M. Polymorphism in liver-stage malaria vaccine candidate proteins: immune evasion and implications for vaccine design. *Expert Rev Vaccines* (2016) 15(3):389–99. doi:10.1586/14760584.2016.1125785
2. Plebanski M, Flanagan KL, Lee EA, Reece WH, Hart K, Gelder C, et al. Interleukin 10-mediated immunosuppression by a variant CD4 T cell epitope of *Plasmodium falciparum*. *Immunity* (1999) 10(6):651–60. doi:10.1016/S1074-7613(00)80064-3
3. Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, et al. Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* (1998) 279(5354):1173–7. doi:10.1126/science.279.5354.1173
4. Neafsey DE, Juraska M, Bedford T, Benkeser D, Valim C, Griggs A, et al. Genetic diversity and protective efficacy of the RTS,S/AS01 malaria vaccine. *N Engl J Med* (2015) 373(21):2025–37. doi:10.1056/NEJMoa1505819
5. Krishnarjuna B, Andrew D, MacRaild CA, Morales RA, Beeson JG, Anders RF, et al. Strain-transcending immune response generated by chimeras of the malaria vaccine candidate merozoite surface protein 2. *Sci Rep* (2016) 6:20613. doi:10.1038/srep20613
6. Kester KE, Gray Heppner D Jr, Moris P, Ofori-Anyinam O, Krzych U, Tornieporth N, et al. Sequential phase 1 and phase 2 randomized, controlled trials of the safety, immunogenicity and efficacy of combined pre-erythrocytic vaccine antigens RTS,S and TRAP formulated with AS02 Adjuvant System in healthy, malaria naive adults. *Vaccine* (2014) 32(49):6683–91. doi:10.1016/j.vaccine.2014.06.033
7. Wilson KL, Xiang SD, Plebanski M. A model to study the impact of polymorphism driven liver-stage immune evasion by malaria parasites, to help design effective cross-reactive vaccines. *Front Microbiol* (2016) 7:303. doi:10.3389/fmicb.2016.00303
8. Plebanski M, Gilbert SC, Schneider J, Hannan CM, Layton G, Blanchard T, et al. Protection from *Plasmodium berghei* infection by priming and boosting T cells to a single class I-restricted epitope with recombinant carriers suitable for human use. *Eur J Immunol* (1998) 28(12):4345–55. doi:10.1002/(SICI)1521-4141(199812)28:12<4345::AID-IMMU4345>3.3.CO;2-G
9. Plebanski M, Lee EA, Hannan CM, Flanagan KL, Gilbert SC, Gravenor MB, et al. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. *Nat Med* (1999) 5(5):565–71. doi:10.1038/8444
10. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* (1992) 176(6):1693–702. doi:10.1038/369403a0
11. Bodmer HC, Pemberton RM, Rothbard JB, Askonas BA. Enhanced recognition of a modified peptide antigen by cytotoxic T cells specific for influenza nucleoprotein. *Cell* (1988) 52(2):253–8. doi:10.1016/0092-8674(88)90514-4
12. Morgan CS, Holton JM, Olafson BD, Bjorkman PJ, Mayo SL. Circular dichroism determination of class I MHC-peptide equilibrium dissociation constants. *Protein Sci* (1997) 6(8):1771–3. doi:10.1002/pro.5560060819
13. Romero P, Corradin G, Luescher IF, Maryanski JL. H-2Kd-restricted antigenic peptides share a simple binding motif. *J Exp Med* (1991) 174(3):603–12. doi:10.1084/jem.174.3.603
14. Klenerman P, Rowland-Jones S, McAdam S, Edwards J, Daenke S, Lalloo D, et al. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature* (1994) 369(6479):403–7. doi:10.1038/369403a0
15. Jameson SC, Carbone FR, Bevan MJ. Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. *J Exp Med* (1993) 177(6):1541–50. doi:10.1084/jem.177.6.1541
16. Gascoigne NR, Zal T. Molecular interactions at the T cell-antigen-presenting cell interface. *Curr Opin Immunol* (2004) 16(1):114–9. doi:10.1016/j.coi.2003.11.008
17. Unanue ER. Altered peptide ligands make their entry. *J Immunol* (2011) 186(1):7–8. doi:10.4049/jimmunol.1090118
18. Candia M, Kratzer B, Pickl WF. On peptides and altered peptide ligands: from origin, mode of action and design to clinical application (immunotherapy). *Int Arch Allergy Immunol* (2016) 170(4):211–33. doi:10.1159/000448756

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Comparative Genomics of *Glossina palpalis gambiensis* and *G. morsitans morsitans* to Reveal Gene Orthologs Involved in Infection by *Trypanosoma brucei gambiense*

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Blood-feeding *Glossina palpalis gambiense* (Gpg) fly transmits the single-celled eukaryotic parasite *Trypanosoma brucei gambiense* (Tbg), the second *Glossina* fly African trypanosome pair being *Glossina morsitans*/*T.*brucei rhodesiense. Whatever the *T. brucei* subspecies, whereas the onset of their developmental program in the zoo-anthropophilic blood feeding flies does unfold in the fly midgut, its completion is taking place in the fly salivary gland where does emerge a low size metacyclic trypomastigote population displaying features that account for its establishment in mammals-human individuals included. Considering that the two *Glossina—T. brucei* pairs introduced above share similarity with respect to the developmental program of this African parasite, we were curious to map on the *Glossina morsitans morsitans* (Gmm), the Differentially Expressed Genes (DEGs) we listed in a previous study. Briefly, using the gut samples collected at days 3, 10, and 20 from Gpg that were fed or not at day 0 on Tbg—hosting mice, these DGE lists were obtained from RNA seq—based approaches. Here, post the mapping on the quality controlled DEGs on the Gmm genome, the identified ortholog genes were further annotated, the resulting datasets being compared. Around 50% of the Gpg DEGs were shown to have orthologs in the Gmm genome. Under one of the three *Glossina* midgut sampling conditions, the number of DEGs was even higher when mapping on the Gmm genome than initially recorded. Many Gmm genes annotated as "Hypothetical" were mapped and annotated on many distinct databases allowing some of them to be properly identified. We identify *Glossina* fly candidate genes encoding (a) a broad panel of proteases as well as (b) chitin—binding proteins, (c) antimicrobial peptide production—Pro3 protein, transferrin, mucin, atttacin, cecropin, etc—to further select in functional studies, the objectives being to probe and validated fly genome manipulation that prevents the onset of the developmental program of one or the other *T. brucei* spp. stumpy form sampled by one of the other bloodfeeding *Glossina* subspecies.

Keywords: human African Trypanosomiasis, *Glossina palpalis gambiensis*, *Glossina morsitans morsitans*, *Trypanosoma brucei gambiense*, differentially expressed genes, heterologous genes

INTRODUCTION

Trypanosomes causing either Human African Trypanosomiasis (HAT, i.e., sleeping sickness) or Animal African Trypanosomiasis (AAT, i.e., Nagana) are transmitted by *Glossina* spp. (tsetse flies). These hematophagous flies acquire their parasite during a blood meal on an infected host, and transmit the mature form of the parasite to another host during a subsequent blood meal. Two forms of HAT have been reported: a chronic and an acute form (Hoare, 1972; Aksoy et al., 2014; Beschin et al., 2014). The chronic form, spread throughout 24 sub-Saharan countries of West Africa, is caused by *Trypanosoma brucei gambiense* (Tbg) and is transmitted by *Glossina palpalis*; this form represents over 90% of all sleeping sickness cases (Welburn et al., 2009). The acute form, endemic to 12 East African countries, is caused by *Trypanosoma brucei rhodesiense* (Tbr), and is transmitted by *Glossina morsitans morsitans* (Gmm). Currently the disease persists in sub-Saharan countries (Louis et al., 2002), where more than 60 million people are exposed to the trypanosomiasis risk. Progress in deciphering the mechanisms of host-parasite interactions involves identifying the genes encoding the factors that govern tsetse fly vector competence (Vickerman et al., 1988; Maudlin and Welburn, 1994; Van den Abbeele et al., 1999), which may promote the development of anti-vector strategies that are alternative or complementary to current strategies.

Using a microarray approach, we recently investigated the effect of trypanosome ingestion by *G. palpalis gambiensis* (Gpg) flies on the transcriptome signatures of *Sodalis glossinidius* (Farikou et al., 2010; Hamidou Soumana et al., 2014a) and *Wigglesworthia glossinidia* (Hamidou Soumana et al., 2014b), two symbionts of tsetse flies (Aksoy et al., 2014). The aim of this previous work was to identify the genes that are differentially expressed in trypanosome infected vs. non-infected or self-cured (refractory) flies and that, consequently, can be suspected to positively or negatively control fly infection. Similarly, using the RNA-seq *de novo* assembly approach, we investigated the differential expression of *G. p. gambiensis* genes in flies challenged or not with trypanosomes (Hamidou Soumana et al., 2015). Furthermore, transcriptome profiling of *T. b. brucei* development in Gmm has recently been reported (Savage et al., 2016).

Since the acute form of HAT is caused by the Gmm/Tbr vector/parasite "couple," the identification of molecular targets common to both Gpg and Gmm (i.e., orthologous genes) deserves further consideration. Indeed, identification of these targets would allow the development of common approaches to fight both forms of HAT. As Gpg and Gmm are two separate *Glossina* species, their genomes should display some differences between each other. Furthermore, the Gmm genome and the sequences of the Gpg RNA-seq *de novo* assembled genes have been annotated with reference to two distinct database sets: the first set comprises *Drosophila melanogaster*, *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, and *Phlebotomus papatasi* (International Glossina Genome Initiative, 2014), whereas the second set comprises *Ceratitis capitata*, *Drosophila melanogaster*, *D. willistoni*, *D. virilis*, *D. mojavensis*, *Acyrthosiphon pisum*, *Hydra magnipapillata*, *Anopheles* sp., *Bombyx* sp., *Aedes* sp., and *Glossina morsitans* (data that were available before the publication of the whole genome sequence; Hamidou Soumana et al., 2015). This indicates that only the *D. melanogaster* database was common to the two database sets used to annotate the differentially expressed Gpg genes and the Gmm genome, respectively. Thus, for the present study, it was necessary to map the sequences of the Gpg RNA-seq *de novo* assembled genes on the Gmm genome and annotate them on the corresponding database. This has been achieved, and the Gpg genes that were previously shown to be differentially expressed (i.e., stimulated vs. non-stimulated flies, and infected vs. non-infected flies; Hamidou Soumana et al., 2015) were annotated on the Gmm database. Finally, the data resulting from the best hits annotation, which provide a translation product for each gene (and thus its potential biological function and physiological role), were compared with data resulting from the previous annotation of the same genes on the set of above-mentioned databases. The overall results provide a data platform that can be applied for further identification of candidate genes involved in the vector competence of both fly species. Importantly, these data could represent promising targets in the development of new anti-vector strategies in the fight against the chronic or acute forms of sleeping sickness.

MATERIALS AND METHODS

Ethical Statement

All animal experiments in this report were conducted according to internationally recognized guidelines. The experimental protocols were approved by the Ethics Committee on Animal Experiments and the Veterinary Department of the Centre International de Recherche Agronomique pour le Développement (CIRAD; Montpellier, France).

Sample Processing, RNA-Seq Library Preparation, and Sequencing

Samples for this study were previously used to identify the differentially expressed genes (DEGs) in Gpg. The different steps are described in the corresponding report (Hamidou Soumana et al., 2015), as well as *pro parte* in reports related to the differential expression of *S. glossinidius* and *W. glossinidia* genes (Hamidou Soumana et al., 2014a,b). Sample processing is summarized in **Figure 1**.

Preparation and Sequencing of the RNA-Seq Libraries

The sequential steps consisted of: RNA extraction from the pooled midguts of each biological replicate, resuspension of RNA pellets in nuclease-free water, concentration, RNA quantification, and quality control (to confirm the absence of any DNA contamination).

Generation of RNA-Seq Libraries

RNA-seq libraries were generated using the Illumina TruSeq™ RNA Sample Preparation Kit (Illumina; San Diego, USA). The sequential steps consisted of: mRNA purification from 4 μg total RNA using poly-T oligo-linked magnetic beads; fragmentation of RNA using divalent cations under elevated temperature (Illumina fragmentation buffer); first-strand cDNA

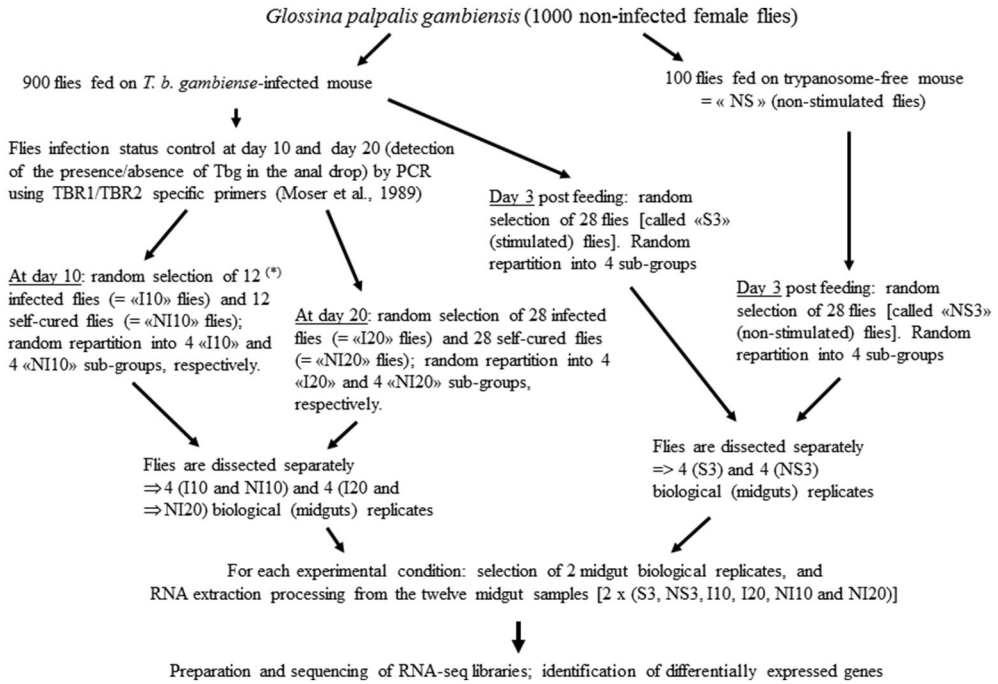

FIGURE 1 | Samples processing. (*) at day 10 the rate of infected flies was law, thus only 12 infected flies ("I10") could be sampled (instead of 28 at day 20). Self-cured flies are flies that have ingested trypanosomes (while they have taken a blood meal on an infected mouse), but the anal drops of which were trypanosome negative.

synthesis using random oligonucleotides and SuperScript II; second-strand cDNA synthesis using DNA Polymerase I and RNase H; conversion of remaining overhangs into blunt ends via exonuclease/polymerase activities and enzyme removal; and adenylation of 3′ ends of cDNA fragments, with ligation of Illumina PE adapter oligonucleotides for further hybridization. Finally, cDNA fragments were selected (preferably 200 bp in length) in which fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail, and the products were purified and quantified using the Agilent DNA assay on the Agilent Bioanalyzer 2100 system.

Brief Summary of the Pipeline for Generating Quality-Controlled Reads

A total of 12 RNA-seq libraries were prepared, sequenced, and compared, including two biological replicates for each of the NS3, S3, I10, NI10, I20, and I20 samples. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2000 platform, and 100-bp paired-end reads were generated. Image analyses and base calling were performed using the Illumina HiSeq Control Software and Real-Time Analysis component. Demultiplexing was performed using CASAVA 1.8.2. The quality of the raw data was assessed using FastQC (Babraham Institute) and the Illumina software SAV (Sequencing Analysis Viewer). Raw sequencing reads from this study were exported in the FASTQ format and were deposited at the NCBI Short Read Archive (SRA) with the accession number SRP046074; aligned BAM files are available on request.

Identification of DEGs Once the Reads Generated from the 12 Gpg Fly Gut RNA Seq Libraries Were Mapped and Annotated on a Panel of Non-insect and Insect Genome Databases, One of Them Being Gmm

The RNA-seq reads that satisfied the quality control (i.e., removal of ambiguous nucleotides, low-quality sequences with quality scores <20, and sequences <15 bp in length) were mapped on the *G. m. morsitans* genome (13,807 scaffolds; International Glossina Genome Initiative, 2014) from VectorBase (www.vectorbase.org) and GenBank (accession no. CCAG010000000). This was achieved via the splice junction mapper TopHat 2.0.13 (Kim et al., 2013) using Bowtie 2.1.0 (Langmead and Salzberg, 2012), to align RNA-seq reads to the *Glossina morsitans* genome (GmorY1 assembly, release date: January 2014). Final read alignments with more than 12 mismatches were discarded.

Gene counting (number of reads aligned on each gene) was performed before statistical analysis, using HTSeq count 0.5.3p9 (union mode; Anders et al., 2014). Genes with <10 reads (cumulating all analyzed samples) were filtered and removed. We used the Bioconductor (Gentleman et al., 2004) software package EdgeR (Robinson et al., 2010) 3.6.7. to identify genes

displaying a modified expression profile as a result of fly infection by trypanosomes. Data were normalized using the upper quartile normalization factors, using the quartiles method (Bullard et al., 2010). Genes with an adjusted $p < 5\%$ according to the False Discovery Rate (FDR) method from Benjamini and Hochberg (1995) were declared differentially expressed.

Bio Informatics-Based Approaches Aimed to Identify Molecular DEGs in Both Gmm and Gpg Once the Latter are Subverted as T. brucei spp Hosts *Per se*

Tsetse fly gene orthologs were tentatively identified using BLAST searches (Mount, 2007) with annotation against the NCBI non-redundant (Nr) sequence database, using an *E*-value cut-off of 10^{-5} ($E < 0.00001$), according to the best hits against known sequences. This was performed to retrieve orthologous genes with the highest sequence similarity to the given unigenes along with putative functional annotations. The official gene symbols of tsetse fly gene orthologs were used for functional annotation. Along with Nr annotations, the "Database for Annotation, Visualization and Integrated Discovery" (DAVID; Dennis et al., 2003) was used to obtain GO annotations of unigenes. The KEGG pathway annotations of tsetse fly gene orthologs were performed using the BLASTX software against the KEGG database (Wixon and Kell, 2000).

Analyzing the two annotation processes of the Gpg DEGs consisted in comparing the list of the "best hits" resulting from the Gpg DEG annotation on the Gmm database with the list resulting from the Gpg DEG annotation previously performed on a set of other databases (*Ceratitis capitata*, *Drosophila melanogaster*, *D. willistoni*, *D. virilis*, *D. mojavensis*, *Acyrthosiphon pisum*, *Hydra magnipapillata*, *Anopheles* sp., *Bombyx* sp., *Aedes* sp., and *Glossina morsitans*; Hamidou Soumana et al., 2015). The first step consisted in mixing the DEGs identified at the three experimental times (3, 10, and 20 days) and removing the duplicates, so as to take into account all recorded DEGs except for one of each. The second step consisted in removing the DEGs in which the annotation (best hit) resulted in "hypothetical" or "uncharacterized" proteins, as well as those identified with a numerical identifier, in order to only consider identified and named proteins. Finally, the names of the proteins (best hits) were standardized and alphabetically classified. This process was performed separately for the DEGs annotated with reference to the Gmm database, as well as those previously annotated on the above-characterized set of other databases. The two final listings were then combined (Microsoft Excel software), and their content was arranged according to the alphabetical order of protein names. This procedure facilitated the detection of the best hits that are common to both annotation processes and their corresponding genes.

RESULTS

Mapping of PolyA+ mRNA

A total of 459,555,846 clusters were generated from the 12 RNA-seq libraries. Quality controls were performed to ensure the reliability of the libraries after removal of ambiguous nucleotides, low-quality sequences (quality scores < 20), and sequences <15 bp in length. Finally, 436,979,101 clean clusters were obtained (**Table 1**). Clean reads had Phred-like quality scores at the Q20 level (i.e., a sequencing error probability of 0.01). These clean sequenced reads with no strand-specificity were mapped to the Gmm reference genome using TopHat (with Bowtie 2) software in order to identify exon-exon splice junctions and to ensure enough sensitivity in mapping reads with polymorphisms.

Filtering and removing any genes with <10 mapped reads allowed mapping 8,286 (stimulated vs. non-stimulated flies; 3 days), 8,032 (infected vs. refractory flies; 10 days) and 8,101 Gpg genes (infected vs. refractory flies; 20 days) on the Gmm reference genome (International Glossina Genome Initiative, 2014). Further, analyses to reveal differential expression (DE) were performed using the bioinformatics tools HTseq and EdgeR from Bioconductor (http://www.bioconductor.org/), which use the R statistical programming language and are widely accepted for modeling the inherent variation between biological replicates. **Figure 2** presents the $\log_2$ fold-change (stimulated vs. non-stimulated flies at day-3 post-infected blood meal) against the $\log_2$ of the reads concentration (log-counts-per-million) for each gene after normalization. The generated cloud shows a log fold-change centered on 0 (ordinate axis), signifying that the libraries are properly normalized. Genes that are differentially expressed between the S and NS samples ($p < 0.05$) are represented in red. Similar results were obtained for the other experimental conditions.

Identification of DEGs and Functional Annotation

The EdgeR method identified a total of 284, 139, and 59 Gmm genes corresponding respectively to the Gpg DEG samples S3

TABLE 1 | Assembly quality of Gpg libraries at the three different sampling times.

Samples	Number of crude clusters (CC)	Number of clusters after filtering (CAF)	% CAF/CC
NS 3-day sample[a]	36,002,596	34,386,734	95.51
NS 3-day sample[b]	41,153,580	39,330,015	95.57
S 3-day sample	32,726,727	31,257,269	95.51
S 3-day sample	33,386,646	31,848,385	95.39
NI 10-day sample	33,159,650	31,593,962	95.28
NI 10-day sample	30,632,671	29,185,036	95.27
I 10-day sample	42,223,049	40,108,756	94.99
I 10-day sample	43,418,918	41,279,341	95.07
NI 20-day sample	41,882,170	39,688,764	94.76
NI 20-day sample	38,192,692	36,205,087	94.80
I 20-day sample	40,587,354	38,401,915	94.62
I 20-day sample	46,189,793	43,693,837	94.60
Total	459,555,846	436,979,101	–
Mean	38,296,320	36,414,925	95.08

The superscipts [a] and [b] are two replicates of the "non-stimulated samples" at day 3. Idem for the other sampling conditions. S, stimulated; NS, non-stimulated; NI, non-infected; I, infected.

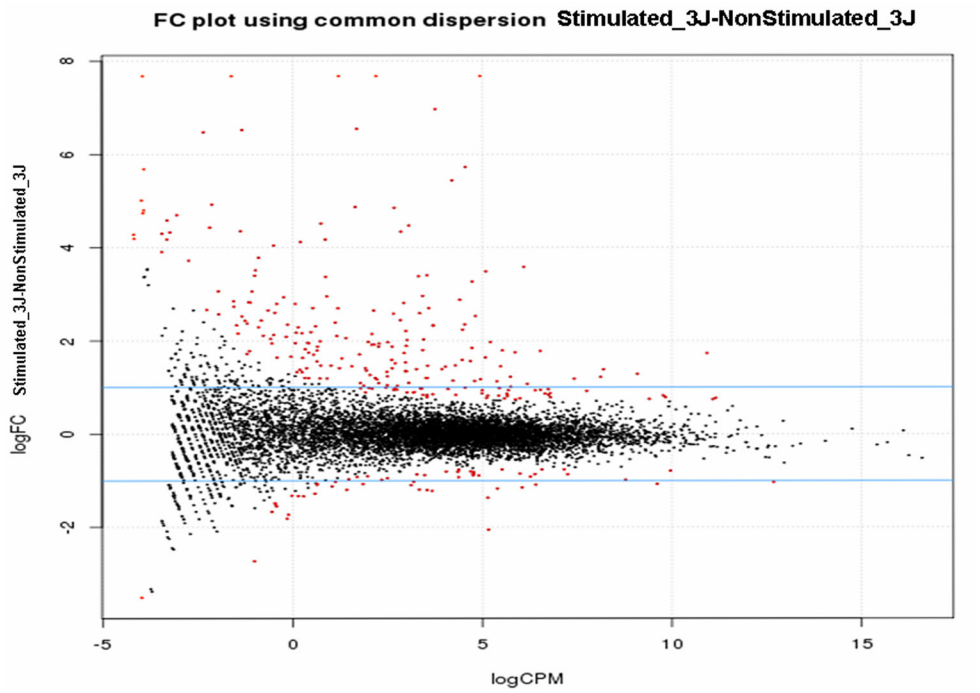

FIGURE 2 | Smear plot of libraries. Samples are from trypanosome stimulated-/-non-stimulated tsetse fly midguts sampled 3 days post-infected blood meal, after normalization using the upper quartile method.

TABLE 2 | Number of differentially expressed genes in Gpg.

Experimental conditions	Number of identified genes	Significantly differentially expressed genes			
		Overall	Overexpressed	Fold-change	
				$2 < \log_2$ FC or $\log_2$ FC < -2	$3 < \log_2$ FC or $\log_2$ FC < -3
S vs. NS (3 days)	8,286	284	229 (80.6%)	97 (34.1%)	44 (15.5%)
I vs. NI (10 days)	8,032	139	119 (85.6%)	60 (43.1%)	35 (25.2%)
I vs. NI (20 days)	8,101	59	37 (62.7%)	19 (32.2%)	6 (10.2%)

S, stimulated; NS, non-stimulated; NI, non-infected; I: infected.

vs. NS3 (Supplementary Table S1), I10 vs. NI10 (Supplementary Table S2), and I20 vs. NI20 (Supplementary Table S3), at a $p < 0.05$. Most of these genes were overexpressed regardless of the experimental condition. Specifically, there were 229 out of 284 genes (80.6%) in the day-3 samples (S3 vs. NS3), 119 out of 139 genes (85.6%) in I-10 vs. NI-10 samples, and 37 out of 59 genes (62.7%) in I20 vs. NI20. Furthermore, the number of DEGs were highly differentially overexpressed ($\log_2$ FC $>$ 2) or underexpressed ($\log_2$ FC < -2). Specifically, there were 97 out of 284 DEGs (34%; S3 vs. NS3), 60 out of 139 DEGs (43%; I10 vs. NI10), and 19 out of 59 DEGs (32%; I20 vs. NI20). These data are summarized in **Table 2**. Genes exhibiting a highly differential overexpression or underexpression under the different experimental conditions (i.e., S vs. NS, I10 vs. NI10, and I20 vs. NI20) are grouped together in **Table 3**. Most DEGs encode a wide range of proteases, although 91 DEGs presented in Supplementary Tables S1–S3 could not be properly annotated (i.e., best hit description = "hypothetical"), signifying that the panel of databases used for the annotation process should be enlarged or that the genes may be specific to the Gmm genome. In addition, several of the DEGs were very highly overexpressed. For example the $\log_2$ FC of GMOY009756, which encodes a trypsin, had a fold-change of 7.14 in S3 vs. NS3 samples, and GMOY002278, which encodes the proteinase inhibitor I2, had a fold-change of 9.47 in I10 vs. NI10. In contrast, some DEGs were underexpressed: the $\log_2$ FC of GMOY005345, which encodes an aspartic peptidase, had a fold-change of –6.51 in I20 vs. NI20 samples. **Table 3** is presented so as to facilitate comparison of differential expression levels for a given gene along the three sampling times. For instance, the levels (in $\log_2$ FC) of GMOY005345, which encodes an aspartic peptidase, are 3.39 (S3 vs. NS3), 2.70 (I10 vs. NI10), and –6.51 (I20 vs. NI20).

Table 3 also provides the functional annotation data for each gene at each sampling time. To obtain an overview of the

TABLE 3 | Annotation on the *Glossina morsitans morsitans* genome of Gpg genes differentially expressed in response to Tbg infection.

Genes	Fold-change log_2 FC	Encoded proteins (best hits)	Gene Ontology (GO)		
			Biological process	Molecular function	Cellular component
PROTEASES AND PROTEASE INHIBITORS					
GMOY005345	3.39	Aspartic peptidase	GO:0006508 proteolysis	GO:0004190 aspartic-type endopeptidase activity	No terms assigned
GMOY005345	2.70	Aspartic peptidase	GO:0006508 proteolysis	GO:0004190 aspartic-type endopeptidase activity	No terms assigned
GMOY005345	–6.51	Aspartic peptidase	GO:0006508 proteolysis	GO:0004190 aspartic-type endopeptidase activity	No terms assigned
GMOY007305	2.09	Destabilase	No terms assigned	GO:0003796 lysozyme activity	No terms assigned
GMOY007305	3.00	Destabilase	No terms assigned	GO:0003796 lysozyme activity	No terms assigned
GMOY000103	2.64	Fat body c-type lysozyme	No terms assigned	No terms assigned	No terms assigned
GMOY000103	2.74	Fat body c-type lysozyme	No terms assigned	No terms assigned	No terms assigned
GMOY002036	–2.75	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY003273	2.30	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY003994	4.19	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY006266	3.72	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY008964	3.32	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY008965	3.52	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY008966	3.35	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY008966	4.41	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY009436	2.11	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY009757	2.76	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY010768	2.73	Peptidase S1A, chymotrypsin-type	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY010768	2.06	Peptidase S1	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY002729	3.01	Serine protease 1	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY000672	6.95	Serine protease 6	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY000672	6.78	Serine protease 6	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY009756	7.14	Trypsin	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY009756	3.48	Trypsin	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY008967	2.55	Trypsin	GO:0006508 proteolysis	GO:0004252 serine-type endopeptidase activity	No terms assigned
GMOY008967	3.37	Trypsin-like cysteine/serine peptid. domain	No terms assigned	GO:0003824 catalytic activity	No terms assigned
GMOY010488	6.83	Imune reactive putative protease inhibitor	No terms assigned	No terms assigned	No terms assigned
GMOY010488	4.69	Immune reactive putative protease inhibitor	No terms assigned	No terms assigned	No terms assigned

(Continued)

TABLE 3 | Continued

Genes	Fold-change log_2 FC	Encoded proteins (best hits)	Gene Ontology (GO)		
			Biological process	Molecular function	Cellular component
GMOY002277	2.31	Proteinase inhibitor I2, Kunitz metazoa	No terms assigned	GO:0004867 serine-type endopeptidase inhibit. Activ.	No terms assigned
GMOY002277	4.10	Proteinase inhibitor I2, Kunitz metazoa	No terms assigned	GO:0004867 serine-type endopeptidase inhibit. Activ.	No terms assigned
GMOY002278	6.54	Proteinase inhibitor I2, Kunitz metazoa	No terms assigned	GO:0004867 serine-type endopeptidase inhibit. Activ.	No terms assigned
GMOY002278	9.47	Proteinase inhibitor I2, Kunitz metazoa	No terms assigned	GO:0004867 serine-type endopeptidase inhibit. Activ.	No terms assigned
GMOY008344	2.94	Trypsin Inhibitor-like	No terms assigned	No terms assigned	No terms assigned
GMOY008344	4.38	Trypsin Inhibitor-like	No terms assigned	No terms assigned	No terms assigned
ESTERASES—HYDROLASES					
GMOY000067	3.35	Alkaline phosphatase	GO:0008152 metabolic process	GO:0016791 phosphatase activity	No terms assigned
GMOY000067	3.60	Alkaline phosphatase	GO:0008152 metabolic process	GO:0003824 catalytic activity	No terms assigned
GMOY004731	2.06	Alkaline phosphatase-like, alpha/beta/alpha	GO:0008152 metabolic process	GO:0003824 catalytic activity	No terms assigned
GMOY006875	–2.02	Alkaline phosphatase-like, alpha/beta/alpha	GO:0008152 metabolic process	GO:0003824 catalytic activity	No terms assigned
GMOY004236	2.36	Acylphosphatase-like	No terms assigned	GO:0003998 acylphosphatase activity	No terms assigned
GMOY006958	2.60	Carboxylesterase	No terms assigned	No terms assigned	No terms assigned
GMOY011249	2.83	Carboxylesterase	No terms assigned	No terms assigned	No terms assigned
GMOY012368	2.53	Exonuclease	No terms assigned	No terms assigned	No terms assigned
GMOY007402	3.85	Extracellular Endonuclease, subunit A	No terms assigned	GO:0016787 hydrolase activity	No terms assigned
GMOY012360	–2.93	Extracellular Endonuclease, subunit A	No terms assigned	GO:0016787 hydrolase activity	No terms assigned
GMOY009375	7.56	Glycoside hydrolase	GO:0005975 carbohyd. metabolic process	GO:0003824 catalytic activity	No terms assigned
GMOY012361	–2.55	Tsal2 protein precursor	No terms assigned	GO:0016787 hydrolase activity	No terms assigned
GMOY004309	2.44	Thiolase-like	GO:0008152 metabolic process	GO:0003824 catalytic activity	No terms assigned
GMOY007148	2.10	Thiolase-like	GO:0008152 metabolic process	GO:0003824 catalytic activity	No terms assigned
BINDING					
GMOY010194	4.12	Araucan	No terms assigned	GO:0003677 DNA binding	No terms assigned
GMOY009525	7.53	Armadillo-type fold	No terms assigned	GO:0005488 binding	No terms assigned
GMOY009611	2.60	Barrier- to-autointegration factor, BAF	No terms assigned	GO:0003677 DNA binding	No terms assigned
GMOY009394	–5.56	Basic-leucine zipper domain	GO:0006355 regulation of transcription	GO:0003700 sequence-specific	No terms assigned
GMOY010195	5.99	Caupolican	GO:0006355 regul. of transcrip,DNA-templated	GO:0003677 DNA binding	GO:0005634 nucleus
GMOY002708	7.89	Chitin binding	GO:0006030 chitin metabolic process	GO:0008061 chitin binding	GO:0005576 extracel
GMOY005278	2.93	Chitin binding domain	GO:0006030 chitin metabolic process	GO:0008061 chitin binding	GO:0005576 extracel
GMOY003840	4.12	Chitin binding domain	GO:0006030 chitin metabolic process	GO:0008061 chitin binding	GO:0005576 extracel
GMOY011054	6.08	Chitin binding domain	GO:0006030 chitin metabolic process	GO:0008061 chitin binding	GO:0005576 extracel
GMOY011810	6.55	Chitin binding domain	GO:0006030 chitin metabolic process	GO:0008061 chitin binding	GO:0005576 extracel

(Continued)

TABLE 3 | Continued

Genes	Fold-change log_2 FC	Encoded proteins (best hits)	Gene Ontology (GO)		
			Biological process	Molecular function	Cellular component
GMOY011809	8.08	Pro1 (Chitin related)	GO:0006030 chitin metabolic process	GO:0008061 chitin binding	GO:0005576 extracel
GMOY004647	4.24	Cupredoxin	No terms assigned	GO:0005507 copper ion binding	No terms assigned
GMOY004364	2.90	Haemolymph juvenile hormone binding	No terms assigned	No terms assigned	No terms assigned
GMOY005487	3.48	Lim3	No terms assigned	GO:0008270 zinc ion binding	No terms assigned
GMOY007084	2.39	NAD(P)-binding domain	No terms assigned	No terms assigned	No terms assigned
GMOY002356	2.31	Nucleotide-binding	No terms assigned	GO:0000166 nucleotide binding	No terms assigned
GMOY002825	4.47	Odorant binding protein 2	No terms assigned	GO:0005549 odorant binding	No terms assigned
GMOY002825	2.03	Odorant binding protein 2	No terms assigned	No terms assigned	No terms assigned
GMOY005548	2.99	Odorant binding protein 7	No terms assigned	No terms assigned	No terms assigned
GMOY001476	2.20	Odorant binding protein 22	No terms assigned	GO:0005549 odorant binding	No terms assigned
GMOY008769	4.95	Small GTPase	GO:0007165 signal transduction	GO:0005525 GTP binding	GO:0016020 membrane
GMOY004228	5.44	Transferrin family	GO:0006879 cellular iron ion homeostasis	GO:0008199 ferric iron binding	GO:0005576 extracel
GMOY004228	2.63	Transferrin family, iron binding site	GO:0006879 cellular iron ion homeostasis	GO:0008199 ferric iron binding	GO:0005576 extracel
GMOY008315	2.05	Winged helix-turn-helix DNA-binding domain	GO:0006355 regul. of transcrip,DNA-templated	GO:0043565 sequence-specific DNA binding Transcription Factor Activity	No terms assigned
TRANSPORT/TRANSFERASE ACTIVITY					
GMOY004684	–2.39	Cellul. retinaldehyde binding/a-tocopherol transport	GO:0006810 transport	GO:0005215 transporter activity	GO:0005622 intracel
GMOY008601	2.58	Fatty acid synthase 3	GO:0008152 metabolic process	GO:0016740 transferase activity	No terms assigned
GMOY008601	4.11	Fatty acid synthase 3	GO:0008152 metabolic process	GO:0016740 transferase activity	No terms assigned
GMOY008602	2.02	Fatty acid synthase 4	GO:0008152 metabolic process	GO:0016740 transferase activity	No terms assigned
GMOY008602	–2.55	Fatty acid synthase 4	GO:0008152 metabolic process	GO:0016740 transferase activity	No terms assigned
GMOY005442	2.35	Lipid transport protein	GO:0006869 lipid transport	GO:0005319 lipid transporter activity	No terms assigned
GMOY005442	2.40	Lipid transport protein	GO:0006869 lipid transport	GO:0005319 lipid transporter activity	No terms assigned
GMOY003490	4.50	Major Facilitator Superfamily transporter	GO:0055085 transmembrane transport	No terms assigned	GO:0016021 integral
GMOY003491	3.97	Major Facilitator Superfamily transporter	GO:0055085 transmembrane transport	No terms assigned	GO:0016021 integral
GMOY005103	2.77	Major Facilitator Superfamily transporter	GO:0055085 transmembrane transport	No terms assigned	GO:0016021 integral
GMOY007627	2.09	Major Facilitator Superfamily transporter	GO:0055085 transmembrane transport	No terms assigned	GO:0016021 integral
GMOY005102	6.28	N-acetylgalactosaminyltransferase	GO:0008152 metabolic process	No terms assigned	No terms assigned
GMOY011877	2.37	Na+ channel, amiloride-sensitive	GO:0006814 sodium ion transport	GO:0005272 sodium channel activity	GO:0016020 membrane
GMOY009903	2.75	Neurotransmitter-gated ion-channel	GO:0006811 ion transport	No terms assigned	GO:0016021 integral
GMOY005934	2.72	Pyridoxal phosphate-dependent transferase	No terms assigned	GO:0003824 catalytic activity	No terms assigned

(Continued)

TABLE 3 | Continued

Genes	Fold-change log_2 FC	Encoded proteins (best hits)	Gene Ontology (GO)		
			Biological process	Molecular function	Cellular component
GMOY009343	8.14	Sodium:neurotransmitter symporter	GO:0006836 neurotransmitter transport	GO:0005328 neurotransmitter:Na symporter act	GO:0016021 integral
GMOY009343	6.43	Sodium:neurotransmitter symporter	GO:0006836 neurotransmitter transport	GO:0005328 neurotransmitter:Na symporter act	GO:0016021 integral
GMOY009386	2.44	Sodium:neurotransmitter symporter	GO:0006836 neurotransmitter transport	GO:0005328 neurotransmitter:Na symporter act	GO:0016021 integral
GMOY002486	2.59	Two pore domain K channel, TASK family	GO:0071805 K ion transmemb, transport	GO:0005267 potassium channel activity	GO:0016020 membrane
GMOY012088	4.05	Tyrosine aminotransferase	GO:0009072 aromatic amino acid family metabolic process	GO:0004838 L-tyrosine:2-oxoglutarate aminotransferase Activity	No terms assigned
OXIDO-REDUCTION PROCESS					
GMOY001939	2.50	Cytochrome P450-4g1	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY002598	2.15	Cytochrome P450	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY006475	2.28	Cytochrome P450-4g1	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY006761	2.42	Cytochrome P450-4g1	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY006761	–2.08	Cytochrome P450-4g1	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY007181	3.49	Cytochrome P450	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY007652	3.43	Cytochrome P450	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY009767	3.96	Cytochrome P450	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY009909	3.35	Cytochrome P450	GO:0055114 oxidation-reduction process	GO:0016705 oxidoreductase activity	No terms assigned
GMOY007529	4.49	Dehydrogenase/reductase	GO:0008152 metabolic process	GO:0016491 oxidoreductase activity	No terms assigned
GMOY004332	2.12	Fatty acyl-CoA reductase	No terms assigned	GO:0080019 fatty-acyl-CoA reductase activity	No terms assigned
GMOY007497	6.25	NADH-cytochrome b-5 reductase 2	GO:0055114 oxidation-reduction process	GO:0016491 oxidoreductase activity	No terms assigned
GMOY010446	2.40	2-oxoglutarate dioxygenase	GO:0055114 oxidation-reduction process	GO:0050353 trimethyllysine dioxygenase activity	No terms assigned
HYPOTHETICAL					
GMOY000215	4.17	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY000215	5.24	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY000257	2.90	Hypothetical	No terms assigned	No terms assigned	No terms assigned

(Continued)

TABLE 3 | Continued

Genes	Fold-change log_2 FC	Encoded proteins (best hits)	Gene Ontology (GO)		
			Biological process	Molecular function	Cellular component
GMOY001239	2.53	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY002434	3.22	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY002933	2.07	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY002986	2.69	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003011	2.30	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003030	4.38	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003034	–2.10	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003158	2.70	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003197	2.04	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003830	2.75	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003830	3.68	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003974	2.28	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY003976	3.89	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY004022	–2.06	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY004337	5.80	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY004337	6.61	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005055	6.08	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005606	6.32	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005797	6.60	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005797	6.49	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005798	3.98	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005798	6.25	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY005799	2.24	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY006671	4.00	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY006276	2.33	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY007187	3.59	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY007637	4.06	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY008016	4.65	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY008016	6.67	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY008627	3.64	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY009539	2.28	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY009540	2.19	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY009541	2.40	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY009951	3.11	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY010224	6.87	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY010224	3.57	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY010232	–2.44	Hypothetical	No terms assigned	No terms assigned	No terms assigned
xGMOY012069	9.07	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY012069	5.33	Hypothetical	No terms assigned	No terms assigned	No terms assigned
GMOY008956	2.90	hypothetical conserved protein	No terms assigned	No terms assigned	No terms assigned
MISCELLANEOUS					
GMOY008458	5.74	Actin-related protein	No terms assigned	No terms assigned	No terms assigned
GMOY008368	2.22	Adipokinetic hormone recept isoform A	No terms assigned	No terms assigned	No terms assigned
GMOY004147	4.32	Apolipophorin-III superfamily	No terms assigned	No terms assigned	No terms assigned
GMOY011562	2.29	Cecropin	No terms assigned	No terms assigned	GO:0005576 extracel
GMOY011562	2.36	Cecropin	No terms assigned	No terms assigned	GO:0005576 extracel
GMOY011563	2.77	Cecropin	No terms assigned	No terms assigned	GO:0005576 extracel
GMOY010882	3.02	Chemosensory protein 3	No terms assigned	No terms assigned	No terms assigned
GMOY007457	2.47	Cytochrome b561/ferric reduct transmembrane	No terms assigned	No terms assigned	GO:0016021 integral

(Continued)

TABLE 3 | Continued

Genes	Fold-change log_2 FC	Encoded proteins (best hits)	Gene Ontology (GO)		
			Biological process	Molecular function	Cellular component
GMOY003354	2.48	Elongase 9	No terms assigned	No terms assigned	GO:0016021 integral
GMOY003354	6.25	Elongase 9	No terms assigned	No terms assigned	GO:0016021 integral
GMOY008821	3.10	Elongase 4	No terms assigned	No terms assigned	GO:0016021 integral
GMOY009277	3.72	Insect cuticle protein	No terms assigned	GO:0042302 structural constituent of cuticle	No terms assigned
GMOY003876	2.08	Insect cuticle protein	No terms assigned	GO:0042302 structural constituent of cuticle	No terms assigned
GMOY011216	2.40	Insect cuticle protein	No terms assigned	GO:0042302 structural constituent of cuticle	No terms assigned
GMOY002258	2.03	Insulin-like	No terms assigned	GO:0005179 hormone activity	GO:0005576 extracel
GMOY003944	9.22	LIM and senesc, cell antigen-like-protein 1	GO:0009987 cellular process	GO:0005198 structural molecule activity	GO:0043226 organelle
GMOY011997	3.02	Mammalian NeuroPept, Y like receptor	No terms assigned	No terms assigned	GO:0016021 integral
GMOY012052	3.40	Mammalian NeuroPept, Y like receptor	No terms assigned	No terms assigned	GO:0016021 integral
GMOY009745	2.02	Milk gland protein 1	No terms assigned	No terms assigned	No terms assigned
GMOY001342	–2.40	Milk gland protein 2	No terms assigned	No terms assigned	No terms assigned
GMOY001342	2.14	Milk gland protein 2	No terms assigned	No terms assigned	No terms assigned
GMOY012125	3.57	Milk gland protein 3	No terms assigned	No terms assigned	No terms assigned
GMOY001343	2.50	Milk gland protein 6	No terms assigned	No terms assigned	No terms assigned
GMOY012016	–4.00	Milk gland protein 8	No terms assigned	No terms assigned	No terms assigned
GMOY012016	2.36	Milk gland protein 8	No terms assigned	No terms assigned	No terms assigned
GMOY012369	2.24	Milk gland protein 10	No terms assigned	No terms assigned	No terms assigned
GMOY010160	2.78	Mpv17/PMP22	No terms assigned	No terms assigned	GO:0016021 integral membrane
GMOY009494	–3.95	Rhodanese-like domain	No terms assigned	No terms assigned	No terms assigned
GMOY010675	2.12	Single domain Von Willebrand factor type C	No terms assigned	No terms assigned	No terms assigned
GMOY007078	2.52	Single domain Von Willebrand factor type C	No terms assigned	No terms assigned	No terms assigned

Gpg genes that were previously shown to be differentially expressed (DEGs) 3, 10, and 20 days after being challenged with Tbg were mapped on the Gmm genome and annotated on this reference genome. The table presents Gmm genes that are heterologs of the Gpg DEGs, in addition to the annotation results and the gene ontology. Only highly differential expressed genes ($log_2FC < -2$ or $log_2FC > 2$) have been considered. ***Black*** *fonts: genes that are differentially expressed in stimulated vs. non-stimulated flies (at day 3 after fly challenge).* ***Blue*** *and* ***Red*** *fonts: genes differentially expressed at day 10 and day 20 after fly challenge, respectively.*

functional groups and categories, we used the GO assignment to classify the functions of the unigenes. According to this process the genes expressed at high levels were classified into three GO groups (**Figure 3**) and further subdivided into categories: biological process (14 categories), molecular functions (22 categories), and cellular component (6 categories). The category "No terms assigned" was predominant across all GO groups at any investigated time.

Comparing Gpg Gene Annotation on the Gmm Genome and on a Previously Used Panel of Genomes

The global and detailed results of this comparative approach are presented in Supplementary Table S4. **Table 4**, which is a refined list of Supplementary Table S4, focuses on the expression of Gmm genes that are similar to Gpg genes previously identified as differentially expressed in response to Tbg infection. The results indicate that a high number of Gpg DEGs have orthologs in the Gmm genome. Furthermore, a large number of Gpg (22) and Gmm genes (23) encoding serine proteases were idenetified. Similarly, nine Gpg and nine Gmm genes were identified as encoding chitin binding proteins. Finally, whereas 14 Gmm genes encoding a "Major Facilitator Superfamily transporter" were identified, only one such gene was characterized in Gpg.

Homologies between Identified Gmm Genes That Are Heterologous to Gpg DEGs with Genes from Other Organisms

In order to identify genes previously annotated as "uncharacterized" or "hypothetical," we used the BLASTx program to identify heterologous genes among various organisms listed in the NCBI databases. Homologies with a

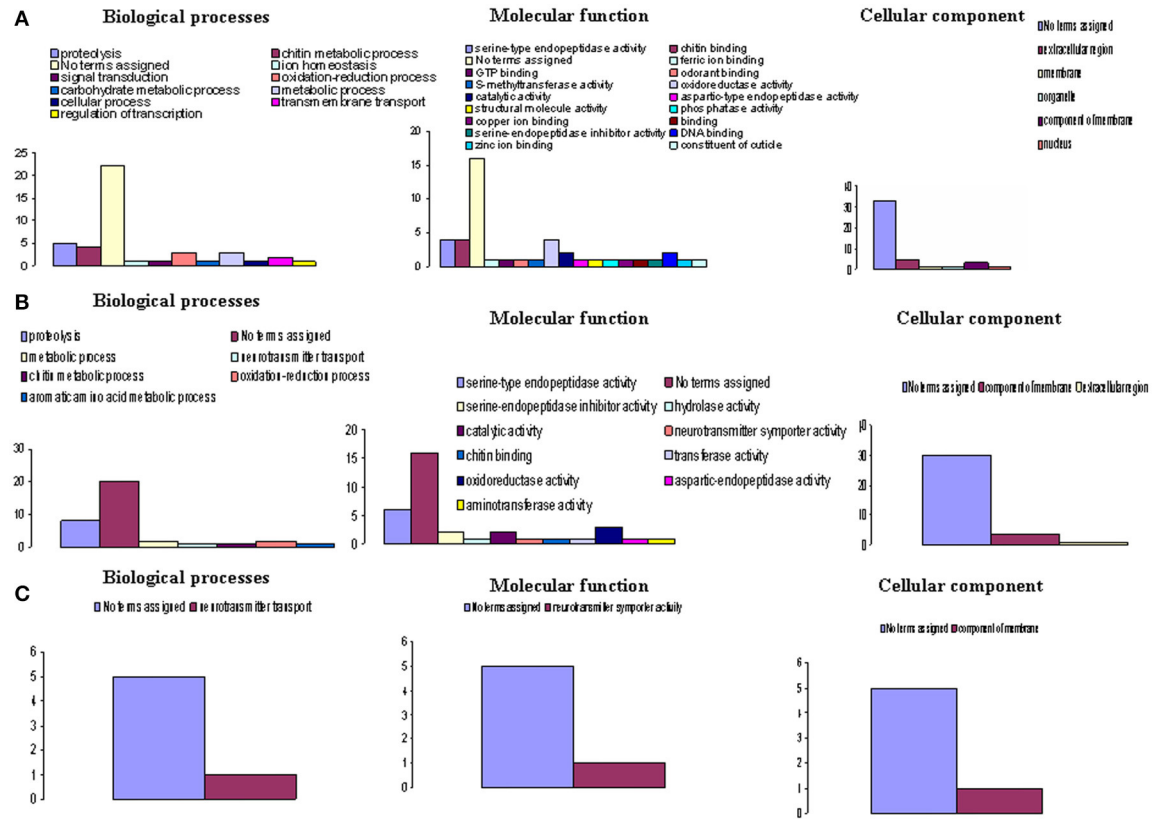

FIGURE 3 | Functional classes of Gmm genes heterologous to highly differentially expressed Gpg genes. Highly differentially expressed genes ($\log_2$ FC > +3 or $\log_2$ FC < −3) were observed in **(A)**. Tbg stimulated vs. non-stimulated Gpg flies (day-3 sampling); **(B)** Tbg infected vs. non-infected Gpg flies (day-10 sampling); and **(C)** Tbg infected vs. non-infected Gpg flies (day-20 sampling). The X-axis designates the Gene Ontology (GO) category, while the Y-axis provides the number of genes in each GO category.

cut-off $E < 10^{-5}$ and-/-or displaying the highest hits score were selected; the minimum accepted homology level was 60%. **Table 5** presents the results of the recorded annotation, and **Figure 4** presents the species from which genomes the genes to be annotated displayed the best match.

Among the 284 Gmm genes heterologous to the day-3 Gpg DEGs samples, 54 genes showed significant matches with other organisms in the investigated databases. The top homology matches were *Drosophila* sp. (11.1%), *Ceratitis capitata* (13%), and *Musca domestica* (68.5%). The remaining 7.4% of genes matched with either *Homo sapiens* (1.8%), *Lucilia sericata* (3.5%), or *Volvox carteri* (2.1%). Similarly, among the 139 Gmm genes heterologous to the day-10 Gpg DEGs samples, 33 genes showed significant matches with other organisms. The top homology matches were *Drosophila* (18.2%), *Ceratitis capitata* (21.3%), and *Musca domestica* (51.5%). The remaining 9% of genes matched with either *Loxodonta africana* (2.9%) or *Volvox carteri* (6.1%). Finally, among the 59 DEGs from day-20 samples, 12 DEGs displayed significant matches with *Musca domestica* (58.7%), *Ceratitis capitata* (8.3%), or *Drosophila* sp (33%).

Several trends appear when comparing results from the annotation reported in **Table 5** with those reported in Supplementary Tables S1–S3 (or in **Table 3**, regarding the genes in which the differential expression level was $-2 < \log_2$ FC or $\log_2$ FC > 2). First, many genes were not annotated; second, for genes that were annotated, the fold-change was identical; and finally, several genes that were annotated as "Hypothetical" when mapped on the Gmm genome could be identified when mapped on other databases. This was the case regarding the genes GMOY003830 (i.e., => Pherophorin-dz1 protein, when annotated on *Volvox carteri*), GMOY004337 (i.e., => Bardet –Biedl syndrome 4 protein homolog, when annotated on *D. willistoni* or *Ceratitis capitata*), GMOY005606 (i.e., => leucine-rich repeat-containing protein 15-like, when annotated on *Musca domestica*), GMOY007560 (i.e., => N-acetylgalactosaminyl transferase 2-like, when annotated on *C. capitata*), GMOY007584 (i.e., => Synaptotagmin-1-like, when annotated on *C capitata*), and GMOY008070 (i.e., => Pherophorin-dz1 protein, when annotated on *Volvox carteri*).

DISCUSSION

The chronic and acute forms of sleeping sickness endemic to sub-Saharan Africa are caused by two *Trypanosoma* sub-species, Tbg and Tbr, which are, respectively, transmitted to their

TABLE 4 | Identification of Gmm gene orthologs of Gpg genes on the basis of their expression products.

Glossina palpalis gambiensis genes	Best hit description-/-name of the encoded proteins	*Glossina morsitans morsitans* genes
GLOS_ARP3.1.1	Actin-related protein [*Drosophila melanogaster*]	
	Actin-related protein	GMOY008458
GLOS_DVIR_GJ17549.1.1	Acyltransferase—GJ17549 [*Drosophila virilis*]	
	Acyl-CoA N-acyltransferase	GMOY003123
GLOS_LOC101462532.1.1	Adenylosuccinate lyase-like [*Ceratitis capitata*]	
	Adenylosuccinase	GMOY002461
GLOS_LOC101450467.1.1	Alkaline phosphatase-like—membrane-bound [*Ceratitis capitata*]	
	Alkaline phosphatase	GMOY000067
GLOS_LOC101455841.1.3	Alpha-2-macroglobuline—CD109 antigen-like isoform X5 [*C. capitata*]	
	Alpha-2-macroglobulin	GMOY010996
GLOS_LOC101461571.2.2	Aspartic protease-like (lysosomal) [*Ceratitis capitata*]	
	Aspartic peptidase	GMOY005345; GMOY010103
GLOS_DVIR_GJ18228.1.1; GLOS_FDL.1.2	Beta-hexosaminidase—GJ18228 [*Drosophila virilis*/*D. melanogaster*]	
	Beta-hexosaminidase domain 2-like	GMOY001794
GLOS_KCC2A.2.2	Ca2+/calmodulin-dependent protein kinase type II	
	Ca2+/calmodulin-dependent protein kinase	GMOY006719
GLOS_CEC.2.2; GLOS_CECC.1.1; GLOS_CG10252.2.2	Cecropin [*G. m. morsitans*/*D. yakuba*/*D. melanogaster*]	
	Cecropin (anti-microbial peptide)	GMOY011562; GMOY011563
GLOS_DANA_GF24496.1.1; GLOS_DANA_GF24494.3.12	Chitin binding—GF24496 [*Drosophila ananassae*]	
GLOS_DGRI_GH11353.5.6; GLOS_DGRI_GH14440.1.1	Chitin binding—GH11353 [*Drosophila grimshawi*]	
GLOS_DMOJ_GI10981.2.2; GLOS_DMOJ_GI13574.3.3	Chitin binding—GI10981 [*Drosophila mojavensis*]	
GLOS_DWIL_GK11657.1.1; GLOS_DWIL_GK13541.1.5	Chitin binding—GK11657 [*Drosophila willistoni*]	
GLOS_DPER_GL15114.1.3	Chitin binding—GL15114 [*Drosophila persimilis*]	
	Chitin binding	GMOY002708; GMOY003840; GMOY005251; GMOY005278; GMOY009806; GMOY009807; GMOY011054; GMOY011809 GMOY011810
GLOS_LOC101462140.1.1	Chitinase 3-like [*Ceratitis capitata*]	
	Chitinase-like protein Idgf5	GMOY009161
GLOS_CP305.1.2; GLOS_C4AC3.1.1; GLOS_CP6G1.1.1; GLOS_CP9F2.9.9; GLOS_CP6W1.1.1	Cytochrome P450 305a1 [*D. melanogaster*] Cytochrome P450 9f2 [*D. melanogaster*]	
	cytochrome P450-4g1	GMOY001150; GMOY001939; GMOY006475; GMOY006761
	Cytochrome P450	GMOY002598; GMOY002627; GMOY005461; GMOY007064; GMOY007181; GMOY007270; GMOY007652; GMOY009767; GMOY009909
GLOS_RN181.1.1	E3 ubiquitin-protein ligase RNF181 homolog	
	E3 ubiquitin-protein ligase SINA like	GMOY002938; GMOY008903
GLOS_ELP2.1.1	Elongator complex protein 2; D. m. GN = Elp2 PE = 1 SV = 1	
	Elongase 4; 9	GMOY008821; GMOY003354
GLOS_LOC101461359.1.1	Endoplasmic reticulum metallopeptidase 1-like isoform X1 [*C. capitata*]	
	Endoplasmic reticulum metallopeptidase 1	GMOY009845; GMOY010241
GLOS_LOC101454791.1.1	Enkurin-like [*Ceratitis capitata*]	
	Enkurin	GMOY009600

(Continued)

TABLE 4 | Continued

Glossina palpalis gambiensis genes	Best hit description-/-name of the encoded proteins	*Glossina morsitans morsitans* genes
GLOS_LOC101459623.1.5	Exonuclease 3′-5′ domain-containing protein 2-like [*C. capitata*]	
	Exonuclease	GMOY012368
GLOS_LOC101459395.1.1	Gamma-glutamyl hydrolase-like [*Ceratitis capitata*]	
	Gamma-glutamyl hydrolase	GMOY000946
GLOS_GSTT1.2.5; GLOS_GST.1.1	Glutathione S-transferase 1-1 [*L. cuprina/Musca domestica*]	
	Glutathione S-transferase	GMOY002000; GMOY009373
GLOS_LOC101449625.1.1	Glyoxalase domain-containing protein 4-like isoform X1 [*C. capitata*]	
	Glyoxylase	GMOY008525
GLOS_DVIR_GJ19325.1.1	G-protein receptor activity—GJ19325 [*Drosophila virilis*]	
	G-protein coupled receptor	GMOY009447
GLOS_DWIL_GK15016.1.2	Haemolynph juvenile hormone binding- GK15016- [*D. willistoni*]	
	Haemolymph juvenile hormone binding	GMOY004364
GLOS_H12.1.1	Histone H1.2 [*Drosophila virilis*]	
	Histone H1	GMOY002746
GLOS_IMDH.2.2	Inosine-5′-monophosphate dehydrogenase [*D. melanogaster*]	
	Inosine-5′-monophosphate dehydrogenase	GMOY006458
GLOS_LECA.10.13	Lectin subunit alpha [*Sarcophaga peregrina*]	
	Lectin-like C-type	GMOY001011; GMOY009274
GLOS_LRRX1.1.4	Leucine-rich repeat-containing protein— [*D. discoideum*]	
	Leucine rich repeat containing protein	GMOY010344
GLOS_LSD1.1.1	Lipid storage droplets surface-binding protein 1	
	lipid storage droplet-1	GMOY007510
GLOS_DWIL_GK13707.1.1	Lipid transporter—GK13707 [*Drosophila willistoni*]	
	Lipid transport protein	GMOY002410; GMOY005442; GMOY005442;
GLOS_LOC101449088.1.1	lysM—peptidoglycan-binding domain-containing protein 1-like [*C. capitata*]	
	LysM domain	GMOY008891
GLOS_DPER_GL12526.1.1	Major Facilitator Superfamily-type transporter / — [*D. persimilis*]	
	Major Facilitator Superfamily transporter	GMOY001742; GMOY003428; GMOY003490; GMOY003491; GMOY003839; GMOY004738; GMOY005103; GMOY005106; GMOY005109; GMOY005501; GMOY007545; GMOY007627; GMOY012075; GMOY012352
GLOS_LOC101462556.1.1	MD-2-related lipid-recognition protein-like [*Ceratitis capitata*]	
	MD-2-related lipid-recognition domain	GMOY006406
GLOS_MYSN.1.1	Myosin heavy chain, non-muscle [*D. melanogaster*]	
	Myosin heavy chain	GMOY007533; GMOY008852
GLOS_DMOJ_GI24301.1.1	Neuropeptide Y receptor—GI24301 [*Drosophila mojavensis*]	
	NeuroPeptide Y like receptor / mammalian (Putative)	GMOY011997; GMOY012052
GLOS_DGRI_GH13991.1.1; GLOS_DVIR_GJ10540.1.1	Odorant binding—GH13991 [*Drosophila grimshawi*]/*Drosophila virilis*	
GLOS_OB99B.1.1	Odorant-binding protein 99b	

(Continued)

TABLE 4 | Continued

***Glossina palpalis gambiensis* genes**	**Best hit description-/-name of the encoded proteins**	***Glossina morsitans morsitans* genes**
	Odorant binding protein 1; 2; 7	GMOY000890; GMOY002825; GMOY005548;
	Odorant binding protein 21; 22	GMOY006418; GMOY001476
GLOS_LOC101453268.1.1	Period circadian protein-like [*Ceratitis capitata*]	
	Period circadian protein	GMOY012110
GLOS_LOC101458811.1.1	Pyridoxal kinase-like [*Ceratitis capitata*]	
	Pyridoxal phosphate-dependent transferase	GMOY005488; GMOY005934
GLOS_DMOJ_GI20119.1.1; GLOS_DMOJ_GI16517.2.2;	Serine proteases (see details in Supplementary Table S4)	GMOY000672; GMOY002036; GMOY002729; GMOY003271
GLOS_DSEC_GM17695.1.1; GLOS_LOC101456159.4.4;		GMOY003273; GMOY003280; GMOY003693; GMOY003994
GLOS_DMOJ_GI18413.1.2; GLOS_DANA_GF15448.2.3;		GMOY006266; GMOY006369; GMOY006991; GMOY008468
GLOS_AAEL_AAEL007969.1.1; GLOS_LOC101461009.2.2;		GMOY008469; GMOY008958; GMOY008962; GMOY008964
GLOS_EAST.2.2; GLOS_LOC101457953.1.5;		GMOY008965; GMOY008966; GMOY009418; GMOY009436
GLOS_LOC101462986.1.1; GLOS_DWIL_GK19454.1.1		GMOY009757; GMOY010502; GMOY010768
GLOS_LOC101459895.9.9; GLOS_DWIL_GK24139.1.1;		
GLOS_LOC101455430.10.10; GLOS_LOC101455604.4.10;		
GLOS_DMOJ_GI21244.4.5; GLOS_DMOJ_GI24442.1.1;		
GLOS_DVIR_GJ21497.1.3; GLOS_DVIR_GJ22718.8.10;		
GLOS_DVIR_GJ21498.1.1; GLOS_DVIR_GJ21499.1.1;		
GLOS_DMOJ_GI19420.1.1; GLOS_DVIR_GJ17584.1.1	Serine protease inhibitor (Serpin) GI19420 [*D. mojavensis/D. virilis*]	
GLOS_DANA_GF14653.1.2; GLOS_DERE_GG24413.1.1;	Serine-type endopeptidase inhibitor— [*D. ananassae/D. erecta*]	
GLOS_DWIL_GK10999.1.1; GLOS_LOC101459846.1.2	Serine-type endopeptidase inhibitor /Metalloendopeptidase [*D. willistoni*]	
	Serine proteinase inhibitors (Kazal domain)	GMOY010058
GLOS_DWIL_GK15974.5.7; GLOS_DMOJ_GI22128.1.1	Single domain von Willebrand factor type C [*D. willistoni*]	
	Single domain Von Willebrand factor type C	GMOY003774; GMOY005237; GMOY007078; GMOY010675
GLOS_DWIL_GK22031.1.1	Sulfate transmembrane transporter—GK22031 [*Drosophila willistoni*]	
	Sulfate transporter	GMOY000550
GLOS_LOC101448839.1.1; GLOS_LOC101454308.1.2	Timeless-like isoform X1 protein (circadian rhythm regulation) [*C. capitata*]	
	Timeless protein	GMOY006112
GLOS_TRF.1.1	Transferrin [*Sarcophaga peregrina*]	
	Transferrin family, iron binding site	GMOY004228
GLOS_LOC101463325.1.1	Trypsin-like [*Ceratitis capitata*]	
	Trypsin-like cysteine/serine peptidase domain	GMOY002535; GMOY008308
GLOS_DWIL_GK18237.1.1	UDP-glucuronosyl/UDP-glucosyltransferase—GK18237 [*D. willistoni*]	
	UDP-glucuronosyl/UDP-glucosyltransferase	GMOY007046
GLOS_LOC101451574.1.1	WD repeat-containing protein 81-like isoform X1 [*Ceratitis capitata*]	
	WD40/YVTN repeat-like-containing domain	GMOY010092
GLOS_LOC101458997.1.1	Yellow-like protein (protein of the gelly) [*Ceratitis capitata*]	
	yolk protein 3	GMOY006227

The table compares the results of the previous Gpg DEG annotation on a panel of various genomes with the corresponding annotation on the Gmm genome. A comparison is presented between the Gpg and the Gmm genes on the basis of the identified proteins they encode.

TABLE 5 | Gmm gene heterologs of Gpg DEGs matching genes from other organism databases.

Genes	log_2 FC	Homology with other organisms (>60%)
GMOY000550	-0.90	*Musca domestica* sodium-independent sulfate anion transporter-like (LOC101893700), mRNA
GMOY001137	1.09	*Musca domestica* aminomethyltransferase, mitochondrial-like (LOC101895864), mRNA
GMOY001742	-0.9	*Ceratitis capitata* synaptic vesicle glycoprotein 2B-like (LOC101452461), transcript variant X3, mRNA
GMOY002004	-1.78	*Musca domestica* putative fatty acyl-CoA reductase CG5065-like (LOC101898308), mRNA
GMOY002004	1.8	*Musca domestica* putative fatty acyl-CoA reductase CG5065-like (LOC101898308), mRNA
GMOY002024	0.96	*Musca domestica* phosphotriesterase-related protein-like (LOC101890186), mRNA
GMOY002356	2.31	*C. capitata* CUGBP Elav-like family member 2-like (LOC101455154), transcript variant X1 to X3, mRNA
GMOY002461	0.82	*Musca domestica* adenylosuccinate lyase-like (LOC101900029), mRNA
GMOY002486	2.6	*Musca domestica* potassium channel subfamily K member 9-like (LOC101895107), mRNA
GMOY002535	1.5	*Ceratitis capitata* serine protease easter-like (LOC101451852), mRNA
GMOY002729	1.91	*Lucilia sericata* clone LScDNA1 putative salivary trypsin mRNA, complete cds
GMOY002729	3.0	*Musca domestica* serine proteinase stubble-like (LOC101890358), mRNA
GMOY002938	-1.10	*Musca domestica* uncharacterized LOC101893009 (LOC101893009), mRNA
GMOY003158	2.70	*Musca domestica* uncharacterized LOC101895341 (LOC101895341), mRNA
GMOY003161	1.44	*Musca domestica* thyrotropin receptor-like (LOC101887582), mRNA
GMOY003354	2.48	*Ceratitis capitata* elongation of very long chain fatty acids protein AAEL008004-like (LOC101449680), mRNA
GMOY003354	6.3	*Musca domestica* elongation of very long chain fatty acids protein AAEL008004-like (LOC101893043), mRNA
GMOY003443	1.27	*Musca domestica* uncharacterized LOC101889318 (LOC101889318), partial mRNA
GMOY003590	1.77	*Musca domestica* collagen alpha-1(IV) chain-like (LOC101897761), transcript variant X3, mRNA
GMOY003590	1.3	*Musca domestica* collagen alpha-1(IV) chain-like (LOC101897761), transcript variant X3, mRNA and variant X1, X2
GMOY003830	2.75	*Homo sapiens* BAC clone CH17-465I15 from chromosome unknown, complete sequence (=hypothetical)
GMOY003830	3.7	*Volvox carteri f*, *nagariensis* mRNA for pherophorin-dz1 protein
GMOY003839	0.95	*Musca domestica* putative inorganic phosphate cotransporter-like (LOC101889974), mRNA
GMOY003949	1.01	*M. domestica* glutamine-fructose-6-phosphate aminotransferase [isomerizing] 2-like (LOC101889985), transcript
GMOY004309	2.44	*Musca domestica* fatty acid synthase-like (LOC101893120), mRNA
GMOY004332	2.1	*Drosophila willistoni* GK20732 (Dwil\GK20732), mRNA /Fatty acyl-CoA reductase
GMOY004337	5.8	*D. willistoni* GK20950 (Dwil\GK20950), mRNA Bardet-Biedl syndrome 4 protein homolog (=hypothetical on Gmm genome)
GMOY004337	6.6	*Ceratitis capitata* Bardet-Biedl syndrome 4 protein homolog (LOC101449311), mRNA (=hypothetical on Gmm genome)
GMOY004589	1.09	*Musca domestica* muscle M-line assembly protein unc-89-like (LOC101890868) mRNA
GMOY004712	1.65	*Musca domestica* acyl-protein thioesterase 1-like (LOC101890399), mRNA
GMOY004738	-0.78	*Musca domestica* facilitated trehalose transporter Tret1-like (LOC101891733), transcript variant X1, Mrna
GMOY004873	1.6	*Musca domestica* transmembrane and TPR repeat-containing protein CG4341-like (LOC101893859), mRNA
GMOY005102	6.28	*Musca domestica* N-acetylgalactosaminyltransferase 4-like (LOC101894376), mRNA
GMOY005106	-1.22	*Drosophila willistoni* GK13266 (Dwil\GK13266), mRNA / Major facilitator superfamily transporter
GMOY005278	2.93	*Musca domestica* mucin-5AC-like (LOC101899868), mRNA
GMOY005278	1.9	*Musca domestica* mucin-5AC-like (LOC101899868), mRNA
GMOY005345	-6.5	*Musca domestica* lysosomal aspartic protease-like (LOC101894831), mRNA
GMOY005487	3.48	*Musca domestica* LIM/homeobox protein Lhx4-like (LOC101900654), mRNA
GMOY005488	1.81	*Musca domestica* alpha-methyldopa hypersensitive protein-like (LOC101888467), mRNA
GMOY005527	0.87	*Musca domestica* c-1-tetrahydrofolate synthase, cytoplasmic-like (LOC101891351), transcript variant X2, mRNA
GMOY005606	6.3	*Musca domestica* leucine-rich repeat-containing protein 15-like (LOC101899894), mRNA (=hypothetical on Gmm genome)
GMOY005934	2.72	*Ceratitis capitata* cysteine sulfinic acid decarboxylase-like (LOC101455610), mRNA
GMOY006111	1.1	*Drosophila willistoni* GK14673 (Dwil\GK14673), mRNA (Gonadal trypsine)
GMOY006205	1.2	*Ceratitis capitata* DNA replication licensing factor Mcm5-like (LOC101458261), mRNA
GMOY006406	1.69	*Musca domestica* ecdysteroid-regulated 16 kDa protein-like (LOC101898283), mRNA
GMOY006406	1.7	*Musca domestica* ecdysteroid-regulated 16 kDa protein-like (LOC101898283), mRNA
GMOY006458	-0.78	*Musca domestica* inosine-5′-monophosphate dehydrogenase-like (LOC101895820), mRNA
GMOY006671	4.0	*Musca domestica* uncharacterized LOC101890025 (LOC101890025), mRNA
GMOY006761	1.99	*Musca domestica* cytochrome P450 CYP4G13v2 mRNA, complete cds
GMOY006761	2.4	*Musca domestica* cytochrome P450 CYP4G13v2 mRNA, complete cds
GMOY006761	-2.1	*Musca domestica* cytochrome P450 CYP4G13v2 mRNA, complete cds

(Continued)

TABLE 5 | Continued

Genes	log_2 FC	Homology with other organisms (>60%)
GMOY006875	-2.0	*Musca domestica* membrane-bound alkaline phosphatase-like (LOC101896753), mRNA
GMOY006875	-1.8	*Musca domestica* membrane-bound alkaline phosphatase-like (LOC101896753), mRNA
GMOY006979	1.08	*Musca domestica* phosrestin-2-like (LOC101892743), Mrna
GMOY007046	2.0	*Ceratitis capitata* UDP-glucuronosyltransferase 2B13-like (LOC101462823), transcript variant X2, mRNA
GMOY007131	0.97	*Musca domestica* inositol-3-phosphate synthase-like (LOC101889622), mRNA
GMOY007148	2.10	*Ceratitis capitata* fatty acid synthase-like (LOC101463409), mRNA
GMOY007497	6.3	*Musca domestica* NADH-cytochrome b5 reductase 3-like (LOC101897795), transcript variant X2, mRNA
GMOY007523	1.44	*Musca domestica* collagen alpha-1(IV) chain-like (LOC101895032), transcript variant X3, mRNA
GMOY007523	1.1	*Musca domestica* collagen alpha-1(IV) chain-like (LOC101895032), transcript variant X3, mRNA
GMOY007560	-1.0	*C. capitata* polypeptide N-acetylgalactosaminyltransferase 2-like (LOC101448408), mRNA (=hypothetical on Gmm genome)
GMOY007584	1.1	*Ceratitis capitata* synaptotagmin-1-like (LOC101450559), mRNA (=hypothetical on Gmm genome)
GMOY008017	1.88	*Volvox carteri f. nagariensis* mRNA for pherophorin-dz1 protein (=hypothetical when mapped on Gmm genome)
GMOY008017	1.2	*Volvox carteri f, nagariensis* mRNA for pherophorin-dz1 protein (=hypothetical when mapped on Gmm genome)
GMOY008266	-1.3	*Drosophila willistoni* GK24772 organic anion transporter (Dwil\GK24772), mRNA
GMOY008308	1.9	*Drosophila melanogaster* easter (ea), transcript variant A, mRNA
GMOY008458	5.74	*Musca domestica* actin, indirect flight muscle-like (LOC101895248), mRNA
GMOY008525	0.97	*Drosophila willistoni* GK21885 (Dwil\GK21885), mRNA
GMOY008601	2.58	*Musca domestica* fatty acid synthase-like (LOC101893120), mRNA
GMOY008601	4.1	*Drosophila willistoni* GK12914 (Dwil\GK12914), mRNA
GMOY008602	2.02	*Drosophila pseudoobscura pseudoobscura* GA26263 (Dpse\GA26263), mRNA
GMOY008602	-2.5	*Drosophila willistoni* GK12914 (Dwil\GK12914), mRNA
GMOY008852	0.93	*Musca domestica* myosin heavy chain, non-muscle-like (LOC101892851), transcript variant X1 to X3, Mrna
GMOY008966	4.4	*Loxodonta africana* kallikrein-11-like (LOC100667195), mRNA
GMOY008973	0.80	*Lucilia cuprina* alpha esterase (LcaE7) mRNA, implicated in organophosphate resistance, complete cds
GMOY009018	1.59	*Ceratitis capitata* uncharacterized LOC101448539 (LOC101448539), transcript variant
GMOY009018	1.8	*Musca domestica* uncharacterized LOC101899326 (LOC101899326), mRNA
GMOY009079	1.23	*Musca domestica* fatty acid synthase-like (LOC101893120), mRNA
GMOY009375	7.56	*Musca domestica* uncharacterized LOC101900740 (LOC101900740), mRNA
GMOY009394	-5.56	*Musca domestica* CCAAT/enhancer-binding protein-like (LOC101898926), mRNA
GMOY009447	-1.22	*Ceratitis capitata* calcitonin gene-related peptide type 1 receptor-like (LOC101462563), mRNA
GMOY009600	1.20	*Musca domestica* enkurin-like (LOC101897351), mRNA
GMOY009845	0.6	*Musca domestica* endoplasmic reticulum metallopeptidase 1-like (LOC101898765), transcript variant X3, mRNA
GMOY009903	2.75	*M. domestica* strain rspin nicotinic acetylcholine receptor beta 3 subunit (nAChRbeta3) gene, nAChRbeta3-C allele, complete cds
GMOY009983	1.7	*Drosophila grimshawi* GH17190 (Dgri\GH17190), mRNA
GMOY010224	6.9	*Musca domestica* uncharacterized LOC101889990 (LOC101889990), partial mRNA
GMOY010224	3.6	*Musca domestica* uncharacterized LOC101889990 (LOC101889990), partial mRNA **(=> Hypothetical)**
GMOY010241	0.8	*Musca domestica* endoplasmic reticulum metallopeptidase 1-like (LOC101898765), transcript variant X3, mRNA
GMOY010481	-1.53	*Musca domestica* protein Wnt-5-like (LOC101892275), mRNA
GMOY010972	0.73	*Musca domestica* phenoloxidase subunit A3-like (LOC101897997), transcript variant X1 and X2, mRNA
GMOY011232	1.32	*Drosophila melanogaster* PAPS synthetase (Papss), transcript variant A, mRNA variant A to H
GMOY011418	0.87	*Drosophila willistoni* GK13980 (Dwil\GK13980), mRNA / glycogen synthase
GMOY011618	-0.9	*Ceratitis capitata* putative fatty acyl-CoA reductase CG5065-like (LOC101456246), transcript variant X2, mRNA
GMOY012052	3.4	*Drosophila pseudoobscura pseudoobscura* GA30114 Neuropeptide Y (Dpse\GA30114), mRNA
GMOY012069	9.1	*Musca domestica* uncharacterized LOC101891108 (LOC101891108), mRNA
GMOY012069	5.3	*Musca domestica* uncharacterized LOC101891108 (LOC101891108), mRNA
GMOY012075	1.56	*Drosophila melanogaster* CG31663 (CG31663), transcript variant B, mRNA (Major facilitator superfamily transporter)
GMOY012075	-1.7	*Drosophila willistoni* GK15555 (Dwil\GK15555), mRNA (Major facilitator superfamily transporter)
GMOY012352	0.94	*Ceratitis capitata* monocarboxylate transporter 10-like (LOC101448353), transcript variant X1, mRNA

Black fonts, day-3 samples; Blue fonts, day-10 samples; Red fonts, day-20 samples.

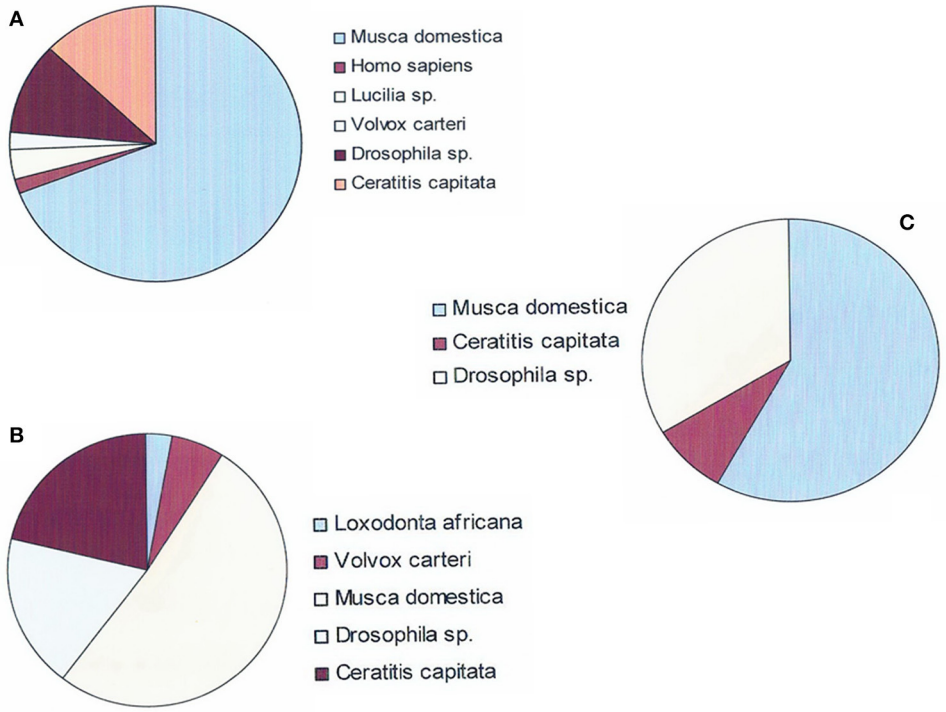

FIGURE 4 | Species on which Gmm genes were mapped in order to characterize genes previously annotated as "hypothetical." (A) Mapping of the 284 Gmm genes heterologous to Gpg DEGs in trypanosome stimulated vs. non-stimulated flies (day-3 sampling). **(B)** Mapping of the 139 Gmm genes heterologous to Gpg DEGs in trypanosome infected vs. non-infected flies (day-10 sampling). **(C)** Mapping of the 59 Gmm genes heterologous to Gpg DEGs in trypanosome infected vs. non-infected flies (day-20 sampling).

vertebrate hosts by the *Glossina* species Gpg and Gmm (Aksoy et al., 2014; Beschin et al., 2014). Nevertheless, the biological cycles, vertebrate transmission processes, and pathogenicity development of the two parasites are similar. Recently, in the context of an anti-vector strategy project to fight the disease, we performed a global transcriptomic analysis of Gpg gene expression associated with fly infection by Tbg. More precisely, we attempted to characterize genes that were differentially expressed according to the status of the fly at several sampling times (i.e., non-infected, infected, or self-cured). This included genes that could be involved in the fly's vector competence, and consequently genes that could possibly be manipulated in order to reduce or even suppress this competence.

The similarities between the Tbg and Tbr life cycles prompted us to determine whether the Gmm genome carried genes that could be heterologous to the Gpg DEGs, which could then allow the development of common molecular approaches. Accordingly, the Gpg sequences resulting from the previous RNA-seq *de novo* assembly (Hamidou Soumana et al., 2015) were mapped on the Gmm genome, the DEGs were characterized, and the corresponding genes were annotated.

When the Gpg sequences were mapped and annotated on a panel of various databases (*C. capitata*, *D. melanogaster*, *D. willistoni*, *D. virilis*, *D. mojavensis*, *Acyrthosiphon pisum*, *Hydra magnipapillata*, *Anopheles* sp., *Bombyx* sp., *Aedes* sp., and *G. morsitans*; Hamidou Soumana et al., 2015) we identified 553 (S vs. NS), 52 (I10 vs. NI10), and 143 (I20 vs. NI20) DEGs. In contrast, we identified 284 (S vs. NS), 139 (I10 vs. NI10) and 59 (I20 vs. NI20) DEGs when sequences were mapped and annotated on the *G. m. morsitans* database (using its whole genome annotated on the *Drosophila melanogaster*, *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, and *Phlebotomus papatasi* databases; International Glossina Genome Initiative, 2014). The differences in the number of identified DEGs, as well as the high number of "uncharacterized" genes, could be due to differences in the database panels used to annotate Gpg or Gmm. We cannot exclude the possibility that some of the Gpg DEGs do not have heterologous genes in Gmm, or that some of them could be specific to either Gpg or Gmm and consequently cannot be annotated yet. Nevertheless, regarding I10 vs. NI10 sampling (and in contrast to the two other experimental conditions), the number of recorded DEGs was more than 2-fold higher when the Gpg transcripts were mapped on the Gmm genome, prompting questions of how this is possible. However, at this stage of our research we cannot offer a satisfactory explanation.

We examined the potential influence of database panel composition by annotating the Gmm DEGs on a separate set of databases that included *D. melanogaster* as an internal control. The results (**Table 5**) clearly demonstrate the validity of the annotation process, since all Gmm genes (GMOY, etc.) were annotated (best hit description and fold-change) on the novel set of databases as they had been annotated on the former

set (**Table 3**), and that several genes could be identified thanks to their annotation primarily on the *Volvox carteri* or *Musca domestica* databases which had never been used before, and despite the fact these organisms (algae and mouse) are genetically distant from the tsetse fly.

The most important observation regarding our objective is that almost all of the Gpg genes previously considered to be potentially involved in tsetse fly vector competence (cf. Hamidou Soumana et al., 2015) had a "countrepart" (i.e., heterologous genes) in the Gmm genome, despite the fact that none of the Gpg DEGs matched with any Gmm genes. This was the case for the large array of genes encoding peptidases, especially serine peptidases (represented by more than 20 genes), identified in the genomes of both fly species. This was similarly observed for ∼10 genes present in both genomes that encode chitin binding proteins, since chitin metabolism is involved in the ability of tsetse flies to host trypanosomes (Maudlin and Welburn, 1994; Welburn and Maudlin, 1999), in addition to cecropin (an antimicrobial peptide), among others (Weiss et al., 2014).

Here, we were particularly interested in detecting the presence or not of genes with a reported role in the immunity of tsetse flies or other organisms (Weiss et al., 2014). Genes encoding Pro3 protein (GMOY009756, GMOY000672) and transferrin (GMOY004228) were identified. Pro3 has a potential function as a serine protease (tyrosinase) and is specifically produced by the proventriculus, an organ that plays an important role in the tsetse immune response. This protein could be involved in the immune response via activation of the cascade of prophenol oxidase and melanization (Jiang et al., 1998). Moreover, the gene GMOY0010488 was identified as encoding an "immuno reactive putative protease inhibitor" that is overexpressed in trypanosome stimulated or infected Gpg flies. The transferrin gene was overexpressed in both stimulated and infected Gpg flies; this result is in agreement with Geiser and Winzerling (2012), who reported on the role of transferrin in the immune response of insects, as well as its role in iron transport. By reducing the oxidative stress in tsetse fly guts, transferrin may promote the survival of trypanosomes. Guz et al. (2012) observed transferrin overexpression after challenge with bacteria, even at a higher level than what is typically observed in the case of infection by trypanosomes.

The gene GMOY011809 encodes Pro 1 peritrophin, which is a constituent of the peritrophic membrane (PM). The PM is established after the fly takes its first blood meal, and it is permanently renewed by the proventriculus (Moloo et al., 1970; Tellam et al., 1999). The PM primarily functions to envelop the blood meal and protect the intestinal epithelium against abrasion by ingested matter, although it can also represent an obstacle to the passage of ingested parasites into the ectoperitrophic space (Lehane, 1997; Hegedus et al., 2009). The gene GMOY005278 encodes mucin, which participates with peritrophin in the composition of the PM.

We have also identified genes encoding antimicrobial peptides: in Supplementary Table S1, GMOY01052 through GMOY010524 encode attacin, whereas GMOY0011562 and GMOY0011563 encode cecropin. Furthermore, both attacin and cecropin are overexpressed in Gpg trypanosome stimulated or infected flies.

Our work is the first comparison of its kind between the two *Glossina* species. This is primarily due to the fact that the different scientific teams working on HAT commonly focus on investigating either Gmm (and the acute form of trypanosomiasis) or Gpg (and the chronic form of trypanosomiasis), but not both together. Indeed, one of our most relevant findings is the observation that Gmm has the same genes at its disposal that Gpg may use to control its vector competence. Importantly, this comparison will assist future studies in revealing common molecular targets to increase the refractoriness of either fly species to infection by trypanosomes.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: IH, AG. Performed the experiments: IH, BT, SR, HP. Analyzed the data: IH, SR, HP, AG. Wrote the paper: AG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00540/full#supplementary-material

REFERENCES

Aksoy, S., Weiss, B. L., and Attardo, G. M. (2014). Trypanosome transmission dynamics in tsetse. *Curr. Opin. Insect Sci.* 3, 43–49. doi: 10.1016/j.cois.2014.07.003

Anders, S., Pyl, P. T., and Huber, W. (2014). HTSeq - A Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300.

Beschin, A., Van Den Abbeele, J., De Baetselier, P., and Pays, E. (2014). African trypanosome control in the insect vector and mammalian host. *Trends Parasitol.* 30, 538–547. doi: 10.1016/j.pt.2014.08.006

Bullard, J. H., Purdom, E., Hansen, K. D., and Dudoit, S. (2010). Upper quartile: evaluation of statistical methods for normalization and differential expression in mRNASeq experiments. *BMC Bioinformatics* 11:94. doi: 10.1186/1471-2105-11-94

Dennis G. Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., et al. (2003). DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* 4:P3. doi: 10.1186/gb-2003-4-5-p3

Farikou, O., Njiokou, F., Mbida Mbida, J. A., Njitchouang, G. R., Djeunga, H. N., Asonganyi, T., et al. (2010). Tripartite interactions between tsetse flies, Sodalis

glossinidius and trypanosomes-an epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. *Infect. Genet. Evol.* 10, 115–121. doi: 10.1016/j.meegid.2009.10.008

Geiser, D. L., and Winzerling, J. J. (2012). Insect transferrins: multifunctional proteins. *Biochim. Biophys. Acta* 1820, 437–451. doi: 10.1016/j.bbagen.2011.07.011

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5:R80. doi: 10.1186/gb-2004-5-10-r80

Guz, N., Kilincer, N., and Aksoy, S. (2012). Molecular characterization of *Ephestia kuehniella* (Lepidoptera: Pyralidae) transferrin and its response to parasitoid Venturia canescens (Hymenoptera: Ichneumonidae Gravenhorst). *Insect Mol. Biol.* 21, 139–147. doi: 10.1111/j.1365-2583.2011.01129.x

Hamidou Soumana, I., Klopp, C., Ravel, S., Nabihoudine, I., Tchicaya, B., Parrinello, H., et al. (2015). RNA-seq *de novo* assembly reveals differential gene expression in *Glossina palpalis* gambiensis infected with *Trypanosoma brucei* gambiense vs. non-infected and self-cured flies. *Front. Microbiol.* 6:1259. doi: 10.3389/fmicb.2015.01259

Hamidou Soumana, I., Loriod, B., Ravel, S., Tchicaya, B., Simo, G., Rihet, P., et al. (2014a). The transcriptional signatures of *Sodalis glossinidius* in the *Glossina palpalis* gambiensis flies negative for *Trypanosoma brucei* gambiense contrast with those of this symbiont in tsetse flies positive for the parasite: possible involvement of a Sodalis-hosted prophage in fly Trypanosoma refractoriness? *Infect. Genet. Evol.* 24, 41–56. doi: 10.1016/j.meegid.2014.03.005

Hamidou Soumana, I., Tchicaya, B., Simo, G., and Geiger, A. (2014b). Comparative gene expression of Wigglesworthia inhabiting non-infected and *Trypanosoma brucei* gambiense-infected *Glossina palpalis* gambiensis flies. *Front. Microbiol.* 5:620. doi: 10.3389/fmicb.2014.00620

Hegedus, D., Erlandson, M., Gillott, C., and Toprak, U. (2009). New insights into peritrophic matrix synthesis, architecture, and function. *Annu. Rev. Entomol.* 54, 285–302. doi: 10.1146/annurev.ento.54.110807.090559

Hoare, C. A. (1972). *The Trypanosomes of Mammals, A Zoological Monograph*. Oxford: Blackwell Scientific Publications.

International Glossina Genome Initiative (2014). Genome sequence of the tsetse fly (*Glossina morsitans*): vector of African trypanosomiasis. *Science* 344, 380–386. doi: 10.1126/science.1249656

Jiang, H., Wang, Y., and Kanost, M. R. (1998). Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to Drosophila easter. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12220–12225. doi: 10.1073/pnas.95.21.12220

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 14:R36. doi: 10.1186/gb-2013-14-4-r36

Langmead, B., and Salzberg, S. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42, 525–550. doi: 10.1146/annurev.ento.42.1.525

Louis, F. J., Simarro, P. P., and Lucas, P. (2002). Sleeping sickness: one hundred years of control strategy evolution. *Bull. Soc. Pathol. Exot.* 95, 331–336.

Maudlin, I., and Welburn, S. C. (1994). Maturation of trypanosome infections in tsetse. *Exp. Parasitol.* 79, 202–205. doi: 10.1006/expr.1994.1081

Moloo, S. K., Steiger, R. F., and Hecker, H. (1970). Ultrastructure of the peritrophic membrane formation in Glossina Wiedemann. *Acta Trop.* 274, 378–383.

Mount, D. W. (2007). Using the Basic Local Alignment Search Tool (BLAST). *CSH Protoc.* 2007:pdb.top17. doi: 10.1101/pdb.top17

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Savage, A. F., Kolev, N. G., Franklin, J. B., Vigneron, A., Aksoy, S., and Tschudi, C. (2016). Transcriptome profiling of *Trypanosoma brucei* development in the tsetse fly vector *Glossina morsitans*. *PLoS ONE* 11:e0168877. doi: 10.1371/journal.pone.0168877

Tellam, R. L., Wijffels, G., and Willadsen, P. (1999). Peritrophic matrix proteins. *Insect Biochem. Mol. Biol.* 29, 87–101. doi: 10.1016/S0965-1748(98)00123-4

Van den Abbeele, J., Claes, Y., van Bockstaele, D., Le Ray, D., and Coosemans, M. (1999). *Trypanosoma brucei* spp. development in the tsetse fly: characterization of the post mesocyclic stages in the foregut and proboscis. *Parasitology* 118, 469–478. doi: 10.1017/S0031182099004217

Vickerman, K., Tetley, L., Hendry, K. A., and Turner, C. M. (1988). Biology of African trypanosomes in the tsetse fly. *Biol. Cell* 64, 109–119. doi: 10.1016/0248-4900(88)90070-6

Weiss, B. L., Savage, A. F., Griffith, B. C., Wu, Y., and Aksoy, S. (2014). The peritrophic matrix mediates differential infection outcomes in the tsetse fly gut following challenge with commensal, pathogenic, and parasitic microbes. *J. Immunol.* 193, 773–782. doi: 10.4049/jimmunol.1400163

Welburn, S. C., and Maudlin, I. (1999). Tsetse-trypanosome interactions: rites of passage. *Parasitol. Today* 15, 399–403. doi: 10.1016/S0169-4758(99)01512-4

Welburn, S. C., Maudlin, I., and Simarro, P. P. (2009). Controlling sleeping sickness-a review. *Parasitology* 136, 1943–1949. doi: 10.1017/S0031182009006416

Wixon, J., and Kell, D. (2000). The Kyoto encyclopedia of genes and genomes-KEGG. *Yeast* 17, 48–55. doi: 10.1002/(SICI)1097-0061(200004)17:1<48::AID-YEA2>3.0.CO;2-H

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MINI REVIEW
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Implication of Apoptosis for the Pathogenesis of *Trypanosoma cruzi* Infection

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Apoptosis is induced during the course of immune response to different infectious agents, and the ultimate fate is the recognition and uptake of apoptotic bodies by neighboring cells or by professional phagocytes. Apoptotic cells expose specific ligands to a set of conserved receptors expressed on macrophage cellular surface, which are the main cells involved in the clearance of the dying cells. These scavenger receptors, besides triggering the production of anti-inflammatory factors, also block the production of inflammatory mediators by phagocytes. Experimental infection of mice with the parasite *Trypanosoma cruzi* shows many pathological changes that parallels the evolution of human infection. Leukocytes undergoing intense apoptotic death are observed during the immune response to *T. cruzi* in the mouse model of the disease. *T. cruzi* replicate intensely and secrete molecules with immunomodulatory activities that interfere with T cell-mediated immune responses and secretion of pro-inflammatory cytokine secretion. This mechanism of immune evasion allows the infection to be established in the vertebrate host. Under inflammatory conditions, efferocytosis of apoptotic bodies generates an immune-regulatory phenotype in phagocytes, which is conducive to intracellular pathogen replication. However, the relevance of cellular apoptosis in the pathology of Chagas' disease requires further studies. Here, we review the evidence of leukocyte apoptosis in *T. cruzi* infection and its immunomodulatory mechanism for disease progression.

Keywords: ***Trypanosoma cruzi*, apoptosis, immunomodulation, infection, macrophages**

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INTRODUCTION

Chagas disease or American trypanosomiasis was discovered by Carlos Chagas in the early twentieth century. The disease is caused by the protozoan parasite *Trypanosoma cruzi*. It is estimated that 18–20 million people in Latin America are infected with *T. cruzi*, and approximately 100 million people are living in areas at risk of infection (1). *T. cruzi* is an obligate intracellular parasite that infects a variety of the mammalian host cells but shows preference for cells of the macrophage and muscle lineage.

The biological cycle in man has two evolutionary forms (1) trypomastigotes, the flagellate form of the parasite that invades cells, where they differentiate into (2) amastigotes that have no free flagellum and replicate inside almost all nucleated cells of the vertebrate host (2). Soon after infection, trypomastigotes have the ability to escape the parasitophorous vacuole and differentiate into amastigotes in the host cell cytoplasm. After several rounds of binary divisions inside the infected cells, amastigotes differentiate to trypomastigotes forms, lyse the infected cells, and reinvade adjacent cells (3, 4).

An important successful factor that allows *T. cruzi* to survive in the vertebrate host is the evasion of the cellular immune response (5–7). Many studies in this area describe the existence of different strategies developed by the parasite to modulate the immune response of the vertebrate host in its favor (4, 8–12).

One of the most efficient mechanisms that *T. cruzi* parasites use to establish a persistent infection is the induction of T and B cell apoptosis, and such process has immunomodulatory effects on the host immune response (8, 13).

APOPTOSIS AS A HALLMARK OF *T. cruzi* INFECTION

Apoptosis is crucial for normal tissue homeostasis and for modulation of immune response. During parasitic infections, apoptosis or programmed cell death can be triggered by antigens, factors secreted and/or released by pathogens, by chronic infection, and by intense cellular activation (14–16). Recent evidences have shown that in order to survive in their hosts, intracellular protozoan parasites have to limit the defense mechanisms and are taking advantage of cell death to facilitate parasite spreading. It was described that *T. cruzi* infection triggers activation-induced cell death (AICD) of $CD4^+$ T lymphocytes during the acute phase of infection (8, 17). The AICD occurs after stimulation with anti-TCR and anti-CD3 agonist antibodies *in vitro*. When cultured in the presence of anti-Fas agonist antibody, $CD4^+$ T cells from infected mice, but not from normal mice, undergo apoptosis (17, 18).

The interaction between the Fas molecule (CD95) and its ligand, the Fas ligand (FasL) molecule (CD95L) induces death by apoptosis (19). The involvement of Fas/FasL molecules was confirmed during the acute phase of *T. cruzi* experimentally infected mice. It was found that there is an increased expression and function of the Fas/FasL in $CD4^+$ T cells, and cells from FasL-deficient mice (*gld* mice) do not undergo AICD during *T. cruzi* infection (20). It has been suggested that AICD could have a deleterious role in *T. cruzi* infection, causing early elimination of effector T cells and supporting parasite escape (13). In agreement with these results, Rodrigues et al. (21) observed high levels of FasL in the serum of *T. cruzi* chronically infected patients (21). In addition, studies by Guillermo et al. (22) and Vasconcelos et al. (23) have revealed more information about the kinetics of Fas/FasL expression and T lymphocytes apoptosis during *T. cruzi* experimental infection (22). Splenic $CD4^+$ and $CD8^+$ T cells showed an upregulation in CD95/CD95L expression in a time-dependent manner during *T. cruzi* acute infection, which was associated with activation-induced cell death (AICD). *In vivo* injection of anti-FasL, but not anti-TNF-alpha or anti-TRAIL antibodies, blocked activation-induced cell death of $CD8^+$ T cells, improved type 1 immune responses, and reduced the infection severity as estimated by parasitemia (22, 23). Recently, Chaves et al. (24) demonstrated that T cell apoptosis was related to decreased cell proliferative and modulation of genes associated with apoptosis and caspase family receptors in chagasic patients with heart problems. Thus, the authors concluded that the T cell death was interfering with the clinical manifestations of the disease (24).

Infection caused by *T. cruzi* results in polyclonal lymphocyte activation (24–27), which, by itself, triggers T cell apoptosis (28, 29). *T. cruzi* released molecules such as *trans*-sialidase, an enzyme that catalyzes the transfer of exogenous sialic acid residues from the host acceptor molecules on the *T. cruzi* surface, induces a strong cell death by apoptosis and resulted in increasing parasite infectivity (30–32).

The *T. cruzi* surface expresses an unusual family of glycoinositolphospholipid (GIPL) molecules that are present in all *T. cruzi* evolutionary forms (33). Studies from our group have shown that in the presence of the cytokine IFN-γ, the ceramide portion of the GIPL induced intense macrophage apoptosis, independent of nitric oxide production. This effect was not observed when macrophages were treated with intact *T. cruzi* GIPL or with the GIPL-derived glycan chain. In *T. cruzi*-infected macrophages, apoptosis also increased the release of infective trypomastigotes and spheromastigotes (34). The pro-apoptotic action of ceramide had been described in previous studies, in which permeable ceramides were shown to promote a strong cytotoxic effect of this molecule (35, 36). Recently, the α-galactosylceramide molecule derived from *T. cruzi* induced NK T cell anergy and IL-33-mediated myeloid-derived suppressor cell accumulation (37).

Clearance of apoptotic cells involves the recognition of the dying cell by the phagocytes, internalization, the immunological consequences to host–parasite interaction, and pathogenesis of disease (38). In recent years, the term "efferocytosis" has been introduced to specifically refer to the engulfment or phagocytosis of apoptotic cells (39–42). Macrophages undergo specific molecular and functional changes upon encounter, interaction with, and uptake of apoptotic cells (efferocytosis) that control both phagocytosis and immune signaling (42).

In vitro studies showed that engulfment of apoptotic $CD4^+$ T lymphocytes from infected mice by *T. cruzi* experimentally infected macrophages exacerbate parasite replication. When apoptosis of $CD4^+$ T cells from infected mice was blocked with anti-FasL mAb, parasite growth was also blocked. Prevention of parasite growth was observed in a transwell coculture system, where $CD4^+$ T cells were cultured and separated from infected macrophages, showing that the contact and phagocytosis of apoptotic cells ensures parasite replication. This finding was also associated with decreased IFN-γ production, suggesting that AICD was occurring on Th1 $CD4^+$ cell population (18). Apoptotic cells trigger production of anti-inflammatory cytokines such as IL-10 and TGF-β by the phagocytes (41, 43–47). The process of

apoptotic cells uptake by *T. cruzi*-infected macrophages is associated with the release of TGF-β IL-10 and prostaglandin PGE_2 (8, 9, 12). Together, these mediators would deactivate macrophages and favor the growth of *T. cruzi* amastigotes and other intracellular parasites (8, 18, 48).

Phagocytic cells express on the cell surface a group of receptors that actively participate in the recognition and capture of apoptotic bodies, the vast majority of these receptors have affinity to phosphatidylserine (PtdSer) expressed by apoptotic cells (49). The integrin αVβ3 is one of these receptors, which recognizes PtdSer through molecules that act as bridges, such as lactadherin, glycoprotein produced by phagocytes, and the fat globule of opsonin-factor-8 EGF (MFG-E8) (50). In addition, the αvβ3 integrin express on phagocytes can bind to thrombospondin, helping the recognition of PS on the surface of apoptotic cells (51). Other apoptotic cell recognition receptors, such as the family of receptor tyrosine kinases Tyro3, Axl, and MerTK (TAM), also need to bind to bridge molecule to interact with PtdSer on the surface of apoptotic cells (52–54).

Because *T. cruzi* is an intracellular pathogen that can not produce putrescine, and is an auxotrophic parasite for polyamines, because of its incapacity to produce putrescine due to the lack of both, ornithine decarboxylase (ODC) and arginine. *T. cruzi* needs to capture exogenous putrescine for the proliferation of amastigote forms within the cells (55, 56). Besides the fact that binding of apoptotic lymphocytes to $\alpha_V\beta_3$ expressed by macrophages results in secretion of PGE_2 and TGF-β, the engulfment of apoptotic cells is also followed by induction of ODC and synthesis of putrescine: this functions as a growth factor for intracellular forms of *T. cruzi* (8, 9). In addition, this deleterious effect of apoptotic cells is eradicated by inhibitors of prostaglandin synthesis (NSAIDs drugs) and neutralizing antibodies for TGF-β (11, 12). Injection of apoptotic cells increases parasitemia *in vivo*, and treatment with the cyclooxygenase inhibitors aspirin or indomethacin reduces parasitemia (8).

The use of NSAIDs in *T. cruzi* experimental infection has been used by different groups. However, it is important to emphasize that the use of cyclooxygenase blockers in experimental infection may lead to conflicting results, depending on the experimental model (8, 11, 57, 58). Recently, it was described that aspirin in low doses decreased mortality, parasitemia, and heart damage in *T. cruzi*-infected mice, and they suggested that the protective effect was established to the generation of anti-inflammatory mediator 15-epi-LXA4 (59). However, when aspirin was given in high doses, this protective effect disappeared (59). Also, it has been characterized that the use of COX inhibitors inhibits *T. cruzi* infection of murine cardiac cells (60). The results presented by Michelin and collaborators suggest that the prostaglandins produced mainly by the activation of the COX-2 enzyme favor the immunosuppression of the acute phase of infection (61). These data reinforce our hypothesis that NSAIDs may somehow favor the immune response of the vertebrate host (8, 11).

The contribution of apoptotic T cell pathways in the outcome of *T. cruzi* infection *in vivo* was addressed by injection of caspase 8 inhibitor or by the use of caspase 8-deficient mice. However, blockade of the initiator caspase 8 *in vivo* was unable to inhibit apoptosis, and mice that have received treatment demonstrated a profound $CD8^+$ T cell depletion. Furthermore, caspase 8-deficient mice upregulated Th2 cytokine responses and increased susceptibility to *T. cruzi* infection (62). Therapy with zIETG (caspase 8 inhibitor), initiated 4 days after infection, resulted in inhibition of T cell expansion and increased parasitemia (62). In contrast, administration of zVAD, a pan caspase inhibitor (or anti-FasL antibody) initiated at day 7 after *T. cruzi* infection was shown to reduce T cell death, promotes type 1 immune response and reduced parasitemia. Spleen cells produced more IFN-γ when stimulated with parasite antigens, while peritoneal macrophages showed a reduced parasite load (63). The number of inflammatory cells in the hearts of acutely infected mice injected with zVAD was not affected (63). Treatment with anti-FasL mAb starting at 11 days after infection, but not anti-TNF or anti-TRAIL antibodies, protect CD8 T cells from AICD, improved cytokine production by CD4 T cells, activate CD8 T cells, upregulate Fas expression by CD8 T cells earlier than CD4 T cells, and decreased parasitemia (22). Mice vaccinated with an adenoviral vector expressing two *T. cruzi*-dominant epitopes improved CD8 T cell functionality and decreased parasitemia after parasite challenge, a phenotype attributed to the lack of CD95 expression in parasite-specific CD8 T cells (23). Recently, Cabral-Piccin and collaborators demonstrated that treatment of *T. cruzi*-infected mice with anti-FasL prevents $CD8^+$ T lymphocytes apoptosis, upregulates type 1 responses to parasite antigens, and reduces macrophages infection when cocultured with CD8 T cells (64). Further analysis showed that injection of anti-FasL mAb resulted in a polarized M1 macrophage phenotype, both *in vitro* and *in vivo*. The authors suggested that rescuing CD8 T cells from death with anti-FasL treatment prevents the negative effects of efferocytosis on macrophage activation.

CONCLUDING REMARKS

The role of T cells-mediated immune response in controlling intracellular protozoan parasite infection is well established. In the acute phase of infection, *T. cruzi* proliferates within the cells of the vertebrate host, besides producing and secreting molecules with immunomodulatory activities that obstruct the immune response mediated by T lymphocytes. The final result of this important modulatory mechanism is the propagation of *T. cruzi* to different organs of the vertebrate host (13). It is also clear that T cell apoptosis occurs during human and experimental infection with *T. cruzi* (8, 24). Some studies showed evidences that parasites have adapted to their hosts modulating or even taking advantage of cell death in order to facilitate their own survival in a hostile environment and promoting disease. In this context, elucidation of apoptotic cell-mediated signaling mechanisms would help to discover new effective therapies and vaccines.

The modulation of host immune response is an important approach in the efficacy of treatment against *T. cruzi* in the

experimental acute models of Chagas disease. The quality of response could be an important factor, not only in T cell disease progression but also in chemotherapy responsiveness. Apoptosis modulation has a beneficial therapeutic effect in various cardiovascular diseases, as well as infectious diseases (65). There appears to be an association between apoptosis and heart failure, as well as in disease severity in Chagas patients, such that pharmacological use of apoptosis inhibitors could be an attractive choice for adjuvant therapy in the chronic treatment phase; this restores an efficient T cell immune response to parasite infection, thus diminishing the pathological signals of disease.

AUTHOR CONTRIBUTIONS

DD-R, MN, AM, and CGF-de-L wrote the paper. All authors read and approved the final version of the manuscript.

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REFERENCES

1. Costa J, Peterson AT. Ecological niche modeling as a tool for understanding distributions and interactions of vectors, hosts, and etiologic agents of Chagas disease. *Adv Exp Med Biol* (2012) 710:59–70. doi:10.1007/978-1-4419-5638-5_7
2. Goldenberg S, Avila AR. Aspects of *Trypanosoma cruzi* stage differentiation. *Adv Parasitol* (2011) 75:285–305. doi:10.1016/B978-0-12-385863-4.00013-7
3. Rubin-de-Celis SS, Uemura H, Yoshida N, Schenkman S. Expression of trypomastigote trans-sialidase in metacyclic forms of *Trypanosoma cruzi* increases parasite escape from its parasitophorous vacuole. *Cell Microbiol* (2006) 8:1888–98. doi:10.1111/j.1462-5822.2006.00755.x
4. Cardoso MS, Reis-Cunha JL, Bartholomeu DC. Evasion of the immune response by *Trypanosoma cruzi* during acute infection. *Front Immunol* (2016) 6:659. doi:10.3389/fimmu.2015.00659
5. Akbar AN, Henson SM. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol* (2011) 11:289–95. doi:10.1038/nri2959
6. Gigley JP, Bhadra R, Moretto MM, Khan IA. T cell exhaustion in protozoan disease. *Trends Parasitol* (2012) 28:377–84. doi:10.1016/j.pt.2012.07.001
7. Albareda MC, De Rissio AM, Tomas G, Serjan A, Alvarez MG, Viotti R, et al. Polyfunctional T cell responses in children in early stages of chronic *Trypanosoma cruzi* infection contrast with monofunctional responses of long-term infected adults. *PLoS Negl Trop Dis* (2013) 7:e2575. doi:10.1371/journal.pntd.0002575
8. Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, et al. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* (2000) 403:199–203. doi:10.1038/35003208
9. Lopes MF, Freire-de-Lima CG, DosReis GA. The macrophage haunted by cell ghosts: a pathogen grows. *Immunol Today* (2000) 21:489–94. doi:10.1016/S0167-5699(00)01713-8
10. DosReis GA, Freire-de-Lima CG, Nunes MP, Lopes MF. The importance of aberrant T-cell responses in Chagas disease. *Trends Parasitol* (2005) 21:237–43. doi:10.1016/j.pt.2005.03.008
11. D'Avila H, Freire-de-Lima CG, Roque NR, Teixeira L, Barja-Fidalgo C, Silva AR, et al. Host cell lipid bodies triggered by *Trypanosoma cruzi* infection and enhanced by the uptake of apoptotic cells are associated with prostaglandin E(2) generation and increased parasite growth. *J Infect Dis* (2011) 204:951–61. doi:10.1093/infdis/jir432
12. Luna-Gomes T, Filardy AA, Rocha JD, Decote-Ricardo D, LaRocque-de-Freitas IF, Morrot A, et al. Neutrophils increase or reduce parasite burden in *Trypanosoma cruzi*-infected macrophages, depending on host strain: role of neutrophil elastase. *PLoS One* (2014) 9:e90582. doi:10.1371/journal.pone.0090582
13. DosReis GA. Evasion of immune responses by *Trypanosoma cruzi*, the etiological agent of Chagas disease. *Braz J Med Biol Res* (2011) 44:84–90. doi:10.1590/S0100-879X2011007500005
14. Cohen TS, Jones-Nelson O, Hotz M, Cheng L, Miller LS, Suzich J, et al. *S. aureus* blocks efferocytosis of neutrophils by macrophages through the activity of its virulence factor alpha toxin. *Sci Rep* (2016) 6:35466. doi:10.1038/srep35466
15. Green DR, Oguin TH, Martinez J. The clearance of dying cells: table for two. *Cell Death Differ* (2016) 23:915–26. doi:10.1038/cdd.2015.172
16. Greenlee-Wacker MC. Clearance of apoptotic neutrophils and resolution of inflammation. *Immunol Rev* (2016) 273:357–70. doi:10.1111/imr.12453
17. Lopes MF, da Veiga VF, Santos AR, Fonseca ME, DosReis GA. Activation-induced CD4+ T cell death by apoptosis in experimental Chagas' disease. *J Immunol* (1995) 154:744–52.
18. Nunes MP, Andrade RM, Lopes MF, DosReis GA. Activation-induced T cell death exacerbates *Trypanosoma cruzi* replication in macrophages cocultured with CD4+ T lymphocytes from infected hosts. *J Immunol* (1998) 160:1313–9.
19. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* (1995) 181:71–7. doi:10.1084/jem.181.1.71
20. Lopes MF, Nunes MP, Henriques-Pons A, Giese N, Morse HC III, Davidson WF, et al. Increased susceptibility of Fas ligand-deficient gld mice to *Trypanosoma cruzi* infection due to a Th2-biased host immune response. *Eur J Immunol* (1999) 29:81–9. doi:10.1002/(SICI)1521-4141(199901)29:01<81::AID-IMMU81>3.3.CO;2-P
21. Rodrigues V Jr, Agrelli GS, Leon SC, Silva Teixeira DN, Tostes S Jr, Rocha-Rodrigues DB. Fas/Fas-L expression, apoptosis and low proliferative response are associated with heart failure in patients with chronic Chagas' disease. *Microbes Infect* (2008) 10:29–37. doi:10.1016/j.micinf.2007.09.015
22. Guillermo LV, Silva EM, Ribeiro-Gomes FL, De Meis J, Pereira WF, Yagita H, et al. The Fas death pathway controls coordinated expansions of type 1 CD8 and type 2 CD4 T cells in *Trypanosoma cruzi* infection. *J Leukoc Biol* (2007) 81:942–51. doi:10.1189/jlb.1006643
23. Vasconcelos JR, Bruna-Romero O, Araujo AF, Dominguez MR, Ersching J, de Alencar BC, et al. Pathogen-induced proapoptotic phenotype and high CD95 (Fas) expression accompany a suboptimal CD8+ T-cell response: reversal by adenoviral vaccine. *PLoS Pathog* (2012) 8:e1002699. doi:10.1371/journal.ppat.1002699
24. Chaves AT, de Assis Silva Gomes Estanislau J, Fiuza JA, Carvalho AT, Ferreira KS, Fares RC, et al. Immunoregulatory mechanisms in Chagas disease: modulation of apoptosis in T-cell mediated immune responses. *BMC Infect Dis* (2016) 16:191. doi:10.1186/s12879-016-1523-1
25. Minoprio PM, Coutinho A, Joskowicz M, D'Imperio Lima MR, Eisen H. Polyclonal lymphocyte responses to murine *Trypanosoma cruzi* infection. II. Cytotoxic T lymphocytes. *Scand J Immunol* (1986) 24:669–79. doi:10.1111/j.1365-3083.1986.tb02186.x
26. Flavia Nardy A, Santos L, Freire-de-Lima CG, Morrot A. Modulation of intrathymic sphingosine-1-phosphate levels promotes escape of immature thymocytes to the periphery with a potential proinflammatory role in Chagas disease. *Biomed Res Int* (2015) 2015:709846. doi:10.1155/2015/709846
27. Tarleton RL. CD8+ T cells in *Trypanosoma cruzi* infection. *Semin Immunopathol* (2015) 37:233–8. doi:10.1007/s00281-015-0481-9
28. Green DR, Droin N, Pinkoski M. Activation-induced cell death in T cells. *Immunol Rev* (2003) 193:70–81. doi:10.1034/j.1600-065X.2003.00051.x
29. Sikora E. Activation-induced and damage-induced cell death in aging human T cells. *Mech Ageing Dev* (2015) 151:85–92. doi:10.1016/j.mad.2015.03.011
30. Leguizamon MS, Mocetti E, Garcia Rivello H, Argibay P, Campetella O. Trans-sialidase from *Trypanosoma cruzi* induces apoptosis in cells from the immune system in vivo. *J Infect Dis* (1999) 180:1398–402. doi:10.1086/315001
31. Mendonca-Previato L, Penha L, Garcez TC, Jones C, Previato JO. Addition of alpha-O-GlcNAc to threonine residues define the post-translational modification of mucin-like molecules in *Trypanosoma cruzi*. *Glycoconj J* (2013) 30:659–66. doi:10.1007/s10719-013-9469-7
32. Freire-de-Lima L, da Fonseca LM, da Silva VA, da Costa KM, Morrot A, Freire-de-Lima CG, et al. Modulation of cell sialoglycophenotype: a stylish

mechanism adopted by *Trypanosoma cruzi* to ensure its persistence in the infected host. *Front Microbiol* (2016) 7:698. doi:10.3389/fmicb.2016.00698
33. Previato JO, Jones C, Xavier MT, Wait R, Travassos LR, Parodi AJ, et al. Structural characterization of the major glycosylphosphatidylinositol membrane-anchored glycoprotein from epimastigote forms of *Trypanosoma cruzi* Y-strain. *J Biol Chem* (1995) 270:7241–50. doi:10.1074/jbc.270.13.7241
34. Freire-de-Lima CG, Nunes MP, Corte-Real S, Soares MP, Previato JO, Mendonca-Previato L, et al. Proapoptotic activity of a *Trypanosoma cruzi* ceramide-containing glycolipid turned on in host macrophages by IFN-gamma. *J Immunol* (1998) 161:4909–16.
35. Wyllie AH. Apoptosis and carcinogenesis. *Eur J Cell Biol* (1997) 73:189–97.
36. Kuzmenko DI, Klimentyeva TK. Role of ceramide in apoptosis and development of insulin resistance. *Biochemistry (Mosc)* (2016) 81:913–27. doi:10.1134/S0006297916090017
37. Huang JR, Tsai YC, Chang YJ, Wu JC, Hung JT, Lin KH, et al. alpha-Galactosylceramide but not phenyl-glycolipids induced NKT cell anergy and IL-33-mediated myeloid-derived suppressor cell accumulation via upregulation of egr2/3. *J Immunol* (2014) 192:1972–81. doi:10.4049/jimmunol.1302623
38. Schaumburg F, Hippe D, Vutova P, Luder CG. Pro- and anti-apoptotic activities of protozoan parasites. *Parasitology* (2006) 132 Suppl:S69–85. doi:10.1017/S0031182006000874
39. Erwig LP, Henson PM. Clearance of apoptotic cells by phagocytes. *Cell Death Differ* (2008) 15:243–50. doi:10.1038/sj.cdd.4402184
40. Hermetet F, Jacquin E, Launay S, Gaiffe E, Couturier M, Hirchaud F, et al. Efferocytosis of apoptotic human papillomavirus-positive cervical cancer cells by human primary fibroblasts. *Biol Cell* (2016) 108:189–204. doi:10.1111/boc.201500090
41. Janssen WJ, Bratton DL, Jakubzick CV, Henson PM. Myeloid cell turnover and clearance. *Microbiol Spectr* (2016) 4. doi:10.1128/microbiolspec.MCHD-0005-2015
42. Elliott MR, Koster KM, Murphy PS. Efferocytosis signaling in the regulation of macrophage inflammatory responses. *J Immunol* (2017) 198:1387–94. doi:10.4049/jimmunol.1601520
43. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature* (1997) 390:350–1. doi:10.1038/37022
44. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* (1998) 101:890–8. doi:10.1172/JCI1112
45. Freire-de-Lima CG, Xiao YQ, Gardai SJ, Bratton DL, Schiemann WP, Henson PM. Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *J Biol Chem* (2006) 281:38376–84. doi:10.1074/jbc.M605146200
46. Xiao YQ, Freire-de-Lima CG, Janssen WJ, Morimoto K, Lyu D, Bratton DL, et al. Oxidants selectively reverse TGF-beta suppression of proinflammatory mediator production. *J Immunol* (2006) 176:1209–17. doi:10.4049/jimmunol.176.2.1209
47. Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *J Immunol* (2008) 181:3575–85. doi:10.4049/jimmunol.181.5.3575
48. Arcanjo AF, LaRocque-de-Freitas IF, Rocha JD, Zamith D, Costa-da-Silva AC, Nunes MP, et al. The PGE2/IL-10 axis determines susceptibility of B-1 cell-derived phagocytes (B-1CDP) to *Leishmania major* infection. *PLoS One* (2015) 10:e0124888. doi:10.1371/journal.pone.0124888
49. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol* (2007) 7:964–74. doi:10.1038/nri2214
50. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature* (2002) 417:182–7. doi:10.1038/417182a
51. Savill J, Hogg N, Ren Y, Haslett C. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest* (1992) 90:1513–22. doi:10.1172/JCI116019
52. Grabiec AM, Hussell T. The role of airway macrophages in apoptotic cell clearance following acute and chronic lung inflammation. *Semin Immunopathol* (2016) 38:409–23. doi:10.1007/s00281-016-0555-3
53. Penberthy KK, Ravichandran KS. Apoptotic cell recognition receptors and scavenger receptors. *Immunol Rev* (2016) 269:44–59. doi:10.1111/imr.12376
54. Roszer T. Transcriptional control of apoptotic cell clearance by macrophage nuclear receptors. *Apoptosis* (2017) 22:284–94. doi:10.1007/s10495-016-1310-x
55. Ariyanayagam MR, Oza SL, Mehlert A, Fairlamb AH. Bis(glutathionyl)spermine and other novel trypanothione analogues in *Trypanosoma cruzi*. *J Biol Chem* (2003) 278:27612–9. doi:10.1074/jbc.M302750200
56. Reigada C, Saye M, Vera EV, Balcazar D, Fraccaroli L, Carrillo C, et al. *Trypanosoma cruzi* polyamine transporter: its role on parasite growth and survival under stress conditions. *J Membr Biol* (2016) 249:475–81. doi:10.1007/s00232-016-9888-z
57. Moraes KC, Diniz LF, Bahia MT. Role of cyclooxygenase-2 in *Trypanosoma cruzi* survival in the early stages of parasite host-cell interaction. *Mem Inst Oswaldo Cruz* (2015) 110:181–91. doi:10.1590/0074-02760140311
58. Cossentini LA, Da Silva RV, Yamada-Ogatta SF, Yamauchi LM, De Almeida Araujo EJ, Pinge-Filho P. Aspirin treatment exacerbates oral infections by *Trypanosoma cruzi*. *Exp Parasitol* (2016) 164:64–70. doi:10.1016/j.exppara.2016.01.008
59. Molina-Berrios A, Campos-Estrada C, Henriquez N, Faundez M, Torres G, Castillo C, et al. Protective role of acetylsalicylic acid in experimental *Trypanosoma cruzi* infection: evidence of a 15-epi-lipoxin A(4)-mediated effect. *PLoS Negl Trop Dis* (2013) 7:e2173. doi:10.1371/journal.pntd.0002173
60. Malvezi AD, Panis C, da Silva RV, de Freitas RC, Lovo-Martins MI, Tatakihara VL, et al. Inhibition of cyclooxygenase-1 and cyclooxygenase-2 impairs *Trypanosoma cruzi* entry into cardiac cells and promotes differential modulation of the inflammatory response. *Antimicrob Agents Chemother* (2014) 58:6157–64. doi:10.1128/AAC.02752-14
61. Michelin MA, Silva JS, Cunha FQ. Inducible cyclooxygenase released prostaglandin mediates immunosuppression in acute phase of experimental *Trypanosoma cruzi* infection. *Exp Parasitol* (2005) 111:71–9. doi:10.1016/j.exppara.2005.05.001
62. Silva EM, Guillermo LV, Ribeiro-Gomes FL, De Meis J, Pereira RM, Wu Z, et al. Caspase-8 activity prevents type 2 cytokine responses and is required for protective T cell-mediated immunity against *Trypanosoma cruzi* infection. *J Immunol* (2005) 174:6314–21. doi:10.4049/jimmunol.174.10.6314
63. Silva EM, Guillermo LV, Ribeiro-Gomes FL, De Meis J, Nunes MP, Senra JF, et al. Caspase inhibition reduces lymphocyte apoptosis and improves host immune responses to *Trypanosoma cruzi* infection. *Eur J Immunol* (2007) 37:738–46. doi:10.1002/eji.200636790
64. Cabral-Piccin MP, Guillermo LV, Vellozo NS, Filardy AA, Pereira-Marques ST, Rigoni TS, et al. Apoptotic CD8 T-lymphocytes disable macrophage-mediated immunity to *Trypanosoma cruzi* infection. *Cell Death Dis* (2016) 7:e2232. doi:10.1038/cddis.2016.135
65. Favaloro B, Allocati N, Graziano V, Di Ilio C, De Laurenzi V. Role of apoptosis in disease. *Aging (Albany NY)* (2012) 4:330–49. doi:10.18632/aging.100459

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REVIEW
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African Trypanosomes Undermine Humoral Responses and Vaccine Development: Link with Inflammatory Responses?

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African trypanosomosis is a debilitating disease of great medical and socioeconomical importance. It is caused by strictly extracellular protozoan parasites capable of infecting all vertebrate classes including human, livestock, and game animals. To survive within their mammalian host, trypanosomes have evolved efficient immune escape mechanisms and manipulate the entire host immune response, including the humoral response. This report provides an overview of how trypanosomes initially trigger and subsequently undermine the development of an effective host antibody response. Indeed, results available to date obtained in both natural and experimental infection models show that trypanosomes impair homeostatic B-cell lymphopoiesis, B-cell maturation and survival and B-cell memory development. Data on B-cell dysfunctioning in correlation with parasite virulence and trypanosome-mediated inflammation will be discussed, as well as the impact of trypanosomosis on heterologous vaccine efficacy and diagnosis. Therefore, new strategies aiming at enhancing vaccination efficacy could benefit from a combination of (i) early parasite diagnosis, (ii) anti-trypanosome (drugs) treatment, and (iii) anti-inflammatory treatment that collectively might allow B-cell recovery and improve vaccination.

Keywords: B-cell lymphopoiesis, African trypanosomosis, vaccination strategies, inflammation, T-cells, macrophage migration inhibitory factor (MIF)

INTRODUCTION

African trypanosomes are strictly extracellular single-celled protozoan parasites belonging to the genus *Trypanosoma*, which cause debilitating diseases in humans and livestock and consequently significantly affect the socioeconomic development of sub-Saharan Africa (1). About 70 million people distributed over a surface of one and a half million square kilometers are estimated to be at risk for contracting sleeping sickness in Africa (2). The distribution of African trypanosomes coincides mostly with the distribution of the habitat of the hematophagic insect vector, i.e., the tsetse "fly" (*Glossina* sp.), with tsetse meaning "fly" in the Tswana language of Southern Africa (3). Human African trypanosomosis (HAT) or sleeping sickness is caused by *Trypanosoma brucei gambiense* (west and central Africa) and *Trypanosoma brucei rhodesiense* (eastern and southern Africa) (4, 5).

Both parasites cause infections that exhibit clinically diverse patterns and hence require different patient management, with the less prevalent *T. b. rhodesiense* HAT considered to be the more acute and virulent/lethal form of the disease (6, 7). HAT mainly affects remote rural communities where the health infrastructure is often minimal. In general, the disease is characterized by two stages: the early hemolymphatic stage whereby parasites proliferate in the blood and lymphatic system and the late meningoencephalitic stage whereby parasites penetrate the blood–brain barrier and proliferate in the cerebral spinal fluid (8). When patients in the meningoencephalitic stage remain untreated, an encephalitic reaction can occur resulting in coma and subsequent death (9–11). However, it is important to mention that in recent years a number of reports have indicated that HAT is not always lethal and that both *T. b. gambiense* and *T. b. rhodesiense* can result in chronic human infections with little or no symptoms (12, 13). Limited surveillance in particular of non-symptomatic cases, however, make it hard to assess how widespread these non-lethal cases are, or what the molecular and genetic underlying factors are that account for HAT resistance in certain individuals (14).

According to WHO, recent successes in the fight against HAT have brought the annual new cases to less than 10,000 (5, 7, 8). To design and maintain future control strategies, it is important to indicate that *T. b. gambiense* is an anthroponotic disease with a minor role for animal reservoirs that accounts for 98% of the reported HAT cases and causes a chronic, gradually progressing disease, whereby the late meningoencephalitic stage is not reached before months or even years of infection (10, 15). *T. b. rhodesiense* on the other hand is a zoonotic disease affecting mainly animals (livestock and wildlife), with humans being only accidentally infected, and represents only 2% of the reported HAT cases, whereby the infections are acute and progress rapidly (within weeks) to the late meningoencephalitic stage (10, 16). The zoonotic nature of *T. b. rhodesiense* infections make them more difficult to control compared to *T. b. gambiense* infections (15, 17, 18). Animal African trypanosomosis (AAT) also known as Nagana is a second form of trypanosomosis that affects sub-Saharan Africa. It is mainly caused by *Trypanosoma congolense*, *Trypanosoma vivax*, and to a lesser extent *Trypanosoma brucei brucei*, while surra and dourine are also forms of AAT caused by *Trypanosoma evansi* and *Trypanosoma equiperdum*, respectively (19–21). Of note, some parasites acquired a mechanical transmission mode (hence, they can reside outside the tsetse/vector belt) and are also found in South/Latin America (*T. vivax* and *T. evansi*) and Asia (*T. evansi* and *T. equiperdum*) (19, 21–23). Yet, *T. congolense* forms a major constraint on livestock production and remains the leading cause of livestock morbidity and mortality in sub-Saharan Africa. Hereby, cattle succumb to infection primarily due to parasite-induced anemia or complications resulting from secondary, opportunistic infections (24). Progressive disease for a prolonged time will weaken these animals, thereby preventing them to be used as draft animals or for food/milk production. As a result, farming in the tsetse belt remains challenging and hampers the development of poor societies, leading to great economic losses in terms of productivity (25, 26). Indeed, AAT accounts for an estimated annual loss of about US$5 billion, whereby Africa invests every year at least US$30 million to control cattle trypanosomosis in term of curative and prophylactic treatments (27, 28). The total losses for the total tsetse-infested lands in terms of agricultural gross domestic product are US$4.75 billion per year (1). In fact, the impact of AAT on the affected areas is the combined result of environmental, political, sociocultural, entomological, and livestock management factors (29), whereby (i) the political instability of the areas hampers controlled intervention strategies and subsequently discourages commercial investment in control strategies, (ii) pharmaceutical companies are less prone to engage/invest in drug discovery/development against diseases that affect the poorest people, (iii) wild animals function as reservoir of the parasite and therefore hamper the control of the disease, and (iv) the inappropriate use of the available drugs resulting in the emergence of drug resistance (30, 31). Up till now, not a single-field applicable vaccine exists, and chemotherapy is the only strategy available to treat the disease, which is associated with high drug toxicity. Nevertheless, so far chemotherapy remains the only therapeutic choice for these diseases, whereby they target unique organelles of trypanosomes such as glycosomes and the kinetoplast that are absent in the mammalian host or trypanosome metabolic pathways that differ from the host counterparts [carbohydrate metabolism, protein and lipid modifications, and programmed cell death (PCD)] (32–34). Unlike the situation with HAT, where the nifurtimox–eflornithine combination therapy is the preferred first-line treatment for second-stage disease (35, 36), no drug combinations are currently used for AAT (27). Instead, alternating use of compounds, particularly diminazene and isometamidium (called a "sanative pair"), with low risk of cross-resistance, is recommended where possible. Hence, there is an urgent need to optimize trypanocide usage/delivery such as extending the half-life of current trypanocides to use lower quantities of trypanocide in a more effective way and, consequently, pose a decreased risk of toxicity and possibly decreased resistance development (37, 38). However, there is some optimism since progress in HAT/AAT control measures were made over the past decade due to the establishment of the Pan-African tsetse and trypanosome eradication campaign, funded by the African Development Bank, which was established in the year 2000. This organization has set tsetse elimination as its goal and has strengthened renewed interest in the research and development of control/intervention options (29, 39). Overall, "elimination" of *T. b. gambiense* HAT has been targeted for 2020 under leadership of the WHO (40).

One crucial factor that stands in the way of total eradication of trypanosomosis in general is inefficient diagnosis of the infection. To date, microscopy detection of the parasite remains the only available tool to diagnose AAT and *T. b. rhodesiense* HAT in a reliable way. Only for *T. b. gambiense*, monitoring tools are available for both detection of exposure and staging of the disease (41). The latter is important to reduce the risk of treatment-associated complications occurring during treatment of the second stage of the disease (42). In this context, improvement in staging diagnosis and early screening methods are current challenges, which would avoid delayed patient treatment. Diagnosis is often hampered due to lack of positive predictive value of existing field applicable techniques and the fact that antibody (Ab)-based detection cannot

differentiate between active or passed—but cured—infections (41). Immunodiagnostics based on antigen detection in this case would be preferable but are currently non-existent for trypanosomosis in the field (41, 43). An additional complication resides in the recent finding that tsetse-transmissible *T. b. gambiense* parasites can be found in human skin biopsies from undiagnosed individuals (44). Hence, this suggests that the current diagnostic methods and control policies need to be reevaluated.

In the next sections, we will give an overview of (i) the different escape mechanisms used by African trypanosomes to survive within their mammalian host and (ii) their strategies to undermine the entire host immune response, including the humoral response, which in turn hampers vaccine development. This review will focus on two most relevant AAT species *T. brucei* and *T. congolense*, given that for both parasites, established murine models and field studies in the economically and clinically relevant host (cattle) are available (45). While for *T. vivax*, field study information is scanty, hardly any representative experimental data are available as these parasites do not grow in mice unless they are carefully adapted (23, 46, 47). However, there is prospect since Minoprio and coworkers were able to establish a murine model for *T. vivax* (48). Although rodents are not natural hosts for these pathogens, murine models can be considered valuable tools to unravel the interactions and the immune evasion mechanisms of these parasites with their mammalian host.

HOST–PARASITE INTERACTIONS

Life Cycle of African Trypanosomes

African trypanosomes have a digenetic/heteroxenous complex life cycle alternating between the intestine of the tsetse fly vector and the blood/tissues of the mammalian host, whereby they progress through different developmental stages, i.e., procyclic or trypomastigote forms, respectively (49, 50). Yet, to survive in each of these hosts, they undergo essential changes at the level of morphology, energy metabolism, and surface coat protein expression (51, 52). Hereby, trypanosomes feed by absorbing nutrients (proteins, carbohydrates, and fats) as well as iron and oxygen from the body fluids of the host to generate the energy necessary for the vital processes (53). Within the bloodstream of the mammalian host, they subsist as bloodstream forms (BSFs) that are ingested by tsetse flies during a blood meal, wherein they differentiate into procyclic forms in the insect midgut. Next, they migrate to the proboscis (mouth parts) where they differentiate into epimastigote forms and finally into infective metacyclic forms (MCFs) that can be transmitted to a new mammalian host during the next blood meal. Although within the tsetse fly both *T. brucei* and *T. congolense* parasites have a similar migratory life cycle (i.e., initial establishment of midgut infection and invasion of the proventriculus), they exhibit differences in transitional developmental stages with production of infective MCFs in the proboscis for *T. congolense* and in the salivary glands for *T. brucei* (54–56). Within the mammalian host, *T. congolense* is a strictly intravascular parasite, whereby they bind to circulating erythrocytes and endothelial cells through their flagellum, causing damage at the adhesion site (57, 58). In contrast, *T. brucei* can also extravasate blood vessels and invade tissues and cause severe tissue injury (44, 59, 60). Hence, this implies differences in virulence mechanisms, host–pathogen relationships, and pathogenic effects between the two species (61). In addition, *T.* congolense exists strictly as a long slender (LS) dividing form, whereas *T. brucei* parasites are pleomorphic (i.e., can exhibit two forms); a LS dividing form and a short stumpy (SS) non-dividing form that is preadapted for transmission to the fly (62, 63). This transition, which involves a quorum sensing factor (i.e., an enigmatic stumpy inducing factor), is suggested to help control parasitemia and to increase the host survival time, thereby increasing the probability for successful transmission of the trypanosomes to a new host (50, 64). It is suggested that this removal of the majority of the population is an altruistic form of PCD and the counterpart of apoptosis in metazoan (65, 66). Hereby, increased intracellular reactive oxygen species and prostaglandin D2, which is produced principally by stumpy forms, are promoting this PCD that can be considered as a second control point in terminal differentiation to the SS form (67, 68). Moreover, it is suggested that the SS form is heterogeneous, whereby one part is altruistic and undergoes apoptosis-like events, thereby stimulating the host's immune response and eliminating the major LS and SS antigen population, while the other is tsetse infective (69). Both the LS and SS forms are covered by a dense variant surface glycoprotein (VSG) coat, which protects them from both the innate and the adaptive host immune systems. Of note, within the tsetse fly, the parasites are covered by a procyclin coat, and only when they differentiate into the MCF (infective form), they express a metacyclic VSG coat (70).

Parasite Escape Mechanisms in the Mammalian Host

To survive as extracellular parasites within the mammalian host environment (i.e., blood or extravascular tissues), African trypanosomes have developed efficient immune evasion mechanisms, at both the parasite level and the level of modulating host responses. Indeed, during millions of years of coevolution with their mammalian host, these parasites have "learned" to divert and sculpture the host immune system to prevent the generation of an effective response. The most predominant changes at the level of the host occurring during African trypanosomosis are massive splenomegaly coinciding with destruction of the lymphoid architecture and hepatomegaly. These modulations are followed by lymphadenopathy and hypergammaglobulinemia, leading to systemic multiple organ failure and death in experimental mouse models (71). In this section, we will give an overview of the most prominent escape mechanisms trypanosomes developed to allow successful infection within their mammalian host.

Parasite-Associated Escape Mechanisms

Already at the onset of infection, i.e., inoculation of trypanosome-containing saliva upon the bite of a tsetse fly, components present in the saliva are able to (i) dampen local host inflammatory immune responses characterized by the release of trypanolytic molecules, i.e., tumor necrosis factor (TNF) and nitric oxide (NO), thereby favoring parasite development and (ii) trigger mast

cell degranulation resulting in release of histamine and increased vasodilatation, thereby allowing parasite dissemination/extravasation into the blood circulation [reviewed in Stijlemans et al. (72)]. Within the mammalian host, trypanosomes are very proficient in avoiding and subsequently reorchestrating host immune responses. Being extracellular parasites, they are confronted with the host's humoral immune response; hence, to allow infection to occur, they have to overcome this major obstacle. In first instance, these parasites are covered with a very dense coat composed of approximately 5×10^6 identical VSG homodimers of 50–60 kDa subunits that are anchored in the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, which functions as a ~15-nm thick barrier and protects the cell from Abs that might bind to buried conserved proteins (73, 74). Second, to prevent Ab-mediated elimination by Abs raised against the immunodominant/immunogenic VSGs, these parasites acquired a system of antigenic variation, whereby they are equipped with a battery of more than 1,000 different VSG genes and pseudogenes in their genome that in turn can undergo segmental gene recombination to encode an estimated 10,000 different VSG surface coats during infection (75). Hence, at regular time points [i.e., upon recognition by the host's humoral response or when a maximal density is reached (Quorum sensing)], they switch their coat into a different variable antigen type, thereby allowing escaping Ab-mediated elimination (76). This antigenic variation is accomplished by (i) *in situ* switching of transcriptional control (i.e., changing the VSG expression site) or (ii) gene replacement resulting in a switch of the terminal telomeric VSG gene itself (77, 78). Besides antigenic variation, these parasites were shown to express a mosaic VSG during the process of VSG switching (i.e., changing from metacyclic to BSFs, or during the course of infection), which in turn might be an efficient way to prevent effective Ab recognition (79, 80). Also the infective MCFs use this differential VSG expression to generate diversity and counter existing partial immunity/enhance transmission, while BSF use this to prolong infection (see **Figure 1**). Interestingly, the MCFs initiate VSG expression by each cell, activating at random one from a small subset of metacyclic VSG (M-VSG) genes, resulting in a heterogenous population, whereby each trypanosome expresses a single VSG (81, 82). Hereby, the M-VSG expression is regulated exclusively at the transcriptional level, while the bloodstream VSG expression is regulated mainly at the posttranscriptional levels and transcribed polycistronically (83). Third, trypanosomes also exhibit a very high endocytosis rate as an efficient way to acquire nutrients and at the same time to remove Ab-bound VSG molecules and thereby prevent Ab-mediated or even complement-mediated opsonization/elimination (84, 85). This might allow parasites to transiently escape T-cell-independent B-cell-mediated

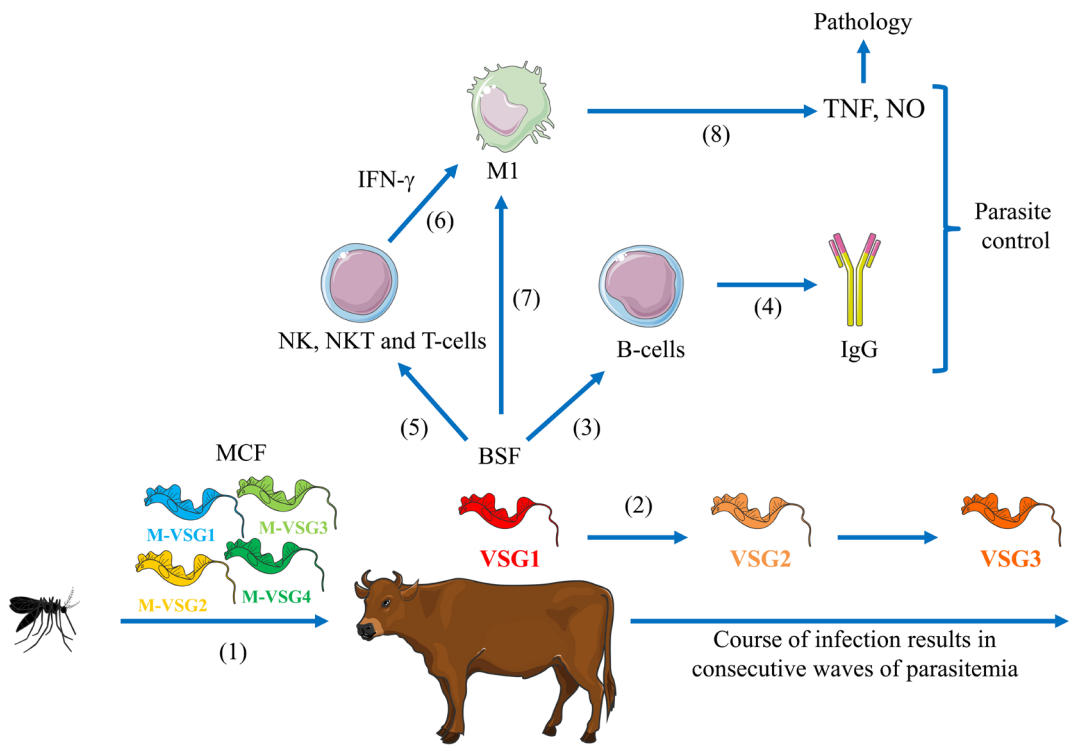

FIGURE 1 | Model for African trypanosomosis within the mammalian host. (1) Upon the bite of a trypanosome-infected tsetse fly, metacyclic form (MCF) parasites are inoculated within the mammalian host. (2) These parasites differentiate into bloodstream forms (BSFs) and switch their metacyclic VSG (M-VSG) into a bloodstream uniform VSG (VSG1) giving rise to the first parasitemia peak. During the course of infection, there is antigenic variation (VSG2, VSG3, etc.) giving rise to different peaks of parasitemia. (3,4) Parasites-derived components trigger B-cell activation and production of antiparasite IgG needed for parasitemia control. (5,6) Parasite-derived components trigger NK, NKT, and T-cell activation resulting in the production of interferon-gamma (IFN-γ). (7) Parasite-derived components in concert with IFN-γ trigger the induction of classically activated macrophages (M1 cells). (8) These M1 cells release pro-inflammatory cytokines [like tumor necrosis factor (TNF)] that are needed for parasite control, but at the same time contribute to pathology development if maintained during the course of infection.

elimination, which is the first line of defense. This immunological escape also gives time to transform into trypomastigote forms, which are adapted to survive in the mammalian host during the initiation of infection and gives the parasites an immunological advantage during the process of antigenic variation.

Although mainly IgMs play a key role during *T. brucei* infection, while in *T. congolense* infections, IgGs are mostly important (86, 87), it could be assumed that complement-mediated lysis is also an important innate defense mechanism. However, trypanosomes have developed efficient mechanisms to avoid complement-mediated elimination. First, trypanosomes are able to avoid elimination *via* the complement pathway, which is typically activated *via* immune complexes with Abs. Indeed, by releasing vast amounts of soluble VSG (mainly at the peak of parasitemia), Abs and complement factors will be scavenged and thereby induce a state of hypocomplementemia that can favor the survival of the parasites (88). Second, besides undermining the classical activation of the complement pathway that could contribute to trypanosome clearance through Ab-mediated trypanolysis and/or phagocytosis, the alternative pathway of complement activation occurring in the absence of specific Abs (i.e., during early stages of infection) is also impaired. Indeed, by masking sites on the VSG plasma membrane, which are capable of promoting alternative pathway activation, the cascade is blocked at the C3 convertase stage, thereby impairing the generation of the terminal complex (C5–C9) that normally induces trypanolysis (89, 90). However, it seems that the later stages of the complement activation cascade do not play a detrimental role in parasite control. Indeed, in AKR mice, which are natural C5 KO mice (91), the absence of the complement lysis pathway does not prevent periodic trypanosome clearance and does not hamper long-term survival in case of *T. congolense* infections (92, 93). However, soluble complement molecules, such as C3a and C5a, secreted during early stages of trypanosome infection, can contribute to the initiation of the early inflammatory immune response and also act as (i) chemotactic agents attracting phagocytes to the site of infection and (ii) release histamine from mast cells, thereby increasing microvascular permeability (94), which would allow/enable parasite extravasation into the blood circulation.

Parasite-Induced Escape Mechanisms

Besides being equipped with tools to avoid elimination by the host's "innate" humoral response, trypanosomes also undermine the host cellular immune responses to allow chronic infection. Moreover, the data in literature suggest that the efficiency to modulate the innate immune response is crucial for the progression of trypanosomosis (95). Hereby, the suppression of cellular immune responses is an efficient mechanism to evade host defense mechanisms and a general feature of trypanosomosis in bovine, human, and murine hosts. To this end, these parasites are equipped with a battery of molecules able to modulate early antiparasite responses to allow establishment. It is important to mention that the course of an African trypanosome infection can be characterized by an early release of interferon-gamma (IFN-γ) by activated NK-, NKT- and T-cells required to induce classically activated macrophages (M1) (see **Figure 1**). In turn, these activated M1 develop upon exposure to parasite-derived molecules such as VSG and CpG a type-1 inflammatory immune response leading to the production of the potential trypanocidal molecules such as TNF and NO that in conjunction with Abs will contribute to parasite control (96, 97). Yet, persistence of this type-1 immune response and hyperactivated M1 cells will culminate in trypanosusceptible animals into immunopathological features such as the systemic immune response syndrome and anemia (98). Trypanotolerant animals on the other hand are able to switch to a more type-2 immune response and the induction of alternatively activate macrophages (M2), whereby the anti-inflammatory cytokine interleukin (IL)-10 was shown to play a pivotal "dampening" role (99, 100).

Undermining Macrophage Functionality

To sustain the development of the first (most prominent) peak of parasitemia in the blood and its control by the host, some parasite-derived molecules are able to dampen pro-inflammatory responses (TNF, NO) by these M1. Most research so far has been performed using *T. brucei* infections and indicate that these parasites release components such as adenylate cyclase (AdC) and kinesin heavy chain (TbKHC-1) to dampen initial host responses, thereby allowing early parasite establishment (101, 102). Indeed, AdC released by altruistic parasites upon parasite phagocytosis by liver-associated myeloid cells prevents production of the trypanolytic cytokine TNF (*via* a protein kinase A pathway), which promotes early establishment of trypanosomes within the mammalian host (101). On the other hand, the release of TbKHC-1 by parasites induces IL-10 and arginase release by myeloid cells in a SIGN-R1-dependent manner and favors initial parasite seeding by inducing the production of polyamines, which constitute trypanosome essential nutrients (102, 103). Recently, it was also shown that metabolites produced by trypanosomes such as indolepyruvate (i.e., a transamination product of tryptophan) can dampen macrophage pro-inflammatory responses that prevent elimination (104). Finally, the order of exposure to parasite-derived versus host-derived macrophage-activating components as well as the relative concentration of these mediators may influence the ability of the host to respond to trypanosome infections. Indeed, early during infection, exposure of macrophages to soluble VSG (encompassing the glycosylinositolphosphate substituent) before IFN-γ priming downregulated the level of signal transducer and activator of transcription 1 phosphorylation, which in turn reduced transcription of pro-inflammatory cytokines such as TNF (105). So far, nothing is known about such mechanisms for *T. congolense*. In summary, it seems that trypanosomes have developed a system whereby altruistic parasites are phagocytosed, thereby disabling the M1-mediated innate immune response required for parasite control and paving the way for initiation and establishment of the first wave of parasitemia.

Modulation of T-Cell Functionality

Besides undermining the antitrypanosomal potential of the myeloid system, the parasite is also impairing T-cell help required to mount a more efficient response during the course of infection. Early during *T. brucei* and *T. congolense* infection, T-cell suppression is occurring *via* suppressive myeloid cells by inhibiting

IL-2 secretion and downregulation of IL-2 receptor expression (106–108), whereby prostaglandins were found to play an important role in the murine model, but not in the bovine model (109). In addition, early data on T cell regulation and trypanosomosis showed that both IFN-γ and TNF play a key role in the suppressive effects on CD4 and CD8 T-cells (107). Furthermore, this suppressive phenotype of the host cells during the early stages of *T. brucei* infection is due to a combination of (i) trypanosome-released macrophage-activating factors leading to secretion of immunosuppressive factors such as NO, prostaglandins, and TNF and (ii) host-derived IFN-γ needed for optimal macrophage activation (110, 111). Moreover, this work also showed that within the *T. brucei* model, there is a compartmentalization of the suppressive effect in murine models during the later stages of infection, whereby NO plays a key role in macrophage-mediated splenic suppression, whereas the macrophage-mediated lymph node suppression occurred in an IFN-γ-dependent manner (110). Hence, at this stage of infection, an IFN-γ-independent suppressive mechanism is elicited in the spleen, whereas in the lymph nodes, IFN-γ is required yet not sufficient to inhibit T cell proliferation. In this context, it was shown that the trypanosome suppression-inducing factor (TSIF) released by *T. brucei* during the course of infection induces TNF and NO secretion by classically activated macrophages (i.e., M1), which is a prerequisite for parasite control. However, at the same time, it blocks T-cell proliferation in a NO, IFN-γ, and cell contact-dependent manner as well as downregulates type-2 immune responses required to dampen M1-mediated pathogenicity (112). Moreover, TSIF was shown to be essential for parasite biology given that TSIF knockdown parasites die within 2 days. In the *T. congolense* model, it was shown that besides IFN-γ, the anti-inflammatory cytokine IL-10 also contributed to T-cell suppression (113). Finally, although both murine and bovine African trypanosomosis induce suppression, it seems that NO does not play a role in the loss of T-cell proliferative function in the bovine trypanosomosis model and that, in contrast to the mouse model, the capacity of monocytes and macrophages to produce NO is actually downregulated in infected cattle (114). In summary, these results suggest that the T-cell suppression is multifactorial, tissue and infection stage dependent, and host/parasite dependent. However, T-cells are dispensable for parasite control, which was evidenced by the fact that mice lacking a functional T-cell compartment are as efficient as immune-competent animals in controlling trypanosome infection (86). These data indicate that T-cell-independent B-cell-mediated elimination is the driving factor implicated in controlling parasitemia. Nevertheless, T-cells play a key role in the development of African trypanosomosis-associated pathogenicity, such as anemia (115).

Undermining B-Cell Functionality

Given that trypanosomes are extracellular parasites, it is not surprising that the host–parasite coevolution resulted in a subtle equilibrium between suppression of B-cell and Ab functionality and parasite persistence. Indeed, besides suppression of myeloid cells and T-cells, B-cells were also found to be negatively affected during the early stages of trypanosome infection. Accordingly, trypanosomes exert full control of the different types of host immune responses to establish chronic infection. In this context, in cattle, it was found that there are also differences in humoral responses between *T. congolense*-infected trypano-resistant (N'Dama) and trypanosusceptible Boran cattle, further highlighting the importance of the humoral immune response in parasitemia control (116). In the following section, the effect of African trypanosome infections at the level of the B-cell compartment will be scrutinized.

In homeostatic conditions, B-cells develop from bone marrow (BM)-derived hematopoietic stem cells (HSCs) that initially differentiate into multipotent progenitor cells and subsequently into common lymphoid progenitor cells (117). Next, B-cell lymphopoiesis occurs through several developmental stages, such as pre-pro-B, pro-B, pre-B, and, finally immature B-cells, which is a highly regulated process with alternating phases of cell proliferation and differentiation (118, 119). During this process, these different B-cell subsets rearrange their immunoglobulin heavy-chain and light-chain gene loci and express different surface markers that can be identified *via* flow cytometry (see **Table 1**; **Figure 2**, left panel). Within the BM, these B-cells also undergo a positive and negative selection procedure, whereby the B-cell receptor (BCR) plays a checkpoint role (120). If the BCRs do not bind their antigen, they stop their development, i.e., during positive selection, while during negative selection, binding of self-antigens to the BCR triggers either clonal deletion, receptor editing, anergy, or ignorance, resulting in central tolerance (121). At the last stage of differentiation within the BM, these immature B-cells exhibit a high IgM expression and low or no expression of the IgD maturation marker. To complete their

TABLE 1 | B-cell surface marker expression used to track cellular alterations during infection.

	Marker expression
Hematopoietic stem cell (Lin^-)	(Terll9, CD3, CDllb, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, IL7r, ckit$^+$, CD34$^+$
Common lymphoid progenitor (Lin^-)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, IL7r$^+$, ckit$^+$, CD34$^-$
Pre-proB (Lin^-)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, CD19$^-$, IgM$^-$, CD43^{high}
ProB (Lin^-)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, CD19$^+$, IgM$^-$, CD43^{high}
PreB (Lin^-)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, CD19$^+$, IgM$^-$, CD43$^{low/-}$
Immature B (BM) (Lin^-)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, CD19$^+$, IgM$^+$, CD43$^{low/-}$
Transitional B (Lin^-) (blood/spleen)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, CD19$^+$, IgM$^+$, IgD$^+$, CD21$^+$
Immature B (spleen) (Lin^-)	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^+$, CD19$^+$, IgM$^+$, IgD$^+$, CD21$^+$
Mature B	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^-$, CD19$^+$, IgM$^+$, IgD$^+$, CD21$^+$
Marginal zone B	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^-$, CD19$^+$, IgM$^+$, IgD$^+$, CD21$^+$, CD1d^{high}
Follicular B	(Ter119, CD3, CD11b, GR1, NK1.1)$^-$, B220$^+$, CD93$^-$, CD19$^+$, IgM$^+$, IgD$^+$, CD21$^+$, CD1d$^-$

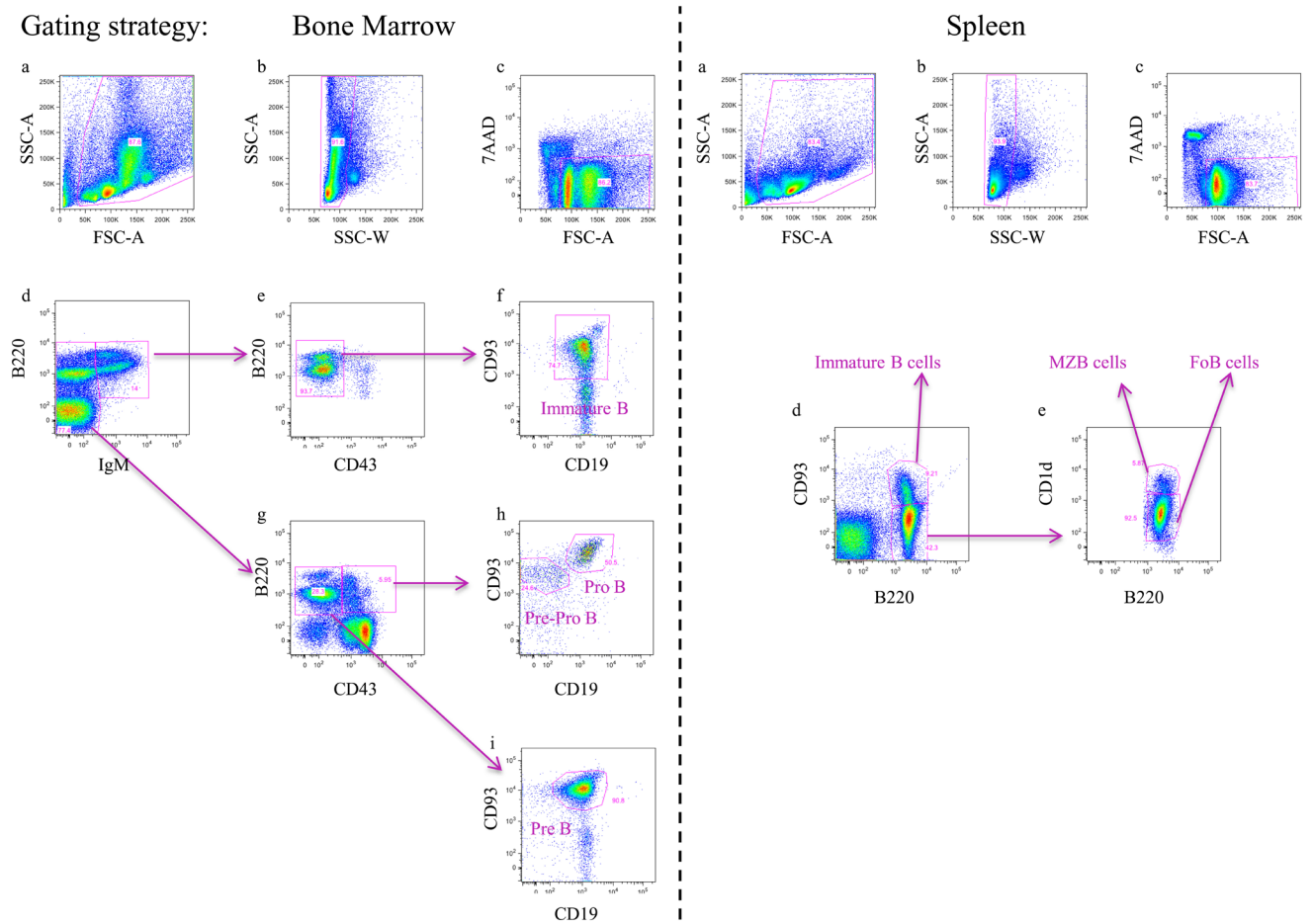

FIGURE 2 | FACS gating strategy to track B-cell alterations during infection within the bone marrow (BM) and spleen. BM (left panel): **(A,B)** In an FSC-A versus SSC-A plot, a life gate was selected followed by gating on singlets in an SSC-A versus SSC-W plot, respectively. **(C,D)** Following gating on 7AAD$^-$ cells and plotting in a B220 versus IgM allow identification of IgM$^+$ and IgM$^-$ cells. **(E,F)** The IgM$^+$ cells are plotted in a B220 versus CD43 plot to identify B220$^+$CD43$^-$ cells and subsequently plotted in a CD93 versus CD19 to identify immature B-cells (CD93$^+$CD19$^+$). **(G)** The IgM$^-$ cells are plotted in a B220 versus CD43 plot to identify B220$^+$CD43$^-$ cells and B220$^+$CD43$^+$ cells. **(H)** The B220$^+$CD43$^-$ cells are plotted in a CD93 versus CD19 to identify pre B-cells (CD93$^+$CD19$^+$). **(I)** The B220$^+$CD43$^+$ cells are plotted in a CD93 versus CD19 to identify pro B-cells (CD93$^+$CD19$^+$) and pre-pro B-cells (CD93$^+$CD19$^-$). Spleen (right panel): **(A,B)** In an FSC-A versus SSC-A plot, a life gate was selected followed by gating on singlets in an SSC-A versus SSC-W plot, respectively. **(C,D)** Following gating on 7AAD$^-$ cells and plotting in a CD93 versus B220 allow identification of immature B-cells (CD93$^+$B220$^+$). **(E)** The CD93$^+$B220$^-$ cells are subsequently plotted in a CD1d versus B220 plot to identify marginal zone B (MZB) cells (CD1d$^+$B220$^+$) and follicular B (FoB) cells (CD1d$^-$B220$^+$). It is important to mention that during infection, the expression levels of typical splenic B-cell subset markers can be modulated, which substantially complicates the identification of the different B-lymphocyte subsets.

development, immature B cells migrate to the spleen *via* the blood as transitional B cells (T1 type). In the spleen, these transitional B (T1) cells differentiate into T2 cells before they develop into two types of mature naïve B-cells (122), namely follicular B (FoB) or marginal zone B (MZB) cells (see **Figure 2**, right panel). In homeostatic conditions, B (FoB) cells are mainly located in the white pulp area of the spleen where they form primary B-cell follicles, preferentially undergo T-cell-dependent activation (upon activation *via* proteins and glycoproteins), and can give rise to both short-lived plasma cells (i.e. plasmablasts) for immediate protection and high-affinity class-switched IgG long-lived plasma cells and memory B cells for persistent protection. In contrast, MZB cells are concentrated outside the splenic marginal sinus surrounding the white pulp. Most of the time, they initiate a fast and preferentially T-cell-independent activation (upon activation *via* polysaccharides or unmethylated CpG DNA) giving rise to not only short-lived plasma cells that rapidly produce low-affinity Abs of IgM isotype but also some populations of long-lived plasma cells (123). Overall, B-cell activation is considered a very efficient defense system against invading "extracellular/blood-borne" pathogens. However, African trypanosomes have developed efficient ways to undermine the host's humoral response to establish chronic infection and allow completion of its life cycle/transmission. Indeed, using murine models, it was shown that African trypanosomes (both *T. brucei* and *T. congolense*) already during the early stages of infection trigger polyclonal B-cell activation in an attempt to dilute-out VSG-specific Abs during the course of infection. For example, it was shown that the CpG motifs of the *T. brucei* trypanosomal genomic DNA triggers TLR-9 signaling events and

contributes to polyclonal B-cell activation (96). This phenomenon might contribute to parasite immune evasion by driving unselective differentiation of B cells into short-lived plasma cells. In addition, Fcγ-receptors on phagocytes become saturated by polyspecific Abs, thereby reducing the efficiency of opsonization-mediated parasite clearance. Besides polyclonal B-cell activation, trypanosomes also undermine the "protective" humoral response by ablating B cell lymphopoiesis in primary and secondary lymphoid organs during both *T. brucei* and *T. congolense* infections. This was reflected by a depletion of all developmental B-cell stages in the BM and the spleen as well as previous effector B cells, such as memory B cells (124–126), thereby preventing the development of a B-cell memory required for permanent elimination (**Figure 3**). Similar results were obtained in the experimental *T. vivax* model (127). In addition, experimental results obtained in mice and livestock animals have shown that trypanosome infections exert detrimental effects on non-pathogen-related vaccines, by preventing the occurrence of memory recall responses (126, 128–130), or on the maintenance of the antigen-specific plasma B cell pool driving the development of collagen-induced arthritis (CIA) in DBA/1 prone mice (131). This destruction of the B-cell compartment at the level of both the BM and the spleen could be attributed to either parasite-derived components and/or host-derived (infection-induced) components. Interestingly, a recent work by Cnops et al. (132), revealed that during murine infection with a chronic low-virulent *T. b. gambiense* field isolate, FoB cells are retained, which coincided with reduced production of TNF and IFN-γ pro-inflammatory cytokines during the acute stage of infection compared to *T. brucei* and *T. congolense* infections. This finding was paralleled by the finding of Lejon et al. (133), which showed that in *T. b. gambiense*, HAT patients' low parasite levels seem to be associated with limited B cell dysfunction, whereby B-cell memory responses are only slightly reduced; however, the functionality of these memory B cells was not verified in rechallenge studies. These findings indicate that in both experimental trypanosomosis and natural infection, the inflammation stage linked to the acuteness of infection could be a major determinant in the

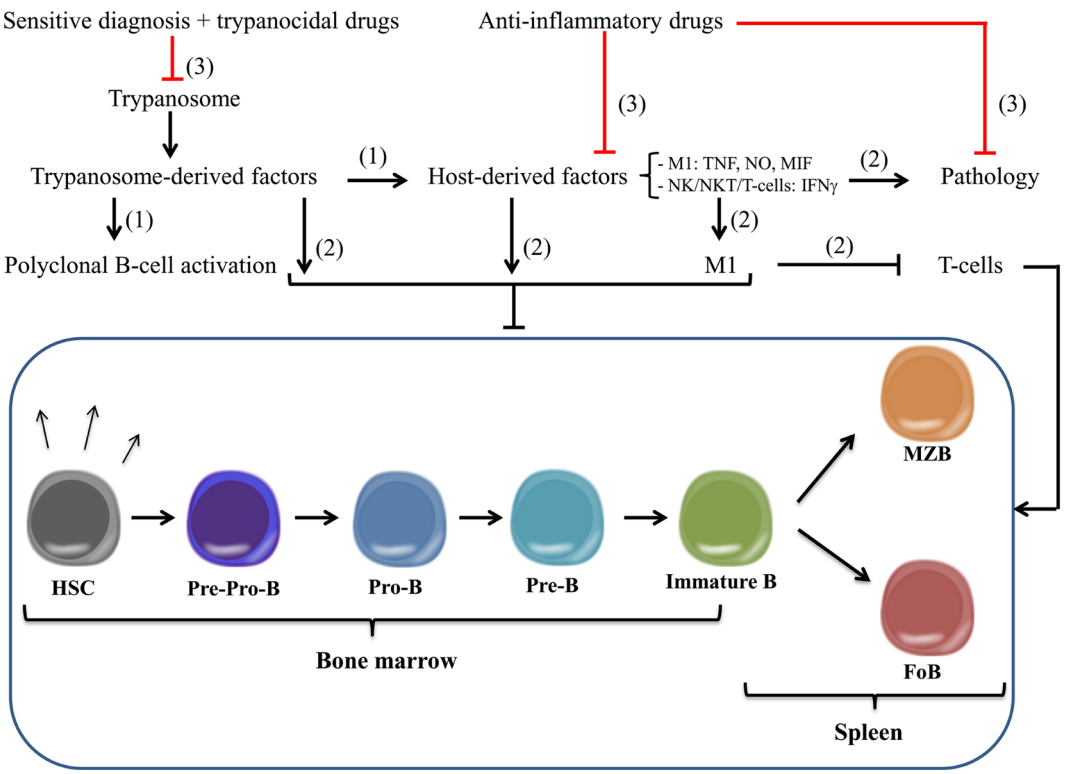

FIGURE 3 | Model for African trypanosomosis-associated impaired B-cell lymphopoiesis and improved vaccine development. (1) During the course of African trypanosomosis (AT), parasite-derived components are released that trigger besides polyclonal B-cell activation also the production of host-derived pro-inflammatory factors (i.e., NK-, NKT-, and T-cell-derived IFN-γ, M1-cell-derived TNF, NO, MIF) needed directly/indirectly for early parasite control. Yet, following control of the first parasitemia peak, polyclonal B-cell activation leads to dilution of parasite-specific antibodies, whereas the persistent pro-inflammatory response contributes to suppression of host responses and pathology. (2) Both parasite- and host-derived components can lead to a general state of impaired B-cell lymphopoiesis in (i) the bone marrow (BM), ranging from pre-pro-B-cell, pro-B-cell, pre-B-cell, and immature B-cell and (ii) the spleen, ranging from immature B-cell till MZB cell and FoB cell. In addition, also host-derived factors (involving M1) can contribute to T-cell suppression that in turn can affect B-cell homeostasis. (3) Hence, a more efficient therapeutic intervention strategy for AT should consist of a combination of (i) more reliable/sensitive diagnosis systems allowing early-stage parasite detection, (ii) more efficient trypanocidal/toxic drugs allowing improved parasite treatment, and (iii) pro-inflammatory-blocking molecules that could lead to a reduced pathology and a restoration of normal B-cell responses, thereby allowing more efficient/optimal vaccination. M1, classically activated myeloid cells; MIF, macrophage migration inhibitory factor; IFN, interferon; TNF, tumor necrosis factor; NO, nitric oxide; MZB, marginal zone B; FoB, follicular B.

processes that drive B-cell compartment destruction. This hypothesis is reinforced by the fact that both IFN-γ$^{(-/-)}$ and IFN-γR$^{(-/-)}$ mice are protected from early trypanosomosis-associated FoB cell depletion (134). This phenotype coincided with a drastic inhibition of B-cell apoptosis and a reduced activation of FoB cells and inflammatory responses during the first week postinfection. These data demonstrated that IFN-γ is an important cytokine involved in undermining trypanosomosis-associated B-cell responses. So far, the cellular source of early IFN-γ production involved in triggering impaired B-cell lymphopoiesis remains to be fully elucidated. However, it was recently suggested that NK-cells, an important early source of IFN-γ, are involved in B-cell killing and suppressing humoral immunity within the *T. brucei* model (115, 135). Yet, it cannot be excluded that other sources of IFN-γ, such as NKT, CD8$^+$, and CD4$^+$ T-cells, are also involved in IFN-γ-mediated B-cell apoptosis given that there is a transition of IFN-γ production by these cells during the course of *T. brucei* infection (115). Interestingly, upon drug treatment [suramin and diminazene aceturate (Berenil)] of *T. brucei*- and *T. congolense*-infected mice, the BM B-cell lymphopoiesis is reinitiated, and the splenic B-cell subsets are repopulated, suggesting that an active chronic infection (i.e., parasite–host interaction) is involved in undermining the humoral responses *via* either parasite-released components and/or inflammatory-based mechanism(s) (136, 137). However, Uzonna and coworkers (137) showed that Berenil besides exerting trypanolytic effects could also modulate the host immune response to the parasite by dampening excessive immune activation and production of pathology-promoting pro-inflammatory cytokines. Hence, it cannot be excluded that the beneficial effects of Berenil for treatment of AT are multifactorial: (i) eliminate parasites thereby resulting in reduced triggering of host inflammatory immune responses and (i) reduce the host's pro-inflammatory potential to respond to pro-inflammatory/parasite-derived components. In both cases, this will result in a more efficient host-mediated parasite control due to a recovery from the impaired B-cell lymphopoiesis and protection from infection-associated pathogenicity due to lower inflammatory responses (138). In this context, it was recently shown within the *T. congolense* experimental model that also host molecules such as macrophage migration inhibitory factor (MIF) can play a key role in regulating trypanosomosis-associated pro-inflammatory responses and B-cell homeostasis (139). In this work, it was shown that *T. congolense*-infected MIF-deficient mice exhibited increased Ab titers that correlated with reduced B-cell apoptosis. Hence, MIF could be considered as a target to alleviate the impaired B-cell lymphopoiesis.

CONCLUSIONS AND PERSPECTIVES

The extracellular African trypanosomes have acquired efficient immune evasion mechanisms to undermine protective host immune responses and allow survival in the host's extracellular environment. Hereby, they are proficient in avoiding elimination *via* the host's humoral immune response by destroying the B-cell compartment/memory and shielding-off conserved epitope, thereby paving the way for chronic infection. This destruction of B-cell memory already very early during infection might explain the failure of developing an effective vaccine. Indeed, immunization with the immunodominant VSG did not yield any universal protection. So far, attempts to immunize with trypanosome molecules (VSG, beta tubulin, etc.), such as conserved membrane proteins or receptors for uptake of nutrients, have resulted in limited protective effects because such molecules are either concealed beneath the surface coat or are expressed at a to low level to induce protective host immunity (140, 141). However, some protection against AT-associated pathological features (i.e., anemia, tissue injury) has been achieved upon vaccination with pathology-inducing factors such as the VSG-derived GPI moiety (*T. brucei*, *T. congolense*) or the cysteine proteinase congopain (*T. congolense*), yet the animals were never fully cured (142, 143). Moreover, as far as the GPI-based strategy in the murine model was concerned, there was no effect on parasitemia but rather the protective effect correlated with reduced pro-inflammatory immune responses and was independent of the Ab response. This is in line with the observation that pathogenicity did not correlate with Ab levels at least for the experimental murine *T. brucei* model (86). In contrast, for the congopain vaccination strategy in experimental bovine *T. congolense* models, there was a correlation between reduced pathogenicity and increased Ab titers (143, 144). This is in line with the observation that *T. congolense*-infected N'Dama cattle (a trypanotolerant breed showing natural resistance to trypanosomosis) exhibited higher antiparasite Ab titers than the susceptible Boran breeds (145, 146), suggesting that there are differences in the frequency of trypanosome-specific Ab-secreting cells in the spleen and in the activation state of B-cells in the blood between both cattle breeds during infection. Interestingly, the sera from *T. congolense*-infected N'Dama cattle specifically recognized dimer-associated epitopes on the congopain antigen (147).

The current research on vaccine development has switched toward identification of invariant surface glycoproteins or conserved *c*-terminal VSG epitopes/peptides that are predicted to contain several MHC II recognition sites (148–150). Whether these latter approaches will lead to the development of an effective protective and antipathology vaccine will be challenging and possibly not achievable given that African trypanosomes undermine B-cell memory responses. In addition, also the whole genome transcriptome analysis (i.e., SAGE technique) that enables to (i) explore the full transcriptome of trypanosusceptible and trypanotolerant cattle might lead to the identification of interesting gene variations linked to the trypanotolerance status of the animal (151–153) and (ii) understand the molecular aspects of the trypanosome dialog with its tsetse and mammalian hosts (i.e., interaction with the salivary glands and LS versus SS differentiation, respectively) might pave the way to develop novel diagnostic/therapeutic intervention strategies (154, 155). Furthermore, although the loss of B-cell responses/memory during AT might rely on either a parasite-induced or a host-induced effect or a combination of both, understanding the molecular mechanisms used by the trypanosomes to dampen B cell responses might lead to the development of new therapeutics not only for AT but also

for other diseases such as autoimmune diseases (i.e., CIA) or malaria, where B-cell dysfunction is contributing to the disease outcome (131, 156–158). In this context, it was shown that the B-cell adaptor molecule Bam32 plays a pivotal role in optimal Ab responses and resistance during *T. congolense* infections in mice (159). Besides parasite-derived molecules, also host-derived molecule could be considered as a potential target for intervention strategies. In this context, MIF can be proposed as potential candidate given that it can play a role both in innate as adaptive immunity *via* interaction with its main receptor CD74 to regulate the host inflammatory response (160, 161). Indeed, MIF was shown to play a pivotal role in stimulating/inflammatory responses and regulating T- and B-cell recruitment as well as B-cell proliferation/survival and thereby contribute to pathology development (162–164). Hence, blocking MIF-signaling could reduce inflammatory responses, thereby alleviating suppression of B-cell lymphopoiesis, which in turn might favor vaccine efficacy.

In the future, most likely, a combination of (i) more sensitive/reliable diagnosis techniques needed for early-stage parasite detection and (ii) anti-parasite intervention strategies (trypanocidal/trypanotoxic drugs) and (iii) antidisease/pathology (anti-inflammatory) intervention strategies will be required to combat AT (see **Figure 3**).

AUTHOR CONTRIBUTIONS

All authors contributed to writing the manuscript. However, BS and MR are co-first and CT and SM share co-last authorship.

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REFERENCES

1. Yaro M, Munyard KA, Stear MJ, Groth DM. Combatting African Animal Trypanosomiasis (AAT) in livestock: the potential role of trypanotolerance. *Vet Parasitol* (2016) 225:43–52. doi:10.1016/j.vetpar.2016.05.003
2. Simarro PP, Cecchi G, Franco JR, Paone M, Diarra A, Ruiz-Postigo JA, et al. Estimating and mapping the population at risk of sleeping sickness. *PLoS Negl Trop Dis* (2012) 6:e1859. doi:10.1371/journal.pntd.0001859
3. Welburn SC, Molyneux DH, Maudlin I. Beyond tsetse – implications for research and control of Human African trypanosomiasis epidemics. *Trends Parasitol* (2016) 32:230–41. doi:10.1016/j.pt.2015.11.008
4. World Health Organization. *Trypanosomiasis, Human African (Sleeping Sickness)*. World Health Organization (2013). 259 p. Available from: http://www.who.int/mediacentre/factsheets/fs259/en/
5. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. *Lancet* (2010) 375:148–59. doi:10.1016/S0140-6736(09)60829-1
6. Simarro PP, Diarra A, Ruiz Postigo JA, Franco JR, Jannin JG. The Human African trypanosomiasis control and surveillance programme of the world health organization 2000–2009: the way forward. *PLoS Negl Trop Dis* (2011) 5(2):e1007. doi:10.1371/journal.pntd.0001007
7. Franco JR, Simarro PP, Diarra A, Jannin JG. Epidemiology of Human African trypanosomiasis. *Clin Epidemiol* (2014) 6:257–75. doi:10.2147/CLEP.S39728
8. Kennedy PG. Clinical features, diagnosis, and treatment of Human African trypanosomiasis (sleeping sickness). *Lancet Neurol* (2013) 12:186–94. doi:10.1016/S1474-4422(12)70296-X
9. Rodgers J. Trypanosomiasis and the brain. *Parasitology* (2010) 137:1995–2006. doi:10.1017/S0031182009991806
10. MacLean L, Reiber H, Kennedy PG, Sternberg JM. Stage progression and neurological symptoms in *Trypanosoma brucei* rhodesiense sleeping sickness: role of the CNS inflammatory response. *PLoS Negl Trop Dis* (2012) 6:e1857. doi:10.1371/journal.pntd.0001857
11. Tiberti N, Matovu E, Hainard A, Enyaru JC, Lejon V, Robin X, et al. New biomarkers for stage determination in *Trypanosoma brucei* rhodesiense sleeping sickness patients. *Clin Transl Med* (2013) 2:1. doi:10.1186/2001-1326-2-1
12. Jamonneau V, Ilboudo H, Kabore J, Kaba D, Koffi M, Solano P, et al. Untreated human infections by *Trypanosoma brucei* gambiense are not 100% fatal. *PLoS Negl Trop Dis* (2012) 6:e1691. doi:10.1371/journal.pntd.0001691
13. MacLean LM, Odiit M, Chisi JE, Kennedy PG, Sternberg JM. Focus-specific clinical profiles in Human African trypanosomiasis caused by *Trypanosoma brucei* rhodesiense. *PLoS Negl Trop Dis* (2010) 4:e906. doi:10.1371/journal.pntd.0000906
14. Bucheton B, Macleod A, Jamonneau V. Human host determinants influencing the outcome of *Trypanosoma brucei* gambiense infections. *Parasite Immunol* (2011) 33:438–47. doi:10.1111/j.1365-3024.2011.01287.x
15. WHO. Control and surveillance of Human African trypanosomiasis. *World Health Organ Tech Rep Ser* (2013) (984):1–237.
16. Lejon V, Bentivoglio M, Franco JR. Human African trypanosomiasis. *Handb Clin Neurol* (2013) 114:169–81. doi:10.1016/B978-0-444-53490-3.00011-X
17. Ruiz-Postigo JA, Franco JR, Lado M, Simarro PP. Human African trypanosomiasis in South Sudan: how can we prevent a new epidemic? *PLoS Negl Trop Dis* (2012) 6:e1541. doi:10.1371/journal.pntd.0001541
18. Hasker E, Mpanya A, Makabuza J, Mbo F, Lumbala C, Kumpel J, et al. Treatment outcomes for Human African trypanosomiasis in the Democratic Republic of the Congo: analysis of routine program data from the world's largest sleeping sickness control program. *Trop Med Int Health* (2012) 17:1127–32. doi:10.1111/j.1365-3156.2012.03042.x
19. Desquesnes M, Dargantes A, Lai DH, Lun ZR, Holzmuller P, Jittapalapong S. *Trypanosoma evansi* and surra: a review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. *Biomed Res Int* (2013) 2013:321237. doi:10.1155/2013/321237
20. Brun R, Hecker H, Lun ZR. *Trypanosoma evansi* and *T. equiperdum*: distribution, biology, treatment and phylogenetic relationship (a review). *Vet Parasitol* (1998) 79:95–107. doi:10.1016/S0304-4017(98)00146-0
21. Claes F, Büscher P, Touratier L, Goddeeris BM. *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol* (2005) 21:316–21. doi:10.1016/j.pt.2005.05.010
22. Jones TW, Dávila AMR. *Trypanosoma vivax* – out of Africa. *Trends Parasitol* (2001) 17:99–101. doi:10.1016/S1471-4922(00)01777-3
23. Osório AL, Madruga CR, Desquesnes M, Soares CO, Ribeiro LR, Costa SC. *Trypanosoma (Duttonella) vivax*: its biology, epidemiology, pathogenesis, and introduction in the New World – a review. *Mem Inst Oswaldo Cruz* (2008) 103:1–13. doi:10.1590/S0074-02762008000100001
24. Naessens J. Bovine trypanotolerance: a natural ability to prevent severe anaemia and haemophagocytic syndrome? *Int J Parasitol* (2006) 36:521–8. doi:10.1016/j.ijpara.2006.02.012
25. Disease T. African Animal trypanosomiasis African Animal trypanosomiasis. *In Vitro* (2009):1–5. Available from: http://www.cfsph.iastate.edu/Factsheets/pdfs/trypanosomiasis_african.pdf

26. Swallow B. *Impacts of Trypanosomiasis on African Agriculture*. Nairobi, Kenya: Int Livest Res Institute (1999). p. 1–46. Available from: http://www.cabdirect.org/abstracts/20003010139.html
27. Giordani F, Morrison LJ, Rowan TG, de Koning HP, Barrett MP. The animal trypanosomiases and their chemotherapy: a review. *Parasitology* (2016) 143(14):1862–89. doi:10.1017/S0031182016001268
28. Gilbert M, Jenner C, Pender J, Rogers D, Slingenbergh J, Wint W. The programme against African trypanosomiasis information system (PAATIS). *The African Trypanosomes*, Vol. 1 (2002). p. 11–24.
29. Bouyer J, Bouyer F, Donadeu M, Rowan T, Napier G. Community- and farmer-based management of animal African trypanosomosis in cattle. *Trends Parasitol* (2013) 29:519–22. doi:10.1016/j.pt.2013.08.003
30. Baker N, de Koning HP, Maser P, Horn D. Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. *Trends Parasitol* (2013) 29:110–8. doi:10.1016/j.pt.2012.12.005
31. Delespaux V, de Koning HP. Drugs and drug resistance in African trypanosomiasis. *Drug Resist Updat* (2007) 10:30–50. doi:10.1016/j.drup.2007.02.004
32. Naula C, Burchmore R. A plethora of targets, a paucity of drugs: progress towards the development of novel chemotherapies for human African trypanosomiasis. *Expert Rev Anti Infect Ther* (2003) 1:157–65. doi:10.1586/14787210.1.1.157
33. Wenzler T, Schumann Burkard G, Schmidt RS, Mäser P, Bergner A, Roditi I, et al. A new approach to chemotherapy: drug-induced differentiation kills African trypanosomes. *Sci Rep* (2016) 6:22451. doi:10.1038/srep22451
34. Haanstra JR, Gerding A, Dolga AM, Sorgdrager FJH, Buist-homan M, Toit F, et al. Targeting pathogen metabolism without collateral damage to the host. *Sci Rep* (2017) 7:40406. doi:10.1038/srep40406
35. Priotto G, Kasparian S, Mutombo W, Ngouama D, Ghorashian S, Arnold U, et al. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei* gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet* (2009) 374:56–64. doi:10.1016/S0140-6736(09)61117-X
36. Alirol E, Schrumpf D, Amici Heradi J, Riedel A, de Patoul C, Quere M, et al. Nifurtimox-eflornithine combination therapy for second-stage gambiense Human African trypanosomiasis: medecins Sans Frontieres experience in the Democratic Republic of the Congo. *Clin Infect Dis* (2013) 56:195–203. doi:10.1093/cid/cis886
37. Unciti-Broceta JD, Arias JL, Maceira J, Soriano M, Ortiz-Gonzalez M, Hernandez-Quero J, et al. Specific cell targeting therapy bypasses drug resistance mechanisms in African trypanosomiasis. *PLoS Pathog* (2015) 11:e1004942. doi:10.1371/journal.ppat.1004942
38. Kroubi M, Karembe H, Betbeder D. Drug delivery systems in the treatment of African trypanosomiasis infections. *Expert Opin Drug Deliv* (2011) 8:735–47. doi:10.1517/17425247.2011.574122
39. Holt HR, Selby R, Mumba C, Napier GB, Guitian J. Assessment of animal African trypanosomiasis (AAT) vulnerability in cattle-owning communities of sub-Saharan Africa. *Parasit Vectors* (2016) 9:53. doi:10.1186/s13071-016-1336-5
40. Simarro PP, Cecchi G, Franco JR, Paone M, Diarra A, Priotto G, et al. Monitoring the progress towards the elimination of gambiense Human African trypanosomiasis. *PLoS Negl Trop Dis* (2015) 9:e0003785. doi:10.1371/journal.pntd.0003785
41. Bonnet J, Boudot C, Courtioux B. Overview of the diagnostic methods used in the field for Human African trypanosomiasis: what could change in the next years? *Biomed Res Int* (2015) 2015:583262. doi:10.1155/2015/583262
42. Kennedy PG. Human African trypanosomiasis-neurological aspects. *J Neurol* (2006) 253:411–6. doi:10.1007/s00415-006-0093-3
43. Odongo S, Sterckx YG, Stijlemans B, Pillay D, Baltz T, Muyldermans S, et al. An anti-proteome nanobody library approach yields a specific immunoassay for *Trypanosoma congolense* diagnosis targeting glycosomal aldolase. *PLoS Negl Trop Dis* (2016) 10:e0004420. doi:10.1371/journal.pntd.0004420
44. Capewell P, Cren-Travaillé C, Marchesi F, Johnston P, Clucas C, Benson RA, et al. The skin is a significant but overlooked anatomical reservoir for vector-borne African trypanosomes. *Elife* (2016) 5:e17716. doi:10.7554/eLife.17716
45. Morrison LJ, Vezza L, Rowan T, Hope JC. Animal African trypanosomiasis: time to increase focus on clinically relevant parasite and host species. *Trends Parasitol* (2016) 32:599–607. doi:10.1016/j.pt.2016.04.012
46. Delafosse A, Thébaud E, Desquesnes M, Michaux Y. Epidemiology of *Trypanosoma vivax* infection in cattle in the tsetse free area of Lake Chad. *Prev Vet Med* (2006) 74:108–19. doi:10.1016/j.prevetmed.2005.10.006
47. La Greca F, Haynes C, Stijlemans B, De Trez C, Magez S. Antibody-mediated control of *Trypanosoma vivax* infection fails in the absence of tumour necrosis factor. *Parasite Immunol* (2014) 36:271–6. doi:10.1111/pim.12106
48. Blom-Potar MC, Chamond N, Cosson A, Jouvion G, Droin-Bergere S, Huerre M, et al. *Trypanosoma vivax* infections: pushing ahead with mouse models for the study of Nagana. II. Immunobiological dysfunctions. *PLoS Negl Trop Dis* (2010) 4:e793. doi:10.1371/journal.pntd.0000793
49. Rotureau B, Van Den Abbeele J. Through the dark continent: African trypanosome development in the tsetse fly. *Front Cell Infect Microbiol* (2013) 3:53. doi:10.3389/fcimb.2013.00053
50. Achcar F, Kerkhoven EJ, Barrett MP. *Trypanosoma brucei*: meet the system. *Curr Opin Microbiol* (2014) 20:162–9. doi:10.1016/j.mib.2014.06.007
51. MacGregor P, Szoor B, Savill NJ, Matthews KR. Trypanosomal immune evasion, chronicity and transmission: an elegant balancing act. *Nat Rev Microbiol* (2012) 10:431–8. doi:10.1038/nrmicro2779
52. Fenn K, Matthews KR. The cell biology of *Trypanosoma brucei* differentiation. *Curr Opin Microbiol* (2007) 10:539–46. doi:10.1016/j.mib.2007.09.014
53. Stijlemans B, Beschin A, Magez S, Van Ginderachter JA, De Baetselier P. Iron homeostasis and *Trypanosoma brucei* associated immunopathogenicity development: a battle/quest for iron. *Biomed Res Int* (2015) 2015:819389. doi:10.1155/2015/819389
54. Peacock L, Cook S, Ferris V, Bailey M, Gibson W. The life cycle of *Trypanosoma (Nannomonas) congolense* in the tsetse fly. *Parasit Vectors* (2012) 5:109. doi:10.1186/1756-3305-5-109
55. Ooi C-P, Bastin P. More than meets the eye: understanding *Trypanosoma brucei* morphology in the tsetse. *Front Cell Infect Microbiol* (2013) 3:71. doi:10.3389/fcimb.2013.00071
56. Van Den Abbeele J, Rotureau B. New insights in the interactions between African trypanosomes and tsetse flies. *Front Cell Infect Microbiol* (2013) 3:63. doi:10.3389/fcimb.2013.00063
57. Banks KL. Injury induced by *Trypanosoma congolense* adhesion to cell membranes. *J Parasitol* (1980) 66:34–7. doi:10.2307/3280584
58. Hemphill A, Frame I, Ross CA. The interaction of *Trypanosoma congolense* with endothelial cells. *Parasitology* (1994) 109(Pt 5):631–41. doi:10.1017/S0031182000076514
59. Trindade S, Rijo-Ferreira F, Carvalho T, Pinto-Neves D, Guegan F, Aresta-Branco F, et al. *Trypanosoma brucei* parasites occupy and functionally adapt to the adipose tissue in mice. *Cell Host Microbe* (2016) 19:837–48. doi:10.1016/j.chom.2016.05.002
60. Mogk S, Meiwes A, Shtopel S, Schraermeyer U, Lazarus M, Kubata B, et al. Cyclical appearance of African trypanosomes in the cerebrospinal fluid: new insights in how trypanosomes enter the CNS. *PLoS One* (2014) 9:e91372. doi:10.1371/journal.pone.0091372
61. Beschin A, Van Den Abbeele J, De Baetselier P, Pays E. African trypanosome control in the insect vector and mammalian host. *Trends Parasitol* (2014) 30:538–47. doi:10.1016/j.pt.2014.08.006
62. MacGregor P, Szöőr B, Savill NJ, Matthews KR. Trypanosomal immune evasion, chronicity and transmission: an elegant balancing act. *Nat Rev Microbiol* (2012) 10:431–8. doi:10.1038/nrmicro2779
63. Rico E, Rojas F, Mony BM, Szoor B, Macgregor P, Matthews KR. Bloodstream form pre-adaptation to the tsetse fly in *Trypanosoma brucei*. *Front Cell Infect Microbiol* (2013) 3:78. doi:10.3389/fcimb.2013.00078
64. Mony BM, Matthews KR. Assembling the components of the quorum sensing pathway in African trypanosomes. *Mol Microbiol* (2015) 96:220–32. doi:10.1111/mmi.12949
65. Welburn SC, Macleod E, Figarella K, Duzensko M. Programmed cell death in African trypanosomes. *Parasitology* (2006) 132(Suppl):S7–18. doi:10.1017/S0031182006000825
66. Duszenko M, Figarella K, Macleod ET, Welburn SC. Death of a trypanosome: a selfish altruism. *Trends Parasitol* (2006) 22:536–42. doi:10.1016/j.pt.2006.08.010
67. Vassella E, Reuner B, Yutzy B, Boshart M. Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. *J Cell Sci* (1997) 110(Pt 2):2661–71.

68. Figarella K, Rawer M, Uzcategui NL, Kubata BK, Lauber K, Madeo F, et al. Prostaglandin D2 induces programmed cell death in *Trypanosoma brucei* bloodstream form. *Cell Death Differ* (2005) 12:335–46. doi:10.1038/sj.cdd.4401564
69. Seed JR, Wenck MA. Role of the long slender to short stumpy transition in the life cycle of the African trypanosomes. *Kinetoplastid Biol Dis* (2003) 2:3. doi:10.1186/1475-9292-2-3
70. Barry JD, Graham SV, Fotheringham M, Graham VS, Kobryn K, Wymer B. VSG gene control and infectivity strategy of metacyclic stage *Trypanosoma brucei*. *Mol Biochem Parasitol* (1998) 91:93–105. doi:10.1016/S0166-6851(97)00193-X
71. Takeya M, Reinwald E, Risse HJ. Pathobiochemical alterations in experimental chronic and acute trypanosomal infection in mice. *Clin Chem Lab Med* (1987) 25:665–74. doi:10.1515/cclm.1987.25.10.665
72. Stijlemans B, Caljon G, Van Den Abbeele J, Van Ginderachter JA, Magez S, De Trez C. Immune evasion strategies of *Trypanosoma brucei* within the mammalian host: progression to pathogenicity. *Front Immunol* (2016) 7:233. doi:10.3389/fimmu.2016.00233
73. Schwede A, Macleod OJ, MacGregor P, Carrington M. How does the VSG coat of bloodstream form African trypanosomes interact with external proteins? *PLoS Pathog* (2015) 11:e1005259. doi:10.1371/journal.ppat.1005259
74. Schwede A, Jones N, Engstler M, Carrington M. The VSG C-terminal domain is inaccessible to antibodies on live trypanosomes. *Mol Biochem Parasitol* (2011) 175:201–4. doi:10.1016/j.molbiopara.2010.11.004
75. Morrison LJ, Marcello L, McCulloch R. Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. *Cell Microbiol* (2009) 11:1724–34. doi:10.1111/j.1462-5822.2009.01383.x
76. *Collection S. HHS Public Access*. (2016) 8:583–92.
77. Pays E, Vanhamme L, Pérez-Morga D. Antigenic variation in *Trypanosoma brucei*: facts, challenges and mysteries. *Curr Opin Microbiol* (2004) 7:369–74. doi:10.1016/j.mib.2004.05.001
78. Borst P. Antigenic variation and allelic exclusion. *Cell* (2002) 109:5–8. doi:10.1016/S0092-8674(02)00711-0
79. Hall JPJ, Wang H, David Barry J. Mosaic VSGs and the scale of *Trypanosoma brucei* antigenic variation. *PLoS Pathog* (2013) 9:e1003502. doi:10.1371/journal.ppat.1003502
80. Dubois ME, Demick KP, Mansfield JM. Trypanosomes expressing a mosaic variant surface glycoprotein coat escape early detection by the immune system. *Infect Immun* (2005) 73:2690–7. doi:10.1128/IAI.73.5.2690-2697.2005
81. Graham SV, Barry JD. Transcriptional regulation of metacyclic variant surface glycoprotein gene expression during the life cycle of *Trypanosoma brucei*. *Mol Cell Biol* (1995) 15:5945–56. doi:10.1128/MCB.15.11.5945
82. Graham SV, Terry S, Barry JD. A structural and transcription pattern for variant surface glycoprotein gene expression sites used in metacyclic stage *Trypanosoma brucei*. *Mol Biochem Parasitol* (1999) 103:141–54. doi:10.1016/S0166-6851(99)00128-0
83. Barry JD, McCulloch R. Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. *Adv Parasitol* (2001) 49:1–70. doi:10.1016/S0065-308X(01)49037-3
84. Engstler M, Pfohl T, Herminghaus S, Boshart M, Wiegertjes G, Heddergott N, et al. Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. *Cell* (2007) 131:505–15. doi:10.1016/j.cell.2007.08.046
85. Field MC, Carrington M. The trypanosome flagellar pocket. *Nat Rev Microbiol* (2009) 7:775–86. doi:10.1038/nrmicro2221
86. Magez S, Schwegmann A, Atkinson R, Claes F, Drennan M, De Baetselier P, et al. The role of B-cells and IgM antibodies in parasitemia, anemia, and VSG switching in *Trypanosoma brucei*-infected mice. *PLoS Pathog* (2008) 4:e1000122. doi:10.1371/journal.ppat.1000122
87. Magez S, Radwanska M, Drennan M, Fick L, Baral TN, Brombacher F, et al. Interferon-gamma and nitric oxide in combination with antibodies are key protective host immune factors during *Trypanosoma congolense* Tc13 Infections. *J Infect Dis* (2006) 193:1575–83. doi:10.1086/503808
88. Balber AE, Bangs JD, Jones SM, Proia RL. Inactivation or elimination of potentially trypanolytic, complement-activating immune complexes by pathogenic trypanosomes. *Infect Immun* (1979) 24:617–27.
89. Ferrante A, Allison AC. Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat. *Parasite Immunol* (1983) 5:491–8. doi:10.1111/j.1365-3024.1983.tb00763.x
90. Devine DV, Falk RJ, Balber AE. Restriction of the alternative pathway of human complement by intact *Trypanosoma brucei* subsp. gambiense. *Infect Immun* (1986) 52:223–9.
91. Ooi YM, Colten HR. Genetic defect in secretion of complement C5 in mice. *Nature* (1979) 282:207–8. doi:10.1038/282207a0
92. Jarvinen JA, Dalmasso A. *Trypanosoma musculi* infections in normocomplementemic, C5-deficient, and C3-depleted mice. *Infect Immun* (1977) 16:557–63.
93. Morrison WI, Roelants GE, Mayor-Withey KS, Murray M. Susceptibility of inbred strains of mice to *Trypanosoma congolense*: correlation with changes in spleen lymphocyte populations. *Clin Exp Immunol* (1978) 32:25–40.
94. Mekori YA, Metcalfe DD. Mast cells in innate immunity. *Immunol Rev* (2000) 173:131–40. doi:10.1016/j.jaci.2004.04.045
95. Paulnock DM, Freeman BE, Mansfield JM. Modulation of innate immunity by African trypanosomes. *Parasitology* (2010) 137:2051–63. doi:10.1017/S0031182010001460
96. Drennan MB, Stijlemans B, Van den Abbeele J, Quesniaux VJ, Barkhuizen M, Brombacher F, et al. The induction of a type 1 immune response following a *Trypanosoma brucei* infection is MyD88 dependent. *J Immunol* (2005) 175:2501–9. doi:10.4049/jimmunol.175.4.2501
97. Mansfield JM, Paulnock DM. Regulation of innate and acquired immunity in African trypanosomiasis. *Parasite Immunol* (2005) 27:361–71. doi:10.1111/j.1365-3024.2005.00791.x
98. Stijlemans B, Guilliams M, Raes G, Beschin A, Magez S, De Baetselier P. African trypanosomosis: from immune escape and immunopathology to immune intervention. *Vet Parasitol* (2007) 148:3–13. doi:10.1016/j.vetpar.2007.05.005
99. Stijlemans B, Vankrunkelsven A, Caljon G, Bockstal V, Guilliams M, Bosschaerts T, et al. The central role of macrophages in trypanosomiasis-associated anemia: rationale for therapeutical approaches. *Endocr Metab Immune Disord Drug Targets* (2010) 10:71–82. doi:10.2174/187153010790827966
100. Bosschaerts T, Morias Y, Stijlemans B, Hérin M, Porta C, Sica A, et al. IL-10 limits production of pathogenic TNF by M1 myeloid cells through induction of nuclear NF-κB p50 member in *Trypanosoma congolense* infection-resistant C57BL/6 mice. *Eur J Immunol* (2011) 41:3270–80. doi:10.1002/eji.201041307
101. Salmon D, Vanwalleghem G, Morias Y, Denoeud J, Krumbholz C, Lhomme F, et al. Adenylate cyclases of *Trypanosoma brucei* inhibit the innate immune response of the host. *Science* (2012) 337:463–6. doi:10.1126/science.1222753
102. De Muylder G, Daulouède S, Lecordier L, Uzureau P, Morias Y, Van Den Abbeele J, et al. A *Trypanosoma brucei* kinesin heavy chain promotes parasite growth by triggering host arginase activity. *PLoS Pathog* (2013) 9:e1003731. doi:10.1371/journal.ppat.1003731
103. Willert E, Phillips MA. Regulation and function of polyamines in African trypanosomes. *Trends Parasitol* (2012) 28:66–72. doi:10.1016/j.pt.2011.11.001
104. McGettrick AF, Corcoran SE, Barry PJ, McFarland J, Crès C, Curtis AM, et al. *Trypanosoma brucei* metabolite indolepyruvate decreases HIF-1α and glycolysis in macrophages as a mechanism of innate immune evasion. *Proc Natl Acad Sci U S A* (2016) 113(48):E7778–87. doi:10.1073/pnas.1608221113
105. Coller SP, Mansfield JM, Paulnock DM. Glycosylinositolphosphate soluble variant surface glycoprotein inhibits IFN-gamma-induced nitric oxide production via reduction in STAT1 phosphorylation in African trypanosomiasis. *J Immunol* (2003) 171:1466–72. doi:10.4049/jimmunol.171.3.1466
106. Sileghem M, Darji A, Hamers R, Van De Winkel M, De Baetselier P. Dual role of macrophages in the suppression of interleukin 2 production and interleukin 2 receptor expression in trypanosome-infected mice. *Eur J Immunol* (1989) 19:829–35. doi:10.1002/eji.1830190508
107. Darji A, Beschin A, Sileghem M, Heremans H, Brys L, De Baetselier P. In vitro simulation of immunosuppression caused by *Trypanosoma brucei*: active involvement of gamma interferon and tumor necrosis factor in the pathway of suppression. *Infect Immun* (1996) 64:1937–43.
108. Uzonna JE, Kaushik RS, Gordon JR, Tabel H. Immunoregulation in experimental murine *Trypanosoma congolense* infection: anti-IL-10 antibodies reverse trypanosome-mediated suppression of lymphocyte proliferation in vitro and moderately prolong the lifespan of genetically susceptible BALB/c mice. *Parasite Immunol* (1998) 20:293–302. doi:10.1046/j.1365-3024.1998.00156.x
109. Sileghem M, Norman Flynn J. Suppression of interleukin 2 secretion and interleukin 2 receptor expression during tsetse-transmitted trypanosomiasis in cattle. *Eur J Immunol* (1992) 22:767–73. doi:10.1002/eji.1830220321

110. Beschin A, Brys L, Magez S, Radwanska M, De Baetselier P. *Trypanosoma brucei* infection elicits nitric oxide-dependent and nitric oxide-independent suppressive mechanisms. *J Leukoc Biol* (1998) 63:429–39.
111. Millar AE, Sternberg J, McSharry C, Wei XQ, Liew FY, Turner CM. T-Cell responses during *Trypanosoma brucei* infections in mice deficient in inducible nitric oxide synthase. *Infect Immun* (1999) 67:3334–8.
112. Gomez-Rodriguez J, Stijlemans B, De Muylder G, Korf H, Brys L, Berberof M, et al. Identification of a parasitic immunomodulatory protein triggering the development of suppressive M1 macrophages during African trypanosomiasis. *J Infect Dis* (2009) 200:1849–60. doi:10.1086/648374
113. Uzonna JE, Kaushik RS, Zhang Y, Gordon JR, Tabel H. Experimental murine *Trypanosoma congolense* infections. II. Role of splenic adherent CD3+Thy1.2+ TCR-alpha beta gamma delta- CD4+8- and CD3+Thy1.2+ TCR-alpha beta gamma delta- CD4-8-cells in the production of IL-4, IL-10, and IFN-gamma and in trypanosom. *J Immunol* (1998) 161:6189–97.
114. Taylor K, Lutje V, Mertens B. Nitric oxide synthesis is depressed in *Bos indicus* cattle infected with *Trypanosoma congolense* and *Trypanosoma vivax* and does not mediate T-cell suppression. *Infect Immun* (1996) 64:4115–22.
115. Cnops J, De Trez C, Stijlemans B, Keirsse J, Kauffmann F, Barkhuizen M, et al. NK-, NKT- and CD8-derived IFNγ drives myeloid cell activation and erythrophagocytosis, resulting in trypanosomosis-associated acute anemia. *PLoS Pathog* (2015) 11:e1004964. doi:10.1371/journal.ppat.1004964
116. Taylor KA, Lutje V, Kennedy D, Authié E, Boulangé A, Logan-Henfrey L, et al. *Trypanosoma congolense*: B-lymphocyte responses differ between trypanotolerant and trypanosusceptible cattle. *Exp Parasitol* (1996) 83:106–16. doi:10.1006/expr.1996.0054
117. Kondo M. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunol Rev* (2010) 238:37–46. doi:10.1111/j.1600-065X.2010.00963.x
118. Boller S, Grosschedl R. The regulatory network of B-cell differentiation: a focused view of early B-cell factor 1 function. *Immunol Rev* (2014) 261:102–15. doi:10.1111/imr.12206
119. Reth M, Nielsen P. Signaling circuits in early B-cell development. *Adv Immunol* (2014) 122:129–75. doi:10.1016/B978-0-12-800267-4.00004-3
120. Mårtensson I-L, Almqvist N, Grimsholm O, Bernardi AI. The pre-B cell receptor checkpoint. *FEBS Lett* (2010) 584:2572–9. doi:10.1016/j.febslet.2010.04.057
121. Lebien TW, Tedder TF. B lymphocytes: how they develop and function. *Blood* (2008) 112:1570–80. doi:10.1182/blood-2008-02-078071
122. Allman D, Pillai S. Peripheral B cell subsets. *Curr Opin Immunol* (2008) 20:149–57. doi:10.1016/j.coi.2008.03.014
123. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. *Nat Rev Immunol* (2015) 15:160–71. doi:10.1038/nri3795
124. Bockstal V, Guirnalda P, Caljon G, Goenka R, Telfer JC, Frenkel D, et al. *T. brucei* infection reduces B lymphopoiesis in bone marrow and truncates compensatory splenic lymphopoiesis through transitional B-cell apoptosis. *PLoS Pathog* (2011) 7:e1002089. doi:10.1371/journal.ppat.1002089
125. Obishakin E, de Trez C, Magez S. Chronic *Trypanosoma congolense* infections in mice cause a sustained disruption of the B-cell homeostasis in the bone marrow and spleen. *Parasite Immunol* (2014) 36:187–98. doi:10.1111/pim.12099
126. Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S. Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. *PLoS Pathog* (2008) 4:e1000078. doi:10.1371/journal.ppat.1000078
127. Chamond N, Cosson A, Blom-Potar MC, Jouvion G, D'Archivio S, Medina M, et al. *Trypanosoma vivax* infections: pushing ahead with mouse models for the study of Nagana. I. Parasitological, hematological and pathological parameters. *PLoS Negl Trop Dis* (2010) 4:e792. doi:10.1371/journal.pntd.0000792
128. Holland WG, Do T'T, Huong N'T, Dung N'T, Thanh NG, Vercruysse J, et al. The effect of *Trypanosoma evansi* infection on pig performance and vaccination against classical swine fever. *Vet Parasitol* (2003) 111:115–23. doi:10.1016/S0304-4017(02)00363-1
129. Holland WG, My LN, Dung TV, Thanh NG, Tam PT, Vercruysse J, et al. The influence of *T. evansi* infection on the immuno-responsiveness of experimentally infected water buffaloes. *Vet Parasitol* (2001) 102:225–34. doi:10.1016/S0304-4017(01)00534-9
130. Sharpe RT, Langley AM, Mowat GN, Macaskill JA, Holmes PH. Immunosuppression in bovine trypanosomiasis: response of cattle infected with *Trypanosoma congolense* to foot-and-mouth disease vaccination and subsequent live virus challenge. *Res Vet Sci* (1982) 32:289–93.
131. De Trez C, Katsandegwaza B, Caljon G, Magez S. Experimental African trypanosome infection by needle passage or natural tsetse fly challenge thwarts the development of collagen-induced arthritis in DBA/1 prone mice via an impairment of antigen specific B cell autoantibody titers. *PLoS One* (2015) 10:e0130431. doi:10.1371/journal.pone.0130431
132. Cnops J, Kauffmann F, De Trez C, Baltz T, Keirsse J, Radwanska M, et al. Maintenance of B cells during chronic murine *Trypanosoma brucei gambiense* infection. *Parasite Immunol* (2016) 38:642–7. doi:10.1111/pim.12344
133. Lejon V, Mumba Ngoyi D, Kestens L, Boel L, Barbé B, Kande Betu V, et al. Gambiense human African trypanosomiasis and immunological memory: effect on phenotypic lymphocyte profiles and humoral immunity. *PLoS Pathog* (2014) 10:e1003947. doi:10.1371/journal.ppat.1003947
134. Cnops J, De Trez C, Bulte D, Radwanska M, Ryffel B, Magez S. IFN-gamma mediates early B-cell loss in experimental African trypanosomosis. *Parasite Immunol* (2015) 37:479–84. doi:10.1111/pim.12208
135. Frenkel D, Zhang F, Guirnalda P, Haynes C, Bockstal V, Radwanska M, et al. *Trypanosoma brucei* co-opts NK cells to kill splenic B2 B cells. *PLoS Pathog* (2016) 12:e1005733. doi:10.1371/journal.ppat.1005733
136. Cnops J, Bockstal V, De Trez C, Miquel MC, Radwanska M, Magez S. Curative drug treatment of trypanosomosis leads to the restoration of B-cell lymphopoiesis and splenic B-cell compartments. *Parasite Immunol* (2015) 37:485–91. doi:10.1111/pim.12209
137. Kuriakose S, Muleme HM, Onyilagha C, Singh R, Jia P, Uzonna JE. Diminazene aceturate (Berenil) modulates the host cellular and inflammatory responses to *Trypanosoma congolense* infection. *PLoS One* (2012) 7:e48696. doi:10.1371/journal.pone.0048696
138. Wellde BT, Preston JM, Kovatch RM, Higgs J, Chumo DA. *Trypanosoma congolense*: erythrocyte indices, plasma iron turnover and effects of treatment in infected cattle. *Ann Trop Med Parasitol* (1989) 83(Suppl 1):201–6. doi:10.1080/00034983.1989.11812425
139. Stijlemans B, Brys L, Korf H, Bieniasz-Krzywiec P, Sparkes A, Vansintjan L, et al. MIF-mediated hemodilution promotes pathogenic anemia in experimental African trypanosomosis. *PLoS Pathog* (2016) 12:e1005862. doi:10.1371/journal.ppat.1005862
140. Magez S, Radwanska M. African trypanosomiasis and antibodies: implications for vaccination, therapy and diagnosis. *Future Microbiol* (2009) 4:1075–87. doi:10.2217/fmb.09.65
141. La Greca F, Magez S. Vaccination against trypanosomiasis: can it be done or is the trypanosome truly the ultimate immune destroyer and escape artist? *Hum Vaccin* (2011) 7:1225–33. doi:10.4161/hv.7.11.18203
142. Stijlemans B, Baral TN, Guilliams M, Brys L, Korf J, Drennan M, et al. A glycosylphosphatidylinositol-based treatment alleviates trypanosomiasis-associated immunopathology. *J Immunol* (2007) 179:4003–14. doi:10.4049/jimmunol.179.6.4003
143. Authié E, Boulangé A, Muteti D, Lalmanach G, Gauthier F, Musoke AJ. Immunisation of cattle with cysteine proteinases of *Trypanosoma congolense*: targetting the disease rather than the parasite. *Int J Parasitol* (2001) 31:1429–33. doi:10.1016/S0020-7519(01)00266-1
144. Lalmanach G, Boulange A, Serveau C, Lecaille F, Scharfstein J, Gauthier F, et al. Congopain from *Trypanosoma congolense*: drug target and vaccine candidate. *Biol Chem* (2002) 383:739–49. doi:10.1515/BC.2002.077
145. Authie E, Duvallet G, Robertson C, Williams DJ. Antibody responses to a 33 kDa cysteine protease of *Trypanosoma congolense*: relationship to "trypanotolerance" in cattle. *Parasite Immunol* (1993) 15:465–74. doi:10.1111/j.1365-3024.1993.tb00632.x
146. Taylor KA, Lutje V, Kennedy D, Authie E, Boulange A, Logan-Henfrey L, et al. *Trypanosoma congolense*: B-lymphocyte responses differ between trypanotolerant and trypanosusceptible cattle. *Exp Parasitol* (1996) 83:106–16. doi:10.1006/expr.1996.0054
147. Boulange AF, Khamadi SA, Pillay D, Coetzer TH, Authie E. Production of congopain, the major cysteine protease of *Trypanosoma (Nannomonas) congolense*, in *Pichia pastoris* reveals unexpected dimerisation at physiological pH. *Protein Expr Purif* (2011) 75:95–103. doi:10.1016/j.pep.2010.09.002

148. Lanca AS, de Sousa KP, Atouguia J, Prazeres DM, Monteiro GA, Silva MS. *Trypanosoma brucei*: immunisation with plasmid DNA encoding invariant surface glycoprotein gene is able to induce partial protection in experimental African trypanosomiasis. *Exp Parasitol* (2011) 127:18–24. doi:10.1016/j.exppara.2010.06.017
149. Dagenais TR, Demick KP, Bangs JD, Forest KT, Paulnock DM, Mansfield JM. T-cell responses to the trypanosome variant surface glycoprotein are not limited to hypervariable subregions. *Infect Immun* (2009) 77:141–51. doi:10.1128/IAI.00729-08
150. Radwanska M, Magez S, Dumont N, Pays A, Nolan D, Pays E. Antibodies raised against the flagellar pocket fraction of *Trypanosoma brucei* preferentially recognize HSP60 in cDNA expression library. *Parasite Immunol* (2000) 22:639–50. doi:10.1046/j.1365-3024.2000.00348.x
151. Berthier D, Chantal I, Thevenon S, Sakande H, Maillard J-C, Bengaly Z, et al. Study of bovine trypanotolerance by whole transcriptome analysis. *Ann N Y Acad Sci* (2008) 1149:71–6. doi:10.1196/annals.1428.062
152. O'Gorman GM, Park SD, Hill EW, Meade KG, Coussens PM, Agaba M, et al. Transcriptional profiling of cattle infected with *Trypanosoma congolense* highlights gene expression signatures underlying trypanotolerance and trypanosusceptibility. *BMC Genomics* (2009) 10:207. doi:10.1186/1471-2164-10-207
153. Auty H, Torr SJ, Michoel T, Jayaraman S, Morrison LJ. Cattle trypanosomosis: the diversity of trypanosomes and implications for disease epidemiology and control. *Rev Sci Tech* (2015) 34:587–98. doi:10.20506/rst.34.2.2382
154. Mulindwa J, Mercé C, Matovu E, Enyaru J, Clayton C. Transcriptomes of newly-isolated *Trypanosoma brucei* rhodesiense reveal hundreds of mRNAs that are co-regulated with stumpy-form markers. *BMC Genomics* (2015) 16:1118. doi:10.1186/s12864-015-2338-y
155. Telleria EL, Benoit JB, Zhao X, Savage AF, Regmi S, e Silva TLA, et al. Insights into the trypanosome-host interactions revealed through transcriptomic analysis of parasitized tsetse fly salivary glands. *PLoS Negl Trop Dis* (2014) 8:e2649. doi:10.1371/journal.pntd.0002649
156. Ng DH, Skehel JJ, Kassiotis G, Langhorne J. Recovery of an antiviral antibody response following attrition caused by unrelated infection. *PLoS Pathog* (2014) 10:e1003843. doi:10.1371/journal.ppat.1003843
157. Bockstal V, Geurts N, Magez S. Acute disruption of bone marrow B lymphopoiesis and apoptosis of transitional and marginal zone B cells in the spleen following a blood-stage plasmodium chabaudi infection in mice. *J Parasitol Res* (2011) 2011:11. doi:10.1155/2011/534697
158. Keitany GJ, Kim KS, Krishnamurty AT, Hondowicz BD, Hahn WO, Dambrauskas N, et al. Blood stage malaria disrupts humoral immunity to the pre-erythrocytic stage circumsporozoite protein. *Cell Rep* (2016) 17:3193–205. doi:10.1016/j.celrep.2016.11.060
159. Onyilagha C, Jia P, Jayachandran N, Hou S, Okwor I, Kuriakose S, et al. The B cell adaptor molecule Bam32 is critically important for optimal antibody response and resistance to *Trypanosoma congolense* infection in mice. *PLoS Negl Trop Dis* (2015) 9:e0003716. doi:10.1371/journal.pntd.0003716
160. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, et al. MIF signal transduction initiated by binding to CD74. *J Exp Med* (2003) 197:1467–76. doi:10.1084/jem.20030286
161. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol* (2003) 3:791–800. doi:10.1038/nri1200
162. Stijlemans B, Leng L, Brys L, Sparkes A, Vansintjan L, Caljon G, et al. MIF contributes to *Trypanosoma brucei* associated immunopathogenicity development. *PLoS Pathog* (2014) 10:e1004414. doi:10.1371/journal.ppat.1004414
163. Klasen C, Ohl K, Sternkopf M, Shachar I, Schmitz C, Heussen N, et al. MIF promotes B cell chemotaxis through the receptors CXCR4 and CD74 and ZAP-70 signaling. *J Immunol* (2014) 192:5273–84. doi:10.4049/jimmunol.1302209
164. Alampour-Rajabi S, El Bounkari O, Rot A, Müller-Newen G, Bachelerie F, Gawaz M, et al. MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis. *FASEB J* (2015) 29:4497–511. doi:10.1096/fj.15-273904

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Canine Macrophage DH82 Cell Line As a Model to Study Susceptibility to *Trypanosoma cruzi* Infection

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Trypanosoma cruzi is an obligatory intracellular protozoan parasite, and it is the etiological agent of Chagas' disease that is endemic in the Americas. In addition to humans, a wide spectrum of mammals can be infected by *T. cruzi*, including dogs. Dogs develop acute and chronic disease, similar to human infection. *T. cruzi* can infect almost all cell types and after cell invasion, the metacyclics trypomastigotes localize in the cytoplasm, where they transform into amastigotes, the replicative form of *T. cruzi* in mammals. After amastigote multiplication and differentiation, parasites lyse host cells and spread through the body by blood circulation. In this work, we evaluated the *in vitro* ability of *T. cruzi* to infect a canine macrophage cell line DH82 compared with RAW264.7, a murine tissue culture macrophage. Our results have shown that the *T. cruzi* is able to infect, replicate and differentiate in DH82 cell line. We observed that following treatment with LPS and IFN-γ DH82 cells were more resistant to infection and that resistance was not related reactive oxygen species production in our system. In this study, we also found that DH82 cells became more susceptible to *T. cruzi* infection when cocultured with apoptotic cells. The analysis of cytokine production has showed elevated levels of the TGF-β, IL-10, and TNF-α produced by *T. cruzi*-infected canine macrophages. Additionally, we demonstrated a reduced expression of the MHC class II and CD80 by infected DH82 cell line.

Keywords: *Trypanosoma cruzi*, chagas disease, dogs, macrophages, immunomodulation, infection model, susceptibility

INTRODUCTION

Chagas' disease is caused by the protozoan hemoflagellate *Trypanosoma cruzi*, which is transmitted by a blood-sucking reduviid bugs (1). Although it was described more than 100 years ago, this disease remains a serious public health problem in South and Central America countries, which come to account for 12 million to 14 million of infected people (2).

The *T. cruzi* infection begins when the infective metacyclic trypomastigotes forms are transmitted to humans or other mammals by the infected feces of triatomine bugs. Trypomastigotes are able to invade cells, phagocytic or non-phagocytic, differentiate, and multiply in almost all mammalian nucleated cells, including macrophages, muscle cells, glial cells, neurons, fibroblasts, adipocytes, and endothelial cells. After cell invasion trypomastigotes transform into amastigotes, that multiply

intensively, then transform into trypomastigotes and finally break the host-infected cells. After reaching the bloodstream and lymphatic vessels, blood trypomastigotes can invade other tissues, penetrate the cells, and differentiate again into amastigotes, forming new multiplying foci. Due to the cyclical sequence of these events, a rapid increase of circulating trypomastigotes is observed. Thus, during the acute phase of Chagas' disease parasitemia is readily detectable by fresh blood examination. After the acute phase, there is a latent phase of infection, also called indeterminate phase, which can last for long periods of time or can be permanent, then develops into a chronic stage (3).

Trypanosoma cruzi can infect a wide range of wild and domestic mammals that serve as parasite reservoirs. Dogs play an important role in domestic cycle of infection representing a risk factor for humans (4, 5). Naturally infected dogs or seropositive dogs were detected in the United States (6), Mexico, Argentina, Venezuela, and Panama (7, 8). In Brazil, the presence of infected animals was also reported in different areas of the country (5, 9). The canine Chagas' disease is becoming a major veterinarian concern in the Americas (8).

Trypanosoma cruzi-infected dogs reproduce several aspects of the infection observed in humans. In the acute phase of infection, dogs develop lymphadenopathy and hepatosplenomegaly and show circulating blood trypomastigotes (10). In animals that progress to the chronic phase of infection, cardiac involvement with cardiomegaly occurs (11). Recently, it was reported that seropositive dogs exhibit high levels of IgG that correlates with the severity of myocarditis, characterized by mononuclear cell infiltrates (12). In experimentally infected dogs of Beagle breed, it was observed high levels of proinflammatory cytokines such as INF-γ, TNF-α, and low IL-10 levels in the acute phase of infection. These data suggest that the development of chronic heart abnormalities may be related to a strong Th1-type response during the acute phase (10). Using Beagle dogs infected by two distinct genetic groups of parasites, Guedes and coworkers (10) showed that the cardiac type 1/2 chemokines and their receptors expression depend on the genetic diversity of parasites that can determine the migration of Th1 or Th2 cells to the heart during the acute and chronic phases of the disease. In addition, they have showed that infected dogs that develop the cardiac form of disease, have increased expression of CCL5, CCL4, and CXCR3 type 1 chemokines receptors when compared with dogs in the indeterminate phase of the disease (13).

Trypanosoma cruzi invasion strategy demonstrates its ability to subvert the macrophage antimicrobial defense mechanisms. The trypomastigotes interact with host cells by inducing a signal that results in lysosomes recruitment in a Ca^{+}-dependent manner. Initially, parasites enter the cells and are located inside parasitophorous vacuoles, at that time *T. cruzi* releases a hemolysin called TcTox to able escape into the host cell cytoplasm (14).

The intracellular signaling of macrophages can be triggered by the innate immunity receptor is also explored by *T. cruzi*. Parasite-derived molecules, like trypomastigote-derived glycosylphosphatidylinositol anchors of mucin-like glycoproteins, can be recognized by toll-like receptors (TLR2) and initiate a cascade of signals that induces the production of IL-12, TNF-α, and nitric oxide (NO) (15–17). The *T. cruzi* GPI-mucin can induce a state of tolerance phenomena in macrophages *in vitro,* similar to that observed when macrophages were stimulated by LPS (18). These researchers showed that macrophages stimulated with low doses of LPS have a lock response to a second stimulus with LPS. This resulted in suppression of IL-12 and TNF-α production, while the production of NO and IL-10 was not affected.

These mechanisms of tolerance induced by endotoxin interfere with the signaling pathways mediated by TLR4, resulting in decreased activation of MAPK and the p50 subunit NFκB. It has been shown that stimulation of macrophages by LPS then mucin-GPI *T. cruzi* produces a tolerogenic effect associated with defects in phosphorylation of IRAK-1, MAPK activation, and degradation IκB (19). An important aspect of these data is the fact that TLR activation can lead to suppression of another TLR, resulting in blocking the activation of macrophages (20). Thus, the interaction between the parasite and the host cell could modulate the activation process that favors the invasion and persistence of the parasite.

Besides, to directly affect macrophages, infection with *T. cruzi* can produce a modulating effect through the deleterious events affecting other cell populations. An important feature observed during experimental infection with *T. cruzi* is an intense $CD4^{+}$ T cell death by apoptosis (21). The interaction of macrophages with apoptotic bodies leads to TGF-β production that induces the deactivation of macrophages making it more susceptible to infection (22).

Various studies have used cell culture systems derived from mammals to understand the interaction between the host and different *T. cruzi* strains. Spleen and peritoneal macrophages obtained from a variety of mouse strains have been widely used to study *T. cruzi* infectivity *in vitro.* Although the importance of dogs as domestic reservoir, as a model for testing of vaccines and new drugs for the treatment of Chagas' disease is recognized, there is a lack of experimental studies with dog macrophages. Here we have investigated the *in vitro* infectivity rate of trypomastigotes and the replication of amastigotes of *T. cruzi* Dm28c in dog DH82 and mouse RAW264.7 tissue culture macrophages. The DH82 cell is a line isolated from a dog presenting malignant histiocytosis, this lineage shows macrophages morphology and is excellent phagocytic cells. Also, express in the cell surface Fc-gamma receptor and are negative for the C3b receptor (23).

In the present study, we demonstrated that DH82 cells are susceptible to *T. cruzi* infection and the parasites were able to modulate expression of the MHC class II, CD80, and cytokines production.

MATERIALS AND METHODS

Cell Culture

To prepare tissue culture cells for infection, DH82 and RAW264.7 cells (ATCC) were grown in 75 cm^2 tissue culture flasks (T75) (Nunc, Roskilde, Denmark) with Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Gibco), 100 μg/mL of streptomycin and 100 Units/mL of penicillin, 1% MEM non-essential amino acids (Sigma-Aldrich), and 2 mM of L-glutamine were cultured at

37°C with 5% CO_2. Subcultures of both cell lines were carried out once a week, when they reached a confluence of 95–100%. Cells were released with 0.25% of trypsin (Difco) and 1 mM of EDTA (Sigma-Aldrich), harvested, and washed twice in HBSS by centrifugation at room temperature for 10 min at 250*g*. The supernatant was discarded, cell pellet was resuspended in DMEM containing 10% FCS, cultured into new T75 flasks, and culture medium was changed after 3 days in culture. Prior to use in infectivity assays, cells' viability was assessed by trypan blue exclusion in a hemocytometer.

Parasites and Infection

Infection of DH82 and RAW264.7 cell lines were carried out in 24-well plates at a concentration of 2.0×10^5 macrophages/well. Cells were infected overnight with chemically induced metacyclic trypomastigotes forms of *T. cruzi* clone Dm28c, obtained as previously described (24), at a 1:1, 3:1, and 5:1 parasite-to-cell ratios in 1 mL of DMEM 10% FCS and incubated at 37°C in 5% CO_2. In the following day (day 1), monolayers were extensively washed to remove extracellular parasites and cultured with complete culture medium containing 1% Nutridoma (Sigma-Aldrich) instead of FCS. Some cultures were stimulated with 400 ng/mL of lipopolysaccharides from *Salmonella enterica* serotype typhimurium (LPS, Sigma-Aldrich) and 1.5 ng/mL of recombinant canine or murine IFN-γ (Serotech) 24 h after infection. DH82 and RAW264.7 were also seeded in 24-well plate containing glass coverslips for parasite burden evaluation. Three days after infection, coverslips were washed with HBSS, fixed with methanol, and stained with Diff-Quick (Thermo Fisher, Waltham, MA, USA). Amastigotes were counted at 100× oil immersion on a (Olympus) microscope. The number of amastigotes was estimated in 100 infected cells per coverslip, and the frequency of infection was compared among six coverslips per time point. The number of viable trypomastigotes released in the supernatants was evaluated each 2 days of culture for 9 days, using a Neubauer chamber. We also conducted experiments using *T. cruzi* of CL strain transfected with green fluorescent protein (GFP) (25) obtained from Vero cells. The DH82 cells were infected overnight (ON). After that, the monolayers were washed, and DH82 cells were stained with DAPI 1.5 μg/mL (VECTASHIELD®) at room temperature for 30 min before examination by confocal microscopy Zeiss Axioplan.

Detection of Reactive Oxygen Species (ROS)

Intracellular levels of ROS were quantified by oxydation of non-fluorescent 2′,7′-dichlorofluorescein probe, delivered as diacetate form (DCFH-DA), to the fluorescent product 2′,7′-dichlorofluorescein (26). DH82 cells were seeded in 96-well plate (5.0×10^4 cells/well), infected with metacyclic trypomastigotes at a ratio of 5:1 (parasite/cell), stimulated or not with LPS (400 ng/mL, Sigma-Aldrich) and recombinant IFN-γ (1.5 ng/mL, Serotech) and after 1, 2, 3, and 4 h later cells were washed and loaded with 10 μM DCFH-DA (Invitrogen) for 20 min at 37°C. Uninfected cells were used as negative control. In different checkpoints, the cells were washed, and fluorescence was measured (excitation = 485 nm excitation; λ emission = 535 nm) in an FLx800 Fluorescence Microplate Reader (BioTek).

Detection of Nitric Oxide (NO)

Quantification of NO DH82 and RAW264.7 (2.5×10^5/mL) was cultivated in the presence of LPS (200 or 400 ng/mL) and INF-γ (1.5 ng/mL). After 12 and 24 h of incubation, NO production was evaluated by the presence of the nitrite accumulated in the supernatant of cultures using Griess colorimetric method.

Apoptotic Lymphocytes and Coculture with DH82

In order to evaluate the effect of apoptotic lymphocytes in *T. cruzi* growth *in vitro*, we have used two approaches. In both approaches, DH82 macrophages were seeded into 24-well plate at a concentration of 2.0×10^5 cells/well, incubated for 1 h at 37°C in a humidified atmosphere with 5% CO_2 and the adherent monolayer was washed to remove non-adherent cells. Apoptotic cells were prepared as previously described (27). Briefly, single cell suspension of the Jurkat human lymphoblastoid cells was prepared and irradiated with 30 Gy (Apo-2) as previous described (27). We first performed experiments with *T. cruzi*-infected DH82 monolayers at a 5:1 parasite/cell ratio. Extracellular parasites were removed by washing the wells with HBSS and then apoptotic lymphocytes were added to the cultures at a 5:1 apoptotic lymphocyte:macrophage ratio, and kept at 37°C with 5% CO_2 for 9 days. In other experiments, prior to *T. cruzi* infection, the DH82 monolayer were incubated with apoptotic lymphocytes at a 5:1 apoptotic lymphocyte:macrophage ratio for 2 h at 37°C with 5% CO_2. After that period non-internalized apoptotic cells were removed and adherent macrophages were infected with *T. cruzi* at 5:1 parasite:macrophage ratio for ON at 37°C. After removing free parasites, the medium was replaced and infected DH82 cells were further incubated at 37°C with 5% CO_2. In both experiments, the extracellular motile trypomastigotes were determined in cell supernatants after 9 days in culture by counting parasites in Neubauer chambers (28).

Cytokine Determination

Cell supernatants were collected at 24 h postinfection for cytokine determination. TNF-α, TGF-β, and IL-10 concentration was estimated by the method of sandwich immunoassay (ELISA) according to methodology recommended by the manufacturer (R&D). The optical density was evaluated by reading in a microplate spectrophotometer (Versamax Microplates Reader Molecular Devices, USA), with filter of 405 nm.

Flow Cytometric Analysis

To assess the expression of MHC class II and CD80 on DH82 macrophage cell line by flow cytometric analysis, cells were infected or not with *T. cruzi* Dm28c and were activated or not in the presence of recombinant IFN-γ and LPS. Cells were detached at 24 h postinfection, washed, adjusted to a concentration of 5.0×10^5 cells/tube and blocked with anti-CD16/CD32 (Calbiochem) at concentration of the 1 μg/10^6 cells to prevent non-specific antibody binding to Fc-receptors. Cells were stained with MHC

class II-FITC, and CD80-PE antibodies (Serotech). All washing steps were performed with phosphate-buffered saline containing 3% bovine serum albumin and 0.02% of sodium azide. Data were acquired (10,000 events), evaluated on FACSCalibur™ cytometer, and analyzed using Cell-Quest® software (BD Biosciences, Heidelberg, Germany). Analyzed gates of scattered dot plots were adjusted not to exceed 2% of positive staining cells related to the particular negative controls (29).

Statistical Analysis

Statistical analysis was performed in the program GraphPad InStat version 3.01 (San Diego, USA). Data were analyzed by the method of Student's *t*-test. Differences at *p*-value 0.05 or lower were reported as significant for a given comparison.

RESULTS

DH82 Canine Macrophages Are Infected by *T. cruzi*

Previously published studies report *T. cruzi* infection of various mouse macrophage cell lines to study the infectivity of *T. cruzi* strains *in vitro* (30), but infection of dog macrophages has not been showed. In order to evaluate the susceptibility of DH82, a canine macrophage cell line, to *T. cruzi* Dm 28c infection, cells were plated and infected at different parasite:cell ratios. We found that canine macrophages were infected and after 5 days in culture parasite intracellular multiplication was observed (**Figures 1A–C**). Analyzing the rate of infected cells, we found that 33% of DH82 cells were infected at a parasite-to-cell ratio of 5:1. (**Figure 1D**). After 7 days in culture a large number of trypomastigotes were counted in the supernatants (**Figures 1E,F**). To further confirm DH82 macrophage cell line infection, we have also used *T. cruzi* of CL strain transfected with GFP and observed the adhesion, internalization (data not shown) and differentiation into intracellular amastigotes 5 days after infection (**Figure 1C**). Our results showed, for the first time, that the DH82, a canine macrophage cell line is susceptible to infection with *T. cruzi*, being able to provide an enabling environment for replication and differentiation of the parasite and the release of viable infective forms.

Canine Macrophages DH82 Are More Susceptible to Infection by *T. cruzi* than Murine Macrophage RAW264.7

Since we have found that dog DH82 tissue culture macrophages could be infected with *T. cruzi* Dm28c, we decided to compare the rate of infection, the replication of amastigotes, and the number of trypomastigotes released in cultures of dog DH82 and mouse RAW264.7 macrophages. For this purpose, DH82 cells and RAW264.7 cells were infected at a parasite/cell ratio of 10:1 and cultivated for 9 days. At the first 3 days of infection, motile trypomastigotes forms were observed in supernatants of DH82 macrophages cell line, but not in RAW264.7 cells. After 8 to 10 days postinfection, it was observed a higher number of trypomastigotes in DH82 culture supernatants than in RAW264.7. It is important to point out that canine DH82 cells infected with *T. cruzi* were able to support the infection for the entire infection period. In contrast, *T. cruzi* replication inside murine macrophage RAW264.7 cells died 7 days after infection whereas the DH82 cells showed loss of viable morphology after 9 days of infection. These findings indicate that DH82 cells are more susceptible to infection by *T. cruzi* than RAW264.7 cell line (**Figure 2**).

Activation of the DH82 Macrophage Decrease the Infectivity of *T. cruzi*

The outcome of infection is determined by the activation status of macrophages. In the present study, we have used classical macrophage activators such as LPS and INF-γ. Therefore, DH82 cells were plated, pretreated or not with LPS or LPS + IFN-γ, and were infected with *T. cruzi*. Following incubation for 9 days, the trypomastigotes forms released in the supernatants were counted. We demonstrated that LPS stimulated macrophages showed a reduced parasite burden in 50%. Furthermore, we demonstrated that pretreatment of mouse macrophages with LPS and IFN-γ resulted in low levels of macrophage infection by *T. cruzi,* suggesting that previous macrophage stimulation was able to effectively control parasite replication (**Figure 3**).

Reactive Species of Oxygen Produced by DH82 Macrophages Is Not Responsible for the Decrease to Infection

Since ROS production is a crucial event associated with *in vitro* microbicidal activity in macrophages (17), we measured the production of ROS to evaluate if it could interfere in intracellular parasite replication of DH82 cells infected by *T. cruzi*. The amount of ROS produced was measured in cultures that were infected or not, in the presence or absence of LPS and IFN-γ. Increased ROS production was similar in activated and non-activated infected cells as well as in uninfected and activated cells (**Figure 4**). Taking into consideration that stimulation of DH82 canine macrophages with LPS and IFN-γ restricted intracellular replication and ROS production was elevated, associated with the fact that ROS release was also observed in *T. cruzi*-infected and unactivated DH82 cells at similar level suggests that, in our system, ROS is not involved in limiting parasite replication inside DH82 cells infected by *T. cruzi.*

Profile of Cytokines of DH82 Cells after Infection with *T. cruzi*

Cytokines play a fundamental role in mediating interaction between macrophages, T lymphocytes, and other cells from the immune system in order to establish an immune response to control disease progression or in maintaining a persistent infection. Depending on the type of cytokines produced, intracellular parasites can be eliminated or can favor its permanence in the infected host (31). Here, we analyzed the TNF-α, TGF-β, and IL-10 cytokines production in the supernatants of *T. cruzi*-infected DH82 cells after overnight stimulation or not with LPS and IFN-γ. Our results showed that secretion of TNF-α production was detected in *T. cruzi*-infected DH82 macrophages at similar levels observed in stimulated

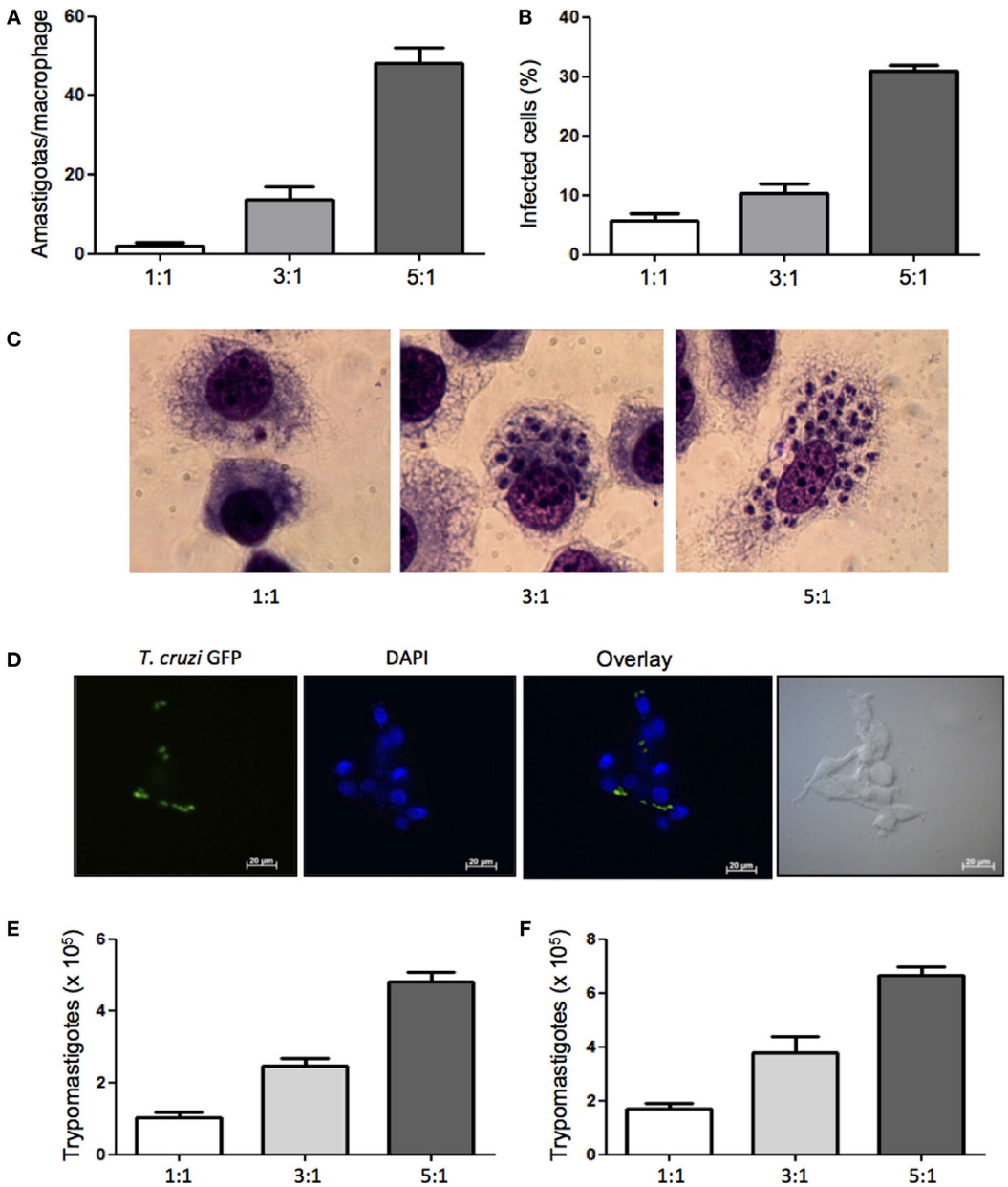

FIGURE 1 | **Infectivity of *Trypanosoma cruzi* in canine macrophage DH82 cell line**. DH82 cells were cultured (2.5 × 10^5/mL) with trypomastigotes forms of *T. cruzi* Dm28c clone. After overnight incubation, the cell culture was washed, and phagocyte was cultured for another 3 days with Dulbecco's Modified Eagle Medium (DMEM) at 37°C. After this period, cells were stained and amastigotes inside the macrophages were counted under the light microscope **(A)** and set the percentage of infected cells **(B)**. Infected macrophage displaying amastigotes after 3 days of infection with *T. cruzi* staining with Diff-Quick **(C)** and infection with CL strain green fluorescent protein (GFP) **(D)** observed by confocal microscope. To quantify trypomastigotes forms in the supernatants, the cells were infected with trypomastigotes forms of *T. cruzi* Dm28c clone. After overnight incubation the cell culture was washed and phagocyte were cultured for another 9 days with DMEM at 37°C. The trypomastigotes forms were quantified in the supernatant of the cultures of infected DH82 macrophages after 7 days **(E)** and 9 days **(F)**. All cultures were performed in triplicate and bars show the mean + SD.

macrophages (**Figure 5A**). However, with stimulation by LPS and IFN-γ, infected cells exhibited TNF-α production and reached higher levels as seen in **Figure 5A**. With regard to IL-10, maximal production was also detected in stimulated and infected DH82 macrophages (**Figure 5C**). LPS and IFN-γ treatment of DH82 resulted in TGF-β secretion but *T. cruzi*-infected and stimulated macrophages triggered a substantially higher level of release TGF-β in DH82 cell than was observed with in *T. cruzi*-infected macrophages only (**Figure 5B**). The increased TGF-β production observed in infected DH82 cells indicate that TGF-β might be involved in the success of DH82 cell infection by *T. cruzi*.

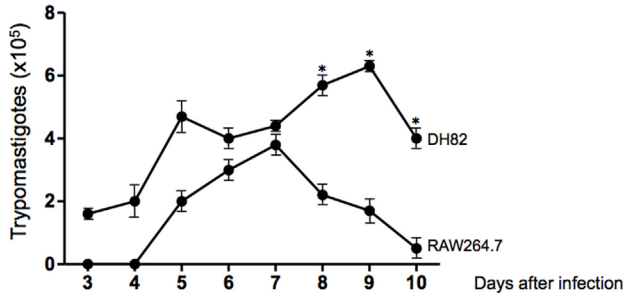

FIGURE 2 | Curve of growth of *Trypanosoma cruzi* in DH82 and RAW264.7 cell line. DH82 and RAW 264.7 macrophages were cultured (2.5×10^5/mL) with trypomastigotes forms of *T. cruzi* Dm28c clone. After overnight incubation, the cell culture was washed, and phagocyte was cultured for another 3 days with Dulbecco's Modified Eagle Medium (DMEM) at 37°C. After 3 days, we start the quantification of the trypomastigotes forms in the supernatants of the cultures. All cultures were performed in triplicate and bars show the mean + SD. Statistical analysis was performed by *t*-test from representative results of three similar experiments ($^*p \leq 0.05$).

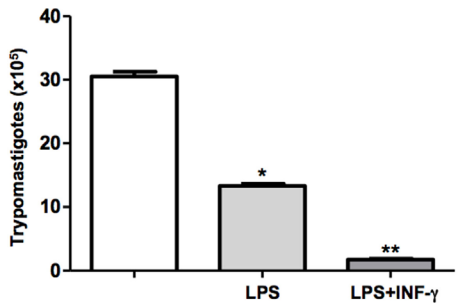

FIGURE 3 | Activated DH82 macrophages decreased trypomastigotes release. DH82 macrophages were cultured (2.5×10^5/mL). The macrophages were infected with *Trypanosoma cruzi* Dm28c clone. Some cultures were stimulated with LPS (400 ng/mL) and INF-γ (1.5 ng/mL) for 24 h. After 9 days of infection was quantified the number of the trypomastigotes forms in the supernatants of the cultures. All cultures were performed in triplicate and bars show the mean + SD. Statistical analysis was performed by *t*-test from representative results of three similar experiments ($^*p \leq 0.05$).

MHC Class II and CD80 Are Modulated after Infection

The ability to express MHC class II and CD80 on surface confer to macrophage the status of antigen-presenting cell (APC). To be efficient APC during infection is very important to maintain unchanged the expression levels of these molecules (32). In order to study possible alterations resulting from *T. cruzi* infection that could lead to any modification in these molecules expression, DH82 canine macrophage cell line was infected or not and cultured in the presence or not of some activating factors, such as LPS and IFN-γ. After 24 h in culture, cells were harvested and labeled with monoclonal antibodies directed against MHC-II and CD80 and the expression was evaluated by flow cytometry analysis. The results showed that upon stimulation, the levels of surface MHC-II molecules increased but when DH82 macrophages were infected with *T. cruzi* the cells expressed lower levels of MHC II (**Figures 6A,B**). Upregulated surface expression of CD80 was also observed in DH82 stimulated and uninfected cells. On the other hand, low level of CD80 expression could be detected in *T. cruzi*-infected cells (**Figures 6C,D**). These data suggest that parasite infection inhibit the ability of DH82 canine macrophage cells to up regulate the expression of both molecules crucial to act as effective APCs.

Taken together, our results indicate that the DH82 canine macrophage cell line is susceptible to infection by *T. cruzi*, able to sustain the infection for long time when compare with RAW264.7, a murine macrophage cell line, support replication and differentiation to the parasite, culminating with the release of the infective forms that can be used as *in vitro* study to explore parasite-host interaction. Furthermore, the infection of activated cells induced production of cytokines known as deactivating factors for macrophage like TGF-β and IL-10, that when associated with decreased expression of MHC II and CD80 represent a powerful mechanism to modulate parasite growth in this cell line.

Interaction between DH2 and Apoptotic Cells Increases Susceptibility to *T. cruzi* Infection

Phagocytosis of apoptotic cells by *T. cruzi*-infected macrophages has the potential to down modulate macrophages leading to an

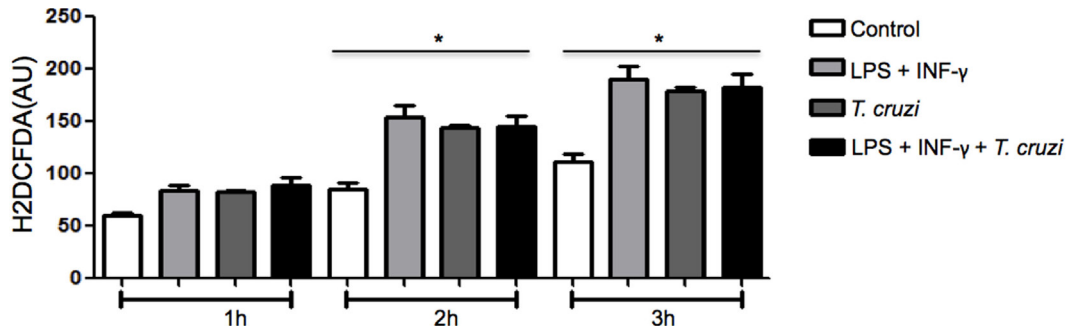

FIGURE 4 | The production of reactive species of oxygen (ROS) does not affect the infectivity of *Trypanosoma cruzi* in DH82 cells. DH82 macrophages were cultured (5.0×10^4/mL) and incubated with H2DCFDA, followed by washing and some cultures were stimulated with LPS (400 ng/mL) or LPS and INF-γ (1.5 ng/mL) in indicated time. All cultures were performed in triplicate are shown as the mean arbitrary fluorescence units (AU) + SD. Statistical analysis was performed by *t*-test from representative results of three similar experiments ($^*p \leq 0.05$).

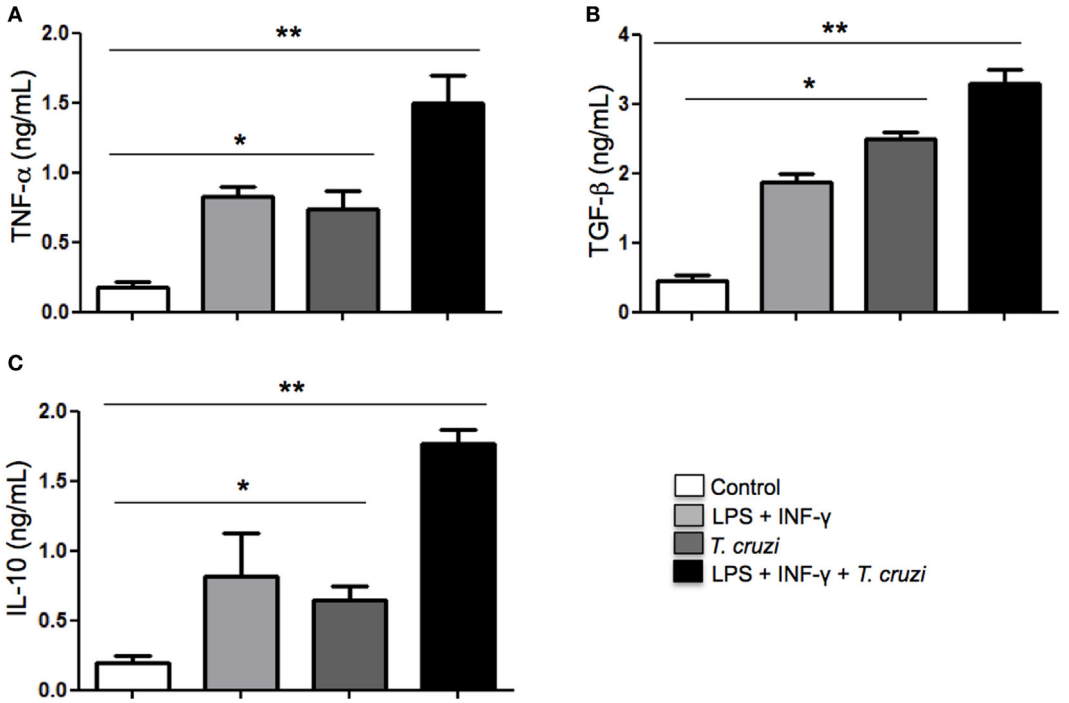

FIGURE 5 | Modulation of cytokines production in DH82 cells by *Trypanosoma cruzi* infection. DH82 macrophages (2.5×10^5/mL) were infected with trypomastigotes forms of *T. cruzi* Dm28c clone. After infection the cells were washed and some cultures were stimulated with LPS (400 ng/mL) and INF-γ (1.5 ng/mL). After 24 h, the supernatant was collected and TNF-α **(A)**, TGF-β **(B)**, and IL-10 **(C)** were measured by ELISA. All cultures were performed in triplicate and bars show the mean + SD. Statistical analysis was performed by *t*-test from representative results of three similar experiments ($^*p \leq 0.05$, $^{**}p \leq 0.01$).

anti-inflammatory response that results in increased parasite replication (22). In this context, we tested the modulatory effect of apoptotic cells on *T. cruzi*-infected DH82 canine macrophages. DH82 cell monolayers were cocultivated with apoptotic Jurkat T cells before or after infection with *T. cruzi*. As seen in **Figure 4**, the quantification of viable parasites released in the supernatants was made 9 days after infection. Cultures that received apoptotic cells before or after *T. cruzi* infection showed a significant increase in the number of released parasites (**Figure 7**), indicating similar modulation as previously described for mouse peritoneal macrophages.

DISCUSSION

Different cultured cells from mammal origin have been extensively used *in vitro* to study host-parasite interaction (33). Various studies have described the use of murine macrophage cell lines to analyze the infection of *T. cruzi* strains *in vitro* (34, 35) but infections of canine macrophages have not been evaluated. In the present work, we investigated whether the canine macrophage cell line DH82 is susceptible to *T. cruzi* infection *in vitro.* To determine the DH82 cells susceptibility to *T. cruzi* Dm 28c infection, macrophages monolayers were infected with metacyclic trypomastigotes differentiated *in vitro.* Our results showed that DH82 cells experienced infection rate of 30%. We found that trypomastigotes differentiated in amastigotes forms, replicated, and transform in trypomastigote before being released in cultures supernatants. To compare intracellular parasitism of *T. cruzi* in macrophages from a different host we also used mouse RAW264.7 tissue cultured macrophages. Microscopically, trypomastigotes free in the supernatants were detected earlier in DH82 than in RAW264.7 cells, as well as the number of parasites was higher in canine than in mouse macrophages. We also noted that DH82 macrophages were able to support infection for a prolonged period of time. This finding favors the use of DH82 cells as a model to study the *in vitro* interaction between *T. cruzi* and canine macrophages. The current study is the first to describe that DH82 cells are infected by *T. cruzi*, reproduce intracellular parasite replication, transformation into trypomastigotes and can be used as model to investigate host-pathogen interaction *in vitro* or can contribute in experiments to identify new drugs for the treatment of Chagas' disease.

In the presence of the cytokines or bacterial products such as LPS or CPG DNA, macrophages exhibited an increase in NO and ROS production that represent a key defensive element in various infectious disease (36). In *in vitro* models of macrophages infection by *T. cruzi,* the resistance to infection is closely associated with activation and consequent increase in the microbicidal activity of macrophages (37, 38). Our results showed that resistance of the DH82 cells to *T. cruzi* infection was increased after stimulation, indicating that pretreatment with classical activators can modulate these macrophages. An opposite effect was seen when *T. cruzi*-infected DH82 cells were cocultured with apoptotic lymphocytes. After interact with apoptotic cells

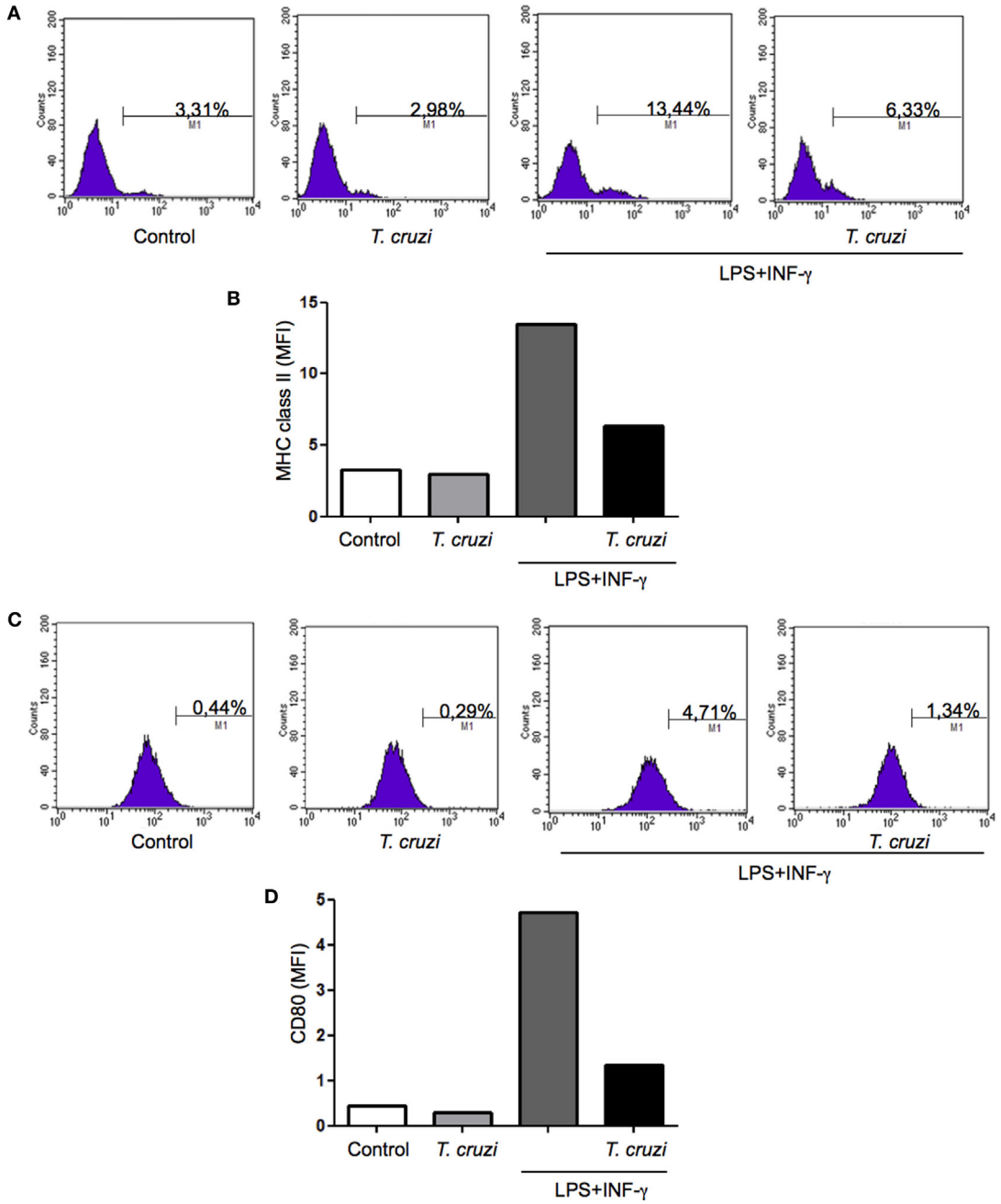

FIGURE 6 | Measurement of MHC class II and CD80 expression in infected DH82 by *Trypanosoma cruzi*. DH82 macrophages (5.0×10^5/mL) were infected with trypomastigotes forms of *T. cruzi* Dm28c clone. After infection the cells were washed and some cultures were stimulated with LPS (400 ng/mL) and INF-γ (1.5 ng/mL). After 24 h, the cells were prepared for analysis by flow cytometry. The M1 marker shows the percentage of cells identified by MHC calss II **(A,B)** or CD80 **(C,D)**. The data are representative of three independent experiments.

canine macrophages became more susceptible to infection, independently if apoptotic lymphocytes were added to the culture before or after infection. Interaction with apoptotic cells lead macrophages to secrete TGF-β, which in turn suppresses their proinflammatory cytokine response. In addition, TGF-β renders both phagocytic and non-phagocytic cells permissive to *T. cruzi* infection (39) and antagonizes INF-γ-induced NO production and macrophage trypanocidal activity (22). Based on these, ours results suggest that canine macrophage DH82 can be driven to the resistance or susceptibility depending on stimulus received that might increase their microbicidal activity or become permissive to cell invasion.

Since our results showed that DH82 cells become more resistant to infection after being stimulated with LPS and

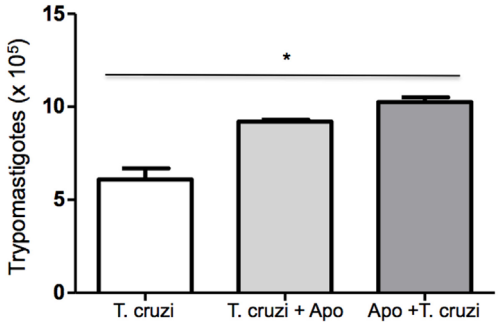

FIGURE 7 | Coculture of apoptotic cells with DH82 cells exacerbates infection by *Trypanosoma cruzi*. DH82 macrophages (2.5×10^5/mL) were exposed to apoptotic T cells (five apoptotic cells for each DH82) 1 h before infection (Apo + *T. cruzi*) or infected and exposed to apoptotic T cells (*T. cruzi* + Apo). After 9 days, the trypomastigotes forms in the supernatants were counted. All cultures were performed in triplicate and bars show the mean + SD. Statistical analysis was performed by *t*-test from representative results of three similar experiments (*$p \leq 0.05$).

IFN-γ, we investigated which molecules could be involved. We attempted to quantify NO production; however, the cells were not able to increase NO even after stimulation with LPS and INF-γ (Figure S1 in Supplementary Material). Similar results were described in a previous study (40) that shown unchanged levels in NO production by DH82 cells. On the other hand, ROS production by canine macrophages was evident after stimulation. In our model, *T. cruzi*-infected cells were capable of producing ROS at similar levels detected in stimulated cells only and the production was markedly enhanced in the presence of LPS + IFN-γ. Despite the production of high levels of ROS by *T. cruzi*-infected DH82 macrophages, intracellular parasite replication was not effectively affected. It is noteworthy to mention that a recent report showed that to withstand such variable sources of oxidative stress, *T. cruzi* has developed complex defense mechanisms. This includes ROS detoxification pathways that are distinct from the ones in the mammalian host, DNA repair pathways and specialized polymerases, which not only protect its genome from the resulting oxidative damage but also contribute to the generation of genetic diversity within the parasite population (41).

When stimulated DH82 macrophages were infected, we detected a strong increase in TNF-α production accompanied by TGF-β and IL-10. This profile suggests that DH82 canine macrophages infected by *T. cruzi* are modulated to produce anti-inflammatory cytokines and this phenomenon can be increased in stimulated cells. In fact, high levels of TGF-β were detected in stimulated and infected cells, as well as in uninfected cells. TGF-β is an important anti-inflammatory mediator and participates in the metabolism of L-arginine, which favors the production of arginase and is involved in the activity of ornithine decarboxylase. The intracellular stage of *T. cruzi* requires this host cell enzyme and the synthesis of polyamines to multiply inside the cell (42). We suggest that this mechanism is involved in the successful infection of DH82 canine macrophage by *T. cruzi*.

Protozoan parasites such as *T. cruzi* can modulate the function of dendritic cells by interfering with recognition, migration, and antigen presentation (43). It is known that *T. cruzi* inhibits the expression of MHC II, CD40, CD80, and CD86, hampers the production and secretion of IL-12, TNF-α, and IL-6, increases the production of the cytokine IL-10, and inhibits antigen presentation in murine and human dendritic cells *in vitro* (44). Corroborating the *in vitro* findings, *in vivo* experiments have also demonstrated that *T. cruzi* is able to impair many aspects of dendritic cells biology. Additionally during acute infection, splenic dendritic cells migration and expression of the CD86 are inhibited in infected mice with *T. cruzi* (45). Interestingly, the same correlation was observed in our study with DH82 canine macrophages. These cells showed a reduction in MHC II and CD80 expression following infection with *T. cruzi*. The MHC II and CD80 expression was increased in LPS and IFN-γ stimulated cells, while infection by *T. cruzi* affected the ability of previously activated cells to increase MHC II and CD80 expression in DH82 cells. Taken together, our results reinforce the relevance of *T. cruzi* infection in modulating canine macrophages and the role in the infection establishment and disease progression. In addition, our results suggest that canine macrophages DH82 are more susceptible to infection by *T. cruzi* when compared with RAW264.7 murine macrophages.

AUTHOR CONTRIBUTIONS

DD-R, CGF-de-L, and PHBM conceived and designed the experiments. PHBM, RFDBR, JBBM, IFLRF, and JL performed the experiments. DD-R, CGF-de-L, MPN, and AM analyzed the data. DD-R and CGF-de-L wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00604/full#supplementary-material.

FIGURE S1 | DH82 canine macrophage do not upregulate nitric oxide. RAW264.7 and DH82 macrophages were cultured (2.5×10^5/mL) in the absence or presence of LPS (200 or 400 ng/mL) and INF-γ (1.5 ng/mL). After 12 and 24 h of incubation, NO production was evaluated by Griess colorimetric method. All cultures were performed in triplicate and bars show the mean + SD. Statistical analysis was performed by *t*-test from representative results of three similar experiments (***$p \leq 0.0001$).

REFERENCES

1. Rodrigues Coura J, De Castro SL. A critical review on Chagas disease chemotherapy. *Mem Inst Oswaldo Cruz* (2002) 97(1):3–24. doi:10.1590/S0074-02762002000100001
2. Rocha MO, Teixeira MM, Ribeiro AL. An update on the management of Chagas cardiomyopathy. *Expert Rev Anti Ifect Ther* (2007) 5(4):727–43. doi:10.1586/14787210.5.4.727
3. Rassi A Jr, Rassi A, Marin-Neto JA. Chagas disease. *Lancet* (2010) 375(9723):1388–402. doi:10.1016/S0140-6736(10)60061-X
4. Cohen JE, Gurtler RE. Modeling household transmission of American trypanosomiasis. *Science* (2001) 293(5530):694–8. doi:10.1126/science.1060638
5. Xavier SC, Roque AL, Lima VS, Monteiro KJ, Otaviano JC, Ferreira, et al. Lower richness of small wild mammal species and Chagas disease risk. *PLoS Negl Trop Dis* (2012) 6(5):e1647. doi:10.1371/journal.pntd.0001647
6. Bradley KK, Bergman DK, Woods JP, Crutcher JM, Kirchhoff LV. Prevalence of American trypanosomiasis (Chagas disease) among dogs in Oklahoma. *J Am Vet Med Assoc* (2000) 217(12):1853–7. doi:10.2460/javma.2000.217.1853
7. Estrada-Franco JG, Bhatia V, Diaz-Albiter H, Ochoa-Garcia L, Barbosa A, Vazquez-Chagoyan JC, et al. Human *Tryapanosoma cruzi* infection and seropositivity in dogs, México. *Emerg Infect Dis* (2006) 12(4):624–30. doi:10.3201/eid1204.050450
8. Esch KJ, Petersen CA. Transmission and epidemiology of zoonotic protozoal diseases of companion animals. *Clin Microbiol Rev* (2013) 26(1):58–85. doi:10.1128/CMR.00067-12
9. Pinto AGS, Schubach TMP, Figueiredo FB, Baptista C, Fagundes A, Barros JHS, et al. Isolation of *Trypanosoma caninum* in domestic dogs in Rio de Janeiro, Brazil. *Parasitology* (2010) 137(11):1653–60. doi:10.1017/S0031182010000417
10. Guedes PMM, Veloso VM, Afonso LCC, Caliari MV, Carneiro CM, Diniz LF, et al. Development of chronic cardiomyopathy in canine Chagas disease correlates with high INF-γ, TNF-α, and low IL-10 production during the acute infection phase. *Vet Immunol Immunopathol* (2009) 130(1–2):43–52. doi:10.1016/j.vetimm.2009.01.004
11. Montenegro VM, Jimenez M, Pinto-Dias JC, Zeledon R. Chagas disease in dogs from endemic areas of Costa Rica. *Mem Inst Oswaldo Cruz* (2002) 97(4):491–4. doi:10.1590/S0074-02762002000400006
12. Cruz-Chan JV, Bolio-González M, Colín-Flores R, Ramirez-Sierra MJ, Quijano-Hernandez I, Dumonteli E. Immunopathology of natural infection with *Trypanosoma cruzi* in dogs. *Vet Parasitol* (2009) 162(1–2):151–5. doi:10.1016/j.vetpar.2009.02.024
13. Guedes PMM, Veloso, Talvini A, Diniz LF, Caldas IS, Do Vale Mata MA, et al. Increased type 1 chemokine expression in experimental Chagas disease correlates with cardiac pathology in Beagles dogs. *Vet Immunol Immunopathol* (2010) 138(1–2):106–13. doi:10.1016/j.vetimm.2010.06.010
14. Idone V, Tam C, Goss JW, Toomore D, Pypaert M, Andrews NW. Repair of injured plasma membrane by rapid $Ca2^{+}$-dependent endocytosis. *J Cell Biol* (2008) 180(5):905–14. doi:10.1083/jcb.200708010
15. Gazzinelli RT, Denkers EY. Protozoan encounters with toll-like receptor signaling pathways: implications for host parasitism. *Nat Rev Immunol* (2006) 6(12):895–906. doi:10.1038/nri1978
16. Kayama H, Takeda K. The innate immune response to *Trypanosoma cruzi* infection. *Microbes Infect* (2010) 12(7):511–7. doi:10.1016/j.micinf.2010.03.005
17. Gomes JA, Campi-Azevedo AC, Teixeira-Carvalho A, Silveira-Lemos D, Vitelli-Avelar D, Sathler-Oliveira R, et al. Impaired phagocytic capacity driven by downregulation of major phagocytosis-related cell surface molecules elicits and overall modulatory cytokine profile in neutrophils and monocytes from the indeterminate clinical form of Chagas disease. *Immunobiology* (2012) 217(10):1006–16. doi:10.1016/j.imbio.2012.01.014
18. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxina-tolerized mouse macrophages: dysregulation of cytokine, chemokine and toll-like receptor 2 and 4 gene expression. *J Immunol* (2000) 164(11):5564–74. doi:10.4049/jimmunol.164.11.5564
19. Ropert C, Closel M, Chaves AC, Gazzinelli RT. Inhibition of a p38/stress-activation protein kinase-2-dependent phosphatase restores function of IL-1 receptor-associate kinase-1 and reverses toll-like receptor 2-and 4-dependent tolerance of macrophages. *J Immunol* (2003) 171(3):1456–65. doi:10.4049/jimmunol.171.3.1456
20. Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. *Trends Immunol* (2003) 24(7):358–63. doi:10.1016/S1471-4906(03)00139-X
21. Lopes MF, da Veiga VF, Santos AR, Fonseca ME, DosReis GA. Activation-induced CD4+ T cell death by apoptosis in experimental Chagas disease. *J Immunol* (1995) 154(2):744–52.
22. Freire de Lima CG, Nascimento DO, Soares MB, Bozza P, Castro-Faria-Neto HC, DosReis AG, et al. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* (2000) 403(6766):199–203. doi:10.1038/35003208
23. Heinrich F, Contioso VB, Stein VM, Carlson R, Tipold A, Reiner U, et al. Passage-dependent morphological and phenotypical changes of a canine histiocytic sarcoma cell line (DH82). *Vet Immunol Immunopathol* (2015) 163(2015):86–92. doi:10.1016/j.vetimm.2014.11.006
24. Contreras VT, Salles JM, Thomas N, Morel CM, Goldenberg S. *In vitro* differentiation of *Trypanosoma cruzi* under chemically defined conditions. *Mol Biochem Parasitol* (1985) 16(3):315–27. doi:10.1016/0166-6851(85)90073-8
25. Gluenz E, Taylor MC, Kelly JM. The *Trypanosoma cruzi* metacyclic-specific protein Met-III associates with the nucleolus and contains independent amino and carboxyl terminal targeting elements. *Int J Parasitol* (2007) 37(6):617–25. doi:10.1016/j.ijpara.2006.11.016
26. Sandaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* (1995) 270(5234):296–9. doi:10.1126/science.270.5234.296
27. Griffith TS, Yu X, Herndon JM, Green DR, Ferguson TA. CD95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance. *Immunity* (1996) 5(1):7–16. doi:10.1016/S1074-7613(00)80305-2
28. Nunes MP, Andrade RM, Lopes MF, DosReis GA. Activation-induced T cell death exacerbates *Trypanosoma cruzi* replication in macrophages cocultured with $CD4^{+}$ T lymphocytes from infected hosts. *J Immunol* (1998) 160(3):1313–9.
29. Stein VM, Czub M, Hansen R, Leibold W, Moore PF, Zurbriggen A, et al. Characterization of canine microglial cells isolated *ex vivo*. *Vet Immunol Immunopathol* (2004) 99(1–2):73–85. doi:10.1016/j.vetimm.2004.01.002
30. Buscaglia CA, Di Noia JM. *Trypanosoma cruzi* clonal diversity and the epidemiology of Chagas disease. *Microbes Infect* (2003) 5(5):419–27. doi:10.1016/S1286-4579(03)00050-9
31. Mege JL, Capo C. Is macrophage polarization the Gordian knot of bacterial infection? *Med Sci (Paris)* (2010) 26(1):83–8. doi:10.1051/medsci/201026183
32. Knowlton ER, Lepone LM, Li J, Rappocciolo G, Jenkins FJ, Rinaldo CR. Professional antigen presenting cells in human herpevirus 8 infection. *Front Immunol* (2013) 3(427):1–17. doi:10.3389/fimmu.2012.00427
33. Martínez-Díaz RA, Escario JA, Nogal-Ruiz JJ, Gómez-Barrio A. Biological characterization of *Trypanosoma cruzi* strains. *Mem Inst Oswaldo Cruz* (2001) 96(1):53–9. doi:10.1590/S0074-02762001000100006
34. De Souza W, De Carvalho TM, Barrias ES. Review on *Trypanosoma cruzi*: host cell interaction. *Int J Cell Biol* (2010) 2010:295394. doi:10.1155/2010/295394
35. Junqueira C, Caetano B, Bartholomeu DC, Melo MB, Ropert C, Rodrigues MM, et al. The endless race between *Trypanosoma cruzi* and host immunity:lessons for beyond Chagas disease. *Expert Rev Mol Med* (2010) 12:e29. doi:10.1017/S1462399410001560
36. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* (2003) 3(1):23–35. doi:10.1038/nri978
37. Garrido VV, Dulgerian LR, Stempin CC, Cerbán FM. The increase in mannose receptor recycling favors arginase induction and *Trypanosoma cruzi* survival in macrophages. *Int J Biol Sci* (2011) 7(9):1257–72. doi:10.7150/ijbs.7.1257
38. Vázquez A, Ruiz-Rosado JD, Terrazas LI, Juárez I, Gomez-Garcia L, Calleja E, et al. Mouse macrophage galactose-type lectin (mMGL) is critical for host resistance against *Trypanosoma cruzi* infection. *Int J Biol Sci* (2014) 10(8):909–20. doi:10.7150/ijbs.9214
39. Silva JS, Twardzik DR, Reed SG. Regulation of *Trypanosoma cruzi* infection *in vitro* and *in vivo* by transforming growth factor beta (TGF-beta). *J Exp Med* (1991) 174(3):539–45. doi:10.1084/jem.174.3.539

40. Wasserman J, Diese L, VanGundy Z, London C, Carson WE, Papenfuss TL. Suppression of canine myeloid cells by soluble factors from cultured canine tumor cells. *Vet Immunol Immunopathol* (2012) 145(1–2):420–30. doi:10.1016/j.vetimm.2011.12.018
41. Machado-Silva A, Cerqueira PG, Grazielle-Silva V, Gadelha FR, Peloso EF, Teixeira SM, et al. How *Trypanosoma cruzi* deals with oxidative stress: antioxidant defence and DNA repair pathways. *Mutat Res Rev Mutat Res* (2016) 767:8–22. doi:10.1016/j.mrrev.2015.12.003
42. Gazzinelli RT, Oswald IP, Hieny S, James SL, Sher A. The microbicidal activity of interferon-gamma-treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. *Eur J Immunol* (1992) 22(10):2501–6. doi:10.1002/eji.1830221006
43. Poncini CV, Soto CDA, Batalla E, Solana ME, Cappa SMG. Trypanosoma cruzi induces regulatory dendritic cells *in vitro*. *Infect Immun* (2008) 76(6):2633–41. doi:10.1128/IAI.01298-07
44. Van Overtvelt L, Vanderheyde N, Verhasselt V, Ismaili J, De Vos L, Goldman M, et al. *Trypanosoma cruzi* infects human dendritic cells and prevents their maturation: inhibition of cytokines, HLA-DR, and costimulatory molecules. *Infect Immun* (1999) 67(8):4033–40.
45. Chaussabel D, Pajak B, Vercruysse V, Bisseyé C, Garzé V, Habib M, et al. Alteration of migration and maturation of dendritic cells and T-cell depletion in the course of experimental *Trypanosoma cruzi* infection. *Lab Invest* (2003) 83(9):1373–82. doi:10.1097/01.LAB.0000087587.93781.6F

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Dependency of B-1 Cells in the Maintenance of Splenic Interleukin-10 Producing Cells and Impairment of Macrophage Resistance in Visceral Leishmaniasis

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Visceral leishmaniasis is a neglected disease caused by *Leishmania* protozoa parasites transmitted by infected sand fly vectors. This disease represents the second in mortality among tropical infections and is associated to a profound immunosuppression state of the host. The hallmark of this infection-induced host immunodeviation is the characteristic high levels of the regulatory interleukin-10 (IL-10) cytokine. In the present study, we investigated the role of B-1 cells in the maintenance of splenic IL-10 levels that could interfere with resistance to parasite infection. Using an experimental murine infection model with *Leishmania (L.) infantum chagasi* we demonstrated an improved resistance of B-1 deficient BALB/XID mice to infection. BALB/XID mice developed a reduced splenomegaly with diminished splenic parasite burden and lower levels of IL-10 secretion of purified splenocytes at 30 days post-infection, as compared to BALB/c wild-type control mice. Interestingly, we found that resident peritoneal macrophages isolated from BALB/XID mice were more effective to control the parasite load in comparison to cells isolated from BALB/c wild-type mice. Our findings point to a role of B-1 cells in the host susceptibility to visceral leishmaniasis.

Keywords: visceral leishmaniasis, *Leishmania (L.) infantum chagasi*, B-1 cells, host protective responses

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INTRODUCTION

Visceral leishmaniasis (VL), also known as Kala Azar is a neglected tropical disease caused by the intracellular protozoan *Leishmania donovani* and *Leishmania (L.) infantum chagasi* parasites (Kaye and Scott, 2011). Over 90% of the annual incidence of new cases occurs in Bangladesh, India, Nepal, Sudan, South Sudan, Ethiopia, and Brazil. In these countries, the outbreaks and prevalence of infection, from which are reported clinical cases, differ in their eco-epidemiology and sand fly vectors involved. This disease is fatal if not treated, and can kill between 20,000 and 40,000 people a year worldwide.

The treatment is often performed on the basis of pentavalent antimony compounds and amphotericin B lipid formulations, and its symptoms include: hepatosplenomegaly, fever, anemia, weight loss, and hyperglobulinemia (Kaye and Scott, 2011; Matlashewski et al., 2011; McCall et al., 2013; Ready, 2014).

The immune system works as a crucial barrier in the hosts to the establishment of natural infections. The initial steps of a immune response against *Leishmania* infection is triggered from the activation of innate receptors pattern recognition receptors (PRRs) by molecules associated with pathogens (MMAPs) such as lipophosphoglycans, glycoinositolphospholipid, and metalloproteinase GP63, all expressed on parasite cell surface (Liu and Uzonna, 2012). Activation of PRRs is crucial for induction of interleukin-12 (IL-12) by antigen presenting cells necessary to promote the secretion of interferon-gamma (IFN-γ) by $CD4^+$ T lymphocytes and natural killer cells. IFN-γ is a type-1 pro-inflammatory cytokine extremely important to activate the microbicidal activity of macrophages, the major reservoir of *Leishmania* parasites. Once activated, macrophages are able to secrete reactive oxygen species (ROS) and nitric oxide (NO), both involved in the destruction of parasites (Kaye and Scott, 2011; Liu and Uzonna, 2012).

In natural and experimental VL infection, cell-mediated immune responses are suppressed causing a decrease in IFN-γ levels. This subversion of the immune response is associated with production of regulatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), associated with the progression of disease (Kumar and Nylén, 2012). In human infection, significantly higher levels of IL-10 produced by regulatory T cells are present in patients that do not respond to chemotherapeutic treatment, suggesting an important role of this cytokine in the suppression of host immunity during disease (Guha et al., 2014). Increased levels of IL-10 negatively modulate innate immunity via macrophage inhibition of ROS and NO expression (Kumar and Nylén, 2012).

The expression of IL-10 is not specific to cells of the innate immune system but also lymphocytes, including B cells that mediate suppressive responses in VL (Murphy et al., 2001; Deak et al., 2010; Gautam et al., 2011; Bankoti et al., 2012). It has been shown that IL-10-derived from B cells is capable to promote the development of suppressive responses associated with susceptibility to infection (Bankoti et al., 2012; Arcanjo et al., 2015). However, the identification of the B cell population involved in the susceptibility to VL is still vague and needs further studies. Recently it has been demonstrated that B-1 cells contribute to susceptibility to infection with *L. (L.) infantum chagasi* (Gonzaga et al., 2015). B-1 cells represent the major population of B lymphocytes in the pleural and peritoneal cavity. These cells are able to secrete high levels of IL-10 that could modulate the phagocytic activity of macrophages (Aziz et al., 2015). The impairment of the mononuclear phagocyte system is a key factor in the disease progression thus contributing to splenic dysfunction and symptoms of splenomegaly (Kaye et al., 2004). In the present study, we aimed to investigate the role of B-1 cells in the resistance of macrophages to *Leishmania* infection.

MATERIALS AND METHODS

Ethics Statement

All mouse studies followed the guidelines set by the National Institutes of Health, United States. The study was approved by the Research Ethics Committee of Federal University of Rio de Janeiro (protocol IMPPG040-07/16). Protocols for animal were approved by the Institutional Ethical Committees in accordance with international guidelines. All animal experimentation was performed in accordance with the terms of the Brazilian guidelines for the animal welfare regulations.

Animals, Infection, and Evaluation of Host Responses

BALB/c wild-type control mice and BALB/XID mice (X-linked BALB/c immunodeficient mice genetically deficient in B-1 cells) originated from breeding colonies kindly donated by Professor Mário Mariano (UNIFESP, Brazil) were maintained in our animal facilities (UFRJ). Experimental infection was performed by inoculating 4- to 8-week-old female BALB/c and B-1 cell-deficient BALB/XID mice intravenously with 5×10^7 *L. (L.) infantum chagasi* amastigotes (IOC-L 3324) obtained from infected hamster spleens. Thirty days after infection, mice were euthanized and the liver and splenic parasite load were evaluated in Giemsa-stained smears and expressed in LDU values (*Leishman–Donovan* units of Stauber = number of amastigotes per 1000 liver cell nuclei/mg of liver weight).

Anti-*Leishmania (L.) infantum chagasi* ELISA

Immunoglobulin isotypes and IgG subtype profiles were monitored by an enzyme-linked immunosorbent assay (ELISA) using the freeze and thawed lysate of stationary phase promastigotes of *L. (L.) infantum chagasi* (MHOM/BR/74/PP/75) as antigen. Whole parasite antigens (2 μg/ml) were plated at 100 μl/well to 96-well plates and, after overnight incubation at 4°C, the plates were washed three times using PBS containing 0.05% (vol/vol) Tween 20 (Sigma, Gillingham, United Kingdom). Serial twofold 1:100 to 1:800 dilutions of serum samples from mice diluted in PBS containing 0.05% Tween were added to the plates and incubated at 37°C for 1 h. Afterward the plates were washed three times with PBS containing 0.05% Tween, and the 1:5,000 dilution of peroxidase-labeled each goat anti-mouse Ig isotypes (Jackson ImmunoResearch, West Grove, United States) were added at 100 μl/well and incubated at 37°C for 1 h. The reaction was developed with 50 mM phosphate/citrate buffer (pH 5.0) containing 2 mM *o*-phenylenediamine HCl and 0.007% (vol/vol) H_2O_2 (Sigma, United Kingdom), and interrupted with the addition of 2 M H_2SO_4 (50 μl/well). The ELISA plates were read at 490 nm (Spectra Max 190, Molecular Devices, Sunnyvale, United States).

Cytokine Assays

Splenocytes (1×10^6/0.5 mL) obtained from control or infected mice at 30 days post-infection (DPI) were cultured in 48 well at

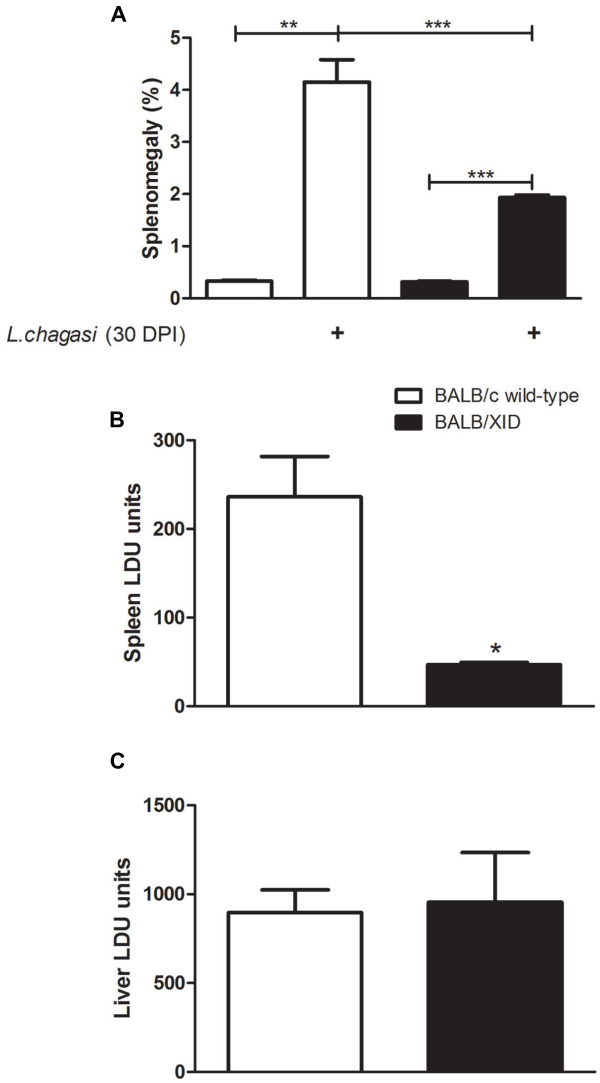

FIGURE 1 | B-1 cell-deficient BALB/XID mice show resistance to visceral leishmaniasis. BALB/c and BALB/XID mice were intravenously injected with 5×10^7 amastigotes of *Leishmania (L.) infantum chagasi*, and 30 days after infection the clinical signs of disease were examined. **(A)** Spleen/body relative weights are attenuated in B-1 cell-deficient BALB/XID mice. The body weight was measured in grams and the spleen/body relative weights (grams of organ weight × 100/grams of body weight) were determined in BALB/c and BALB/XID mice. **(B)** Decreased parasite burden in the spleen of BALB/XID mice. The vertical axis in the histogram represents the average parasite load from spleen or **(C)** liver tissues in Leishman–Donovan units of Stauber (LDU = number of amastigotes/1000 cell nuclei × organ weight in mg) obtained at 30 days post-infection (DPI). Data are means ± SE and represent the results of two independent experiments performed with five to six mice per group. Differences between groups are significant $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

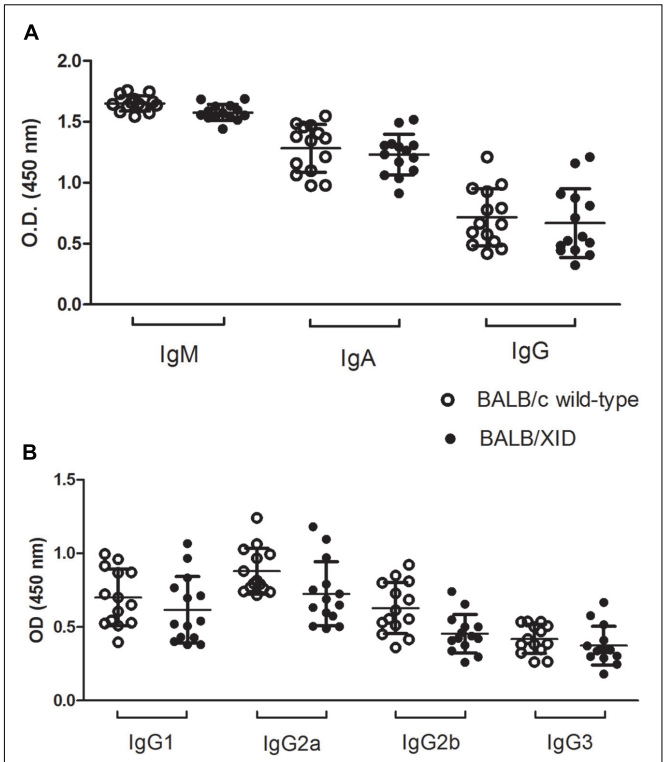

FIGURE 2 | Isotype and IgG subclass profiles of serum antibodies to *Leishmania (L.) infantum chagasi* of B-1 cell-deficient mice. Sera from wild type BALB/c and B-1 cell-deficient BALB/XID mice infected with 5×10^7 amastigote forms of *Leishmania (L.)* infantum chagasi were collected at 30 DPI and the absorbance values of **(A)** antibody Isotypes (IgM, IgA and IgG); and **(B)** IgG antibody subclasses (IgG1, IgG2a, IgG2b and IgG3) were determined by ELISA using *Leishmania (L.) infantum chagasi* promastigote lysates as parasite antigens. Bars show levels of immunoglobulin isotypes and IgG subclasses as the individual absorbancy values of 1/100 diluted sera. Data represent the individual results for each group of mice obtained from two independent experiments.

37°C/5% CO_2 in complete RPMI medium, stimulated or not with 10^6 freeze–thawed stationary phase *L. (L) infantum chagasi* (L579 Fiocruz) promastigotes. After 3 days, supernatants were collected and cytokine levels (IFN-γ, TNF-α, IL-10, and TGF-β) were assayed by ELISA (R&D Systems). Plates were read at 405 nm and values are presented as pg cytokine/mL [mean ± standard error (SE)]. Statistical differences between mean values were evaluated by ANOVA, and pair-wise comparisons were done by the Tukey test.

In Vitro Infection of Macrophages and Detection of ROS

Resident peritoneal macrophages isolated from BALB/c or BALB/XID mice cultured at 1.0×10^5 cells/well in 48-well plates received 10^6 *L. (L.) infantum chagasi* promastigote forms (MHOM/BR/74/PP/75) in the stationary phase at 37°C in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). After 4 h, the cell monolayers were extensively washed for the removal of extracellular parasites, and the cultures were then maintained for 3 days at 37°C. Afterward, infected macrophages were cultured in Schneider medium (Life Technologies) supplemented with 20% FBS at 26°C for an additional 3 days in order to estimate the *L. chagasi* load by counting the promastigotes

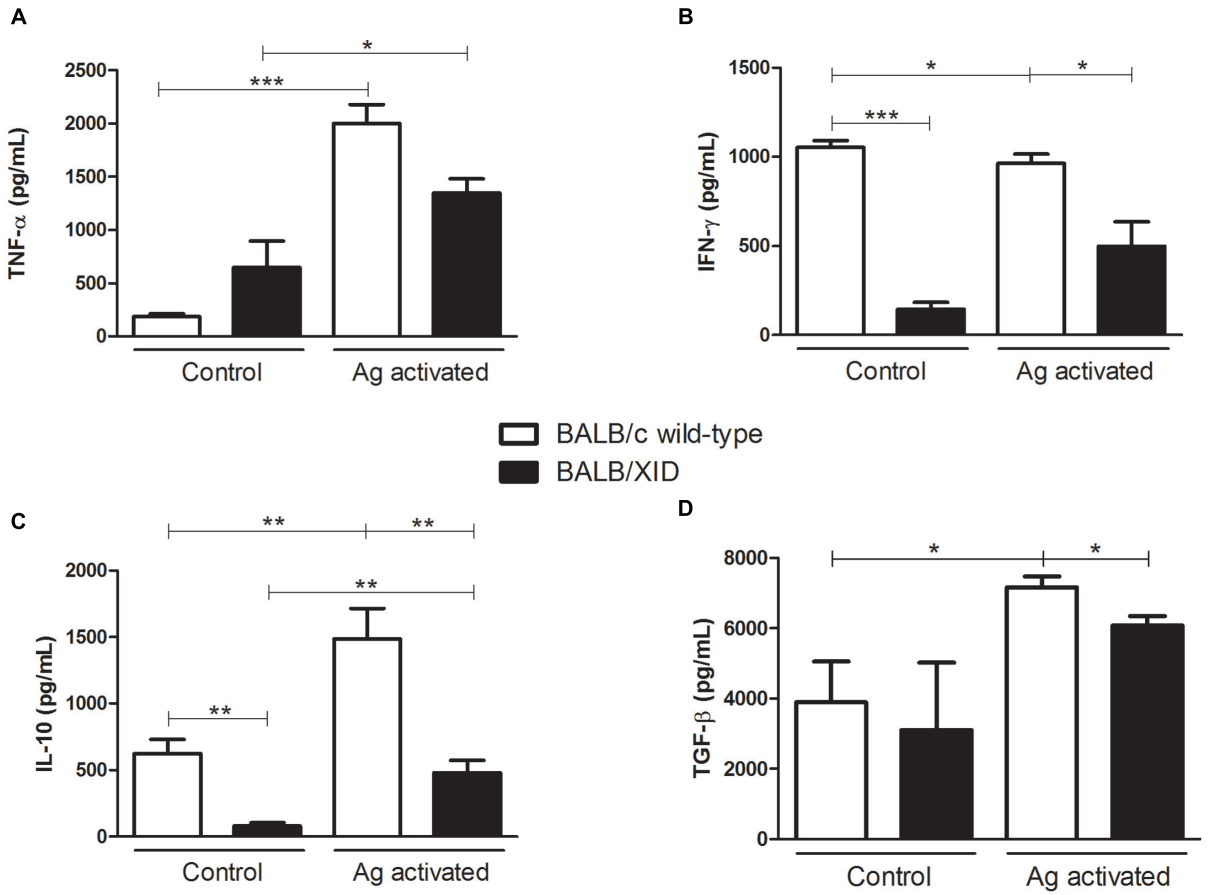

FIGURE 3 | Splenocytes from B-1 cell-deficient BALB/XID mice produce low levels of IL-10. BALB/c and BALB/XID mice were intravenously infected with 5×10^7 amastigotes of *Leishmania (L.) infantum chagasi*, and 30 days after infection splenocytes were isolated and assayed for cytokine production upon parasite antigen (Ag) stimulation. After 3 days of *in vitro* culture with freeze and thawed lysates of *Leishmania (L.) infantum chagasi* promastigotes, as described elsewhere (Popi et al., 2012), splenocyte supernatants were harvested for determination of **(A)** TNF-α, **(B)** IFN-γ, **(C)** IL-10, and **(D)** TGF-β by ELISA. The *y*-axis represents the levels of cytokines, detected by specific ELISA assays, expressed in ng/ml. Data are means ± SE and represent the results of two independent experiments performed with five to six mice per group. Asterisks represent statistical significance between groups $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

forms, as described elsewhere (Ribeiro-Gomes et al., 2004). For detection of ROS, cells were incubated with 10 μM H_2DCFDA probe (Invitrogen) prior to parasite infection and/or activation stimuli (200 ng/ml LPS, 2 ng/ml IFN-γ). A change in fluorescence was assessed with a fluorimeter (Spectramax M3).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 4 software, using one-way ANOVA test. Results were expressed as mean ± SE, differences between control and treated group were considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

In our study, we used a murine model of VL in which infection of BALB/c mice with *L. (L.) infantum chagasi* amastigotes gives rise to a higher parasite load in the first weeks of infection, after which it is controlled by the host immune response. The infection of BALB/c and B-1 cell-deficient BALB/XID mice was performed by inoculating 4- to 8-week-old females intravenously with 5×10^7 *L. (L.) infantum chagasi* amastigotes (IOC-L 3324) obtained from infected hamster spleens. Thirty days after infection, mice were euthanized and the splenic and intrahepatic parasite burdens were evaluated in Giemsa-stained smears and expressed in LDU values. Our results demonstrated an increased resistance of BALB/XID mice correlating with lower increases in the spleen/body weight ratio as compared to BALB/c wild-type control mice (**Figure 1A**). These results were in line with the demonstration of a reduced splenic parasite burden at 30 DPI in B-1 cell-deficient mice (**Figure 1B**). In contrast, infection in both mice groups yielded similar intrahepatic parasite burden indexes 1 month after intravenously inoculation with *L. (L.) infantum chagasi* amastigotes (**Figure 1C**), corroborating previous findings using an infection experimental model with lower parasite dose (Gonzaga et al., 2015). The higher susceptibility of infected BALB/XID mice was not due to

any alteration of antibody-mediated responses as we did not observe any significant change in the immunoglobulin isotypes nor in the IgG subtype profiles from both groups at 30 DPI (**Figure 2**).

A major factor contributing to susceptibility in leishmaniasis is the development of a strong IL-10 response (Murphy et al., 2001; Gautam et al., 2011; Bankoti et al., 2012). We therefore compared the cytokine responses to infection with *L. (L.) infantum chagasi* parasites by analyzing the supernatants of splenocytes isolated from both infected BALB/XID mice and BALB/c wild-type control groups that were stimulated with whole parasite antigens for 3 days. Splenocytes obtained from both infected mice groups at 30 DPI showed increased levels of protective pro-inflammatory response characterized by high levels of TNF-α, when antigen-stimulated (**Figure 3A**). However when compared the ratio of antigen-stimulated/control indexes for the TNF-α values, we observed a higher increase for BALB/c wild-type control groups as compared to BALB/XID mice. Analysis of IFN-γ expression also indicated an increased levels of this pro-inflammatory cytokine upon stimulation of splenocytes with parasite antigens in BALB/XID mice, although basal levels of this cytokine were secreted in the controls of BALB/c wild-type mice (**Figure 3B**). Increased basal levels of IFN-γ may indicate a progressive disease with high parasite load and infection-induced pathology (Goto and Prianti, 2009). In contrast, the splenocytes from infected BALB/XID mice produced lower levels of IL-10 upon stimulation with parasite antigens (**Figure 3C**). The same pattern was obtained for the regulatory TGF-β cytokine, as the splenocytes from infected BALB/XID mice produced slightly lower amounts of TGF-β upon stimulation with parasite antigens in comparison to controls of BALB/c wild-type mice (**Figure 3D**). However, although statistically significant, the differences observed for TGF-β between both mice groups have not yielded striking results.

We next tested the capacity of resident peritoneal macrophages isolated from BALB/XID mice to control de parasite load in comparison to their counterparts isolated from infected BALB/c wild-type mice. In these assays, macrophages were *in vitro* infected with *L. (L.) infantum chagasi* promastigote forms and the parasite load was measured to determine the macrophage resistance to *Leishmania* infection. According to our results, resident peritoneal macrophages obtained from BALB/XID mice were more effective in eliminating *Leishmania* parasites as compared to macrophages isolated from BALB/c wild-type mice (**Figure 4**). The differences in the innate resistance from resident peritoneal macrophages isolated from both mice groups were not due to any chance in the production of ROS, which are important effector agents against intracellular pathogens. Our results showed that *in vitro*-infected IFN-γ/LPS-activated macrophages from both mice groups produced ROS within first hours of infection with *L. (L.) infantum chagasi* promastigotes (**Supplementary Figure S1**). The data point to the interference of inhibitory effect of homeostatic IL-10 produced by B1-cells on the resident peritoneal macrophages derived from BALB/XID mice.

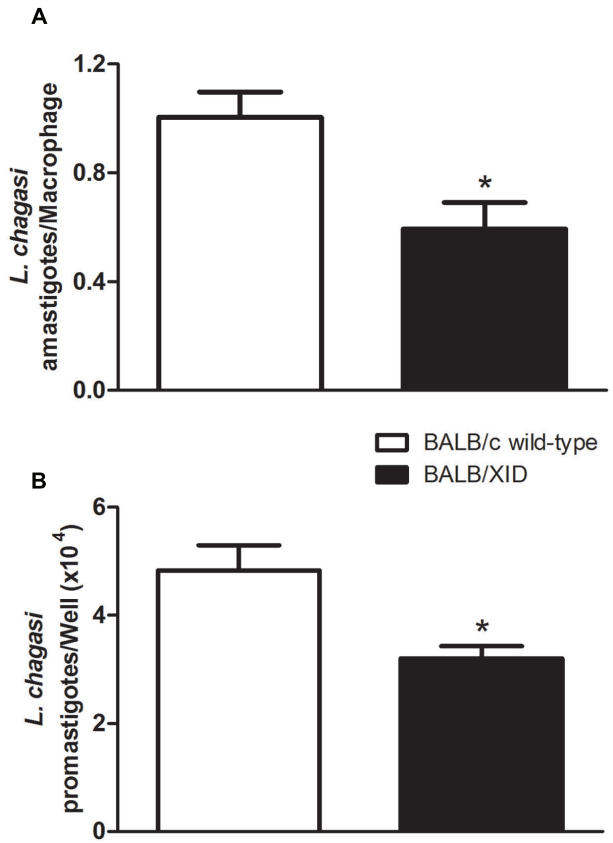

FIGURE 4 | *Leishmania (L.) infantum chagasi* infection of tissue-resident macrophages obtained from BALB/c and BALB/XID mice. Intraperitoneal macrophage cells purified from BALB/c and BALB/XID mice were cultured at 1.0×10^5 cells/well in 48-well plates and infected with 10^6 *Leishmania (L.) infantum chagasi* promastigotes at a 10:1 ratio of parasites:host cells in DMEM containing 10% FBS at 37°C. The infected cells were washed 4 h to remove extracellular parasites and then maintained for 3 days at 37°C to determine the intracellular amastigote load **(A)**. Following intracellular parasitism, infected macrophages were cultured in Schneider medium supplemented with 20% FBS at 26°C for an additional 3 days to estimate the *L. chagasi* load by counting the promastigotes forms derived from released parasites **(B)**. Histograms represent the means ± SE of total number of *Leishmania (L.) infantum chagasi* forms of triplicate assays and are representative from two independent experiments. Differences between groups are significant $^*p < 0.05$.

Taken together, our results clearly indicate that BALB/c mice have an impaired immune response upon infection with *L. (L.) infantum chagasi* as compared to BALB/XID mice. The maintenance of low levels of IL-10 in BALB/XID mice due to the loss of B-1 cells is associated with an improved control of the parasite in spleen tissues and enhanced innate resistance of macrophages to *Leishmania* infection. These results indicate a potentiation of the anti-parasitic activity in B-1 cell-deficient BALB/XID mice. The role of B-1 cells in protective mediated-immunity of the host depends on the nature of the pathogen as well as the infection experimental models (Minoprio et al., 1993; Hoerauf et al., 1994; Babai et al., 1999; Gaubert et al., 1999; Herbert et al., 2002; Popi et al., 2008; Crane et al., 2013;

Szymczak et al., 2013; Gonzaga et al., 2015). Intracelular parasites such as *Trypanosoma cruzi* and *Francisella tularensis* that target macrophage cells are susceptible to negative modulation of the mononuclear phagocyte system by B-1 cells (Minoprio et al., 1993; Crane et al., 2013). In fact these cells can be programmed to differentiate into phagocytes (Popi et al., 2012) that could promote immunosurveillance in the different tissues affected by the parasitism thus contributing to the outcome of infection.

Alternatively, B-1 cells can differentiate into IgM-secreting cells working as an innate-like B cell populations with distinct repertoire and tissue location from the conventional B lymphocytes or B-2 cells (Aziz et al., 2015). Interestingly, it has been proposed that the production and activation of a polyclonal B-lymphocyte responses expressing IgM are the major cause of disease susceptibility in animals infected with *L. infantum* (Deak et al., 2010). In addition, it has been shown that B cells in the marginal zone are able to suppress antigen-specific responses from both $CD8^+$ and $CD4^+$ lymphocytes during the early stages of VL, thereby preventing the generation of protective effector and memory T cells in *L. donovani* infection (Bankoti et al., 2012). The properties of B-1 cells to act as rapid immune responders that promptly migrate and redistribute to secondary lymphoid tissues where they can be resident as cytokine and antibody-secreting differentiated cells (Aziz et al., 2015) could be determinant to the development of infection. Understanding the role of B-1 cells and the control of infection-induced signals that activate these cells in VL could lead to better strategies to control this devastating disease.

AUTHOR CONTRIBUTIONS

AM, CF conceived and designed the experiments. AA, DN, GC, YS, PS performed the experiments. AA, DN, PS, DD, MN, CF and AM analyzed the data. DD, AF, CP and CF contributed reagents/materials/analysis tools. CF and AM wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00978/full#supplementary-material

FIGURE S1 | ROS production during *Leishmania (L.) infantum chagasi* promastigote infection. Resident peritoneal macrophages isolated from BALB/c or BALB/XID mice were infected with *Leishmania (L.) infantum chagasi* promastigote forms (at 1:10 host–parasite ratio. The infection was done in the presence or absence of activation stimuli (200 ng/ml LPS, 2 ng/ml IFN-γ) and the ROS production was measured with fluorometric assay using H_2DCFDA probe. The values represent the mean ± SEM of three independents experiments. Indicated differences between groups are significant $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

REFERENCES

Arcanjo, A. F., La Rocque-de-Freitas, I. F., Rocha, J. D., Zamith, D., Costa-da-Silva, A. C., Nunes, M. P., et al. (2015). The PGE_2/IL-10 axis determines susceptibility of B-1 cell-derived phagocytes (B-1CDP) to *Leishmania major* infection. *PLoS ONE* 10:e0124888. doi: 10.1371/journal.pone.0124888

Aziz, M., Holodick, N. E., Rothstein, T. L., and Wang, P. (2015). The role of B-1 cells in inflammation. *Immunol. Res.* 63, 153–166. doi: 10.1007/s12026-015-8708-3

Babai, B., Louzir, H., Cazenave, P. A., and Dellagi, K. (1999). Depletion of peritoneal CD5+ B cells has no effect on the course of *Leishmania major* infection in susceptible and resistant mice. *Clin. Exp. Immunol.* 117, 123–129. doi: 10.1046/j.1365-2249.1999.00953.x

Bankoti, R., Gupta, K., Levchenko, A., and Stäger, S. (2012). Marginal zone B cells regulate antigen-specific T cell responses during infection. *J. Immunol.* 188, 3961–3971. doi: 10.4049/jimmunol.1102880

Crane, D. D., Griffin, A. J., Wehrly, T. D., and Bosio, C. M. (2013). B1a cells enhance susceptibility to infection with virulent *Francisella tularensis* via modulation of NK/NKT cell responses. *J. Immunol.* 190, 2756–2766. doi: 10.4049/jimmunol.1202697

Deak, E., Jayakumar, A., Cho, K. W., Goldsmith-Pestana, K., Dondji, B., and Lambris, J. D. (2010). Murine visceral leishmaniasis: IgM and polyclonal B-cell activation lead to disease exacerbation. *Eur. J. Immunol.* 40, 1355–1368. doi: 10.1002/eji.200939455

Gaubert, S., Viana da Costa, A., Maurage, C. A., Lima, E. C., Fontaine, J., Lafitte, S., et al. (1999). X-linked immunodeficiency affects the outcome of *Schistosoma mansoni* infection in the murine model. *Parasite Immunol.* 21, 89–101. doi: 10.1046/j.1365-3024.1999.00205.x

Gautam, S., Kumar, R., Maurya, R., Nylén, S., Ansari, N., Rai, M., et al. (2011). IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. *J. Infect. Dis.* 204, 1134–1137. doi: 10.1093/infdis/jir461

Gonzaga, W. F., Xavier, V., Vivanco, B. C., Lopes, J. D., and Xander, P. (2015). B-1 cells contribute to susceptibility in experimental infection with *Leishmania (Leishmania) chagasi*. *Parasitology* 142, 1506–1515. doi: 10.1017/S0031182015000943

Goto, H., and Prianti, M. D. (2009). Immunoactivation and immunopathogeny during active visceral leishmaniasis. *Rev. Inst. Med. Trop. Sao Paulo* 51, 241–246. doi: 10.1590/S0036-46652009000500002

Guha, R., Das, S., Ghosh, J., Sundar, S., Dujardin, J. C., and Roy, S. (2014). Antimony resistant *Leishmania donovani* but not sensitive ones drives greater frequency of potent T-regulatory cells upon interaction with human PBMCs: role of IL-10 and TGF-β in early immune response. *PLoS Negl. Trop. Dis.* 8:e2995. doi: 10.1371/journal.pntd.0002995

Herbert, D. R., Nolan, T. J., Schad, G. A., and Abraham, D. (2002). The role of B cells in immunity against larval *Strongyloides stercoralis* in mice. *Parasite Immunol.* 24, 95–101. doi: 10.1046/j.0141-9838.2001.00441.x

Hoerauf, A., Solbach, W., Lohoff, M., and Röllinghoff, M. (1994). The Xid defect determines an improved clinical course of murine leishmaniasis in susceptible mice. *Int. Immunol.* 6, 1117–1124. doi: 10.1093/intimm/6.8.1117

Kaye, P., and Scott, P. (2011). Leishmaniasis: complexity at the host-pathogen interface. *Nat. Rev. Microbiol.* 9, 604-615. doi: 10.1038/nrmicro2608

Kaye, P. M., Svensson, M., Ato, M., Maroof, A., Polley, R., Stager, S., et al. (2004). The immunopathology of experimental visceral leishmaniasis. *Immunol. Rev.* 201, 239–253. doi: 10.1111/j.0105-2896.2004.00188.x

Kumar, R., and Nylén, S. (2012). Immunobiology of visceral leishmaniasis. *Front. Immunol.* 3:251. doi: 10.3389/fimmu.2012.00251

Liu, D., and Uzonna, J. E. (2012). The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. *Front. Cell. Infect. Microbiol.* 2:83. doi: 10.3389/fcimb.2012.00083

Matlashewski, G., Arana, B., Kroeger, A., Battacharya, S., Sundar, S., Das, P., et al. (2011). Visceral leishmaniasis: elimination with existing interventions. *Lancet Infect. Dis.* 11, 322-325. doi: 10.1016/S1473-3099(10)70320-0

McCall, L. I., Zhang, W. W., and Matlashewski, G. (2013). Determinants for the development of visceral leishmaniasis disease. *PLoS Pathog.* 9:e1003053. doi: 10.1371/journal.ppat.1003053

Minoprio, P., el Cheikh, M. C., Murphy, E., Hontebeyrie-Joskowicz, M., Coffman, R., Coutinho, A., et al. (1993). Xid-associated resistance to experimental Chagas' disease is IFN-gamma dependent. *J. Immunol.* 151, 4200–4208.

Murphy, M. L., Wille, U., Villegas, E. N., Hunter, C. A., and Farrell, J. P. (2001). IL-10 mediates susceptibility to Leishmania donovani infection. *Eur. J. Immunol.* 31, 2848–2856. doi: 10.1002/1521-4141(2001010)31:10<2848::AID-IMMU2848>3.0.CO;2-T

Popi, A. F., Godoy, L. C., Xander, P., Lopes, J. D., and Mariano, M. (2008). B-1 cells facilitate *Paracoccidioides brasiliensis* infection in mice via IL-10 secretion. *Microbes Infect.* 10, 817–824. doi: 10.1016/j.micinf.2008.04.012

Popi, A. F., Osugui, L., Perez, K. R., Longo-Maugéri, I. M., and Mariano, M. (2012). Could a B-1 cell derived phagocyte "be one" of the peritoneal macrophages during LPS-driven inflammation? *PLoS ONE* 7:e34570. doi: 10.1371/journal.pone.0034570

Ready, P. D. (2014). Epidemiology of visceral leishmaniasis. *Clin. Epidemiol.* 6, 147-154. doi: 10.2147/clep.s44267

Ribeiro-Gomes, F. L., Otero, A. C., Gomes, N. A., Moniz-De-Souza, M. C., Cysne-Finkelstein, L., Arnholdt, A. C., et al. (2004). Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J. Immunol.* 172, 4454–4462. doi: 10.4049/jimmunol.172.7.4454

Szymczak, W. A., Davis, M. J., Lundy, S. K., Dufaud, C., Olszewski, M., and Pirofski, L. A. (2013). X-linked immunodeficient mice exhibit enhanced susceptibility to *Cryptococcus neoformans* Infection. *MBio* 4:e00265-13. doi: 10.1128/mBio.00265-13

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frontiers in Microbiology

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Decoding the Role of Glycans in Malaria

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Complications arising from malaria are a concern for public health authorities worldwide, since the annual caseload in humans usually exceeds millions. Of more than 160 species of *Plasmodium*, only 4 infect humans, with the most severe cases ascribed to *Plasmodium falciparum* and the most prevalent to *Plasmodium vivax*. Over the past 70 years, since World War II, when the first antimalarial drugs were widely used, many efforts have been made to combat this disease, including vectorial control, new drug discoveries and genetic and molecular approaches. Molecular approaches, such as glycobiology, may lead to new therapeutic targets (both in the host and the parasites), since all interactions are mediated by carbohydrates or glycan moieties decorating both cellular surfaces from parasite and host cells. In this review, we address the carbohydrate-mediated glycobiology that directly affects *Plasmodium* survival or host resistance.

Keywords: malaria, glycobiology, carbohydrate-targets, *Plasmodium falciparum*

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INTRODUCTION

Malaria is caused by a protozoan of the *Plasmodium* genus, which belongs to the *Apicomplexans* phylum, an obligatory parasite (Kishore et al., 2013). Only in the last two decades has there been a significant reduction in malaria caseloads, and, according to the latest World Health Organization (WHO) records, cases have dropped significantly, i.e., from 262 million in 2000 to 214 million in 2015 (WHO, 2015). It is still a serious condition, however, noteworthy when compared to other pathologies of equal importance worldwide, due to its high prevalence (Binns and Low, 2015). Efforts toward the elimination of malaria involve the extermination of its vectors, and parasites and universal access to prevention (diagnosis and earlier treatment); this includes the discovery of new drugs, to deal with the high number of drug-resistant strains (Tanner and Hommel, 2010; Feng et al., 2016).

The first malaria drug treatments began with the use of quinine as the active ingredient (Parola and Miller, 2002). During World War II, a quinine-derivative, chloroquine, was widely used and treated as a "top secret" (Loff and Cordner, 1999; Skvara, 2004). Chloroquine was associated with many benefits (low cost, efficacy, and safety) (Kofoed et al., 2003; Savarino et al., 2006). Quinine-based drugs were replaced by Artemisinin derivatives and other drugs; and the use of Artemisinin-based Combined Therapies (ACT) is now recommended (Visser et al., 2014; Watsierah and Ouma, 2014; Pousibet-Puerto et al., 2016) to eliminate the blood phases, since in the exoerythrocytic phase (asymptomatic phase), there are no obvious symptoms for early treatment (Imrie et al., 2007).

Most of these drugs are still in use today in different doses, depending on the infective species and host background (Achan et al., 2011). Resistance is usually accompanied by a range of genetic

diversity, and a high level of polymorphism, crucial to spreading these infective parasites but also after the widespread use of drugs, the first resistance-cases have appeared, and it seems that drugs have an "expiration date" this has also been observed in different malaria-infected patients in different regions, such as Thailand and Papua New Guinea (Cui et al., 2003; Brito and Ferreira, 2011). For example, *Plasmodium falciparum* shows high antigenic variation, with more than 60 coding variations of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1), directly related to the virulence and lethality of the infection of this species (Arnot and Jensen, 2011). On the other hand, may present variations in the merozoite surface protein MSP-3α is a multi-gene family important in *P. falciparum* and *Plasmodium vivax*, acting as "decoys" for antigenic diversity during RBCs invasion (Rice et al., 2014). CSP genes or circumsporozoite protein (CSP) in sporozoite surface, thrombospondin-related anonymous protein/sporozoite surface protein 2 (TRAP) or else in *P. vivax* apical membrane antigen 1 (AMA1) in ectodomain and C-terminal region of MSP-1 as a immunodominant antigen that was studied with recombinant protein (MSP119) as a novel potential vaccine (Rocha et al., 2017) and liver stage antigen (LSA1) also studied in malaria vaccine approaches (Pichyangkul et al., 2008).Thus, these and many more key proteins at each *Plasmodium* stage open up the "branches" for studies of this type of interactions, as seen in glycobiology.

CARBOHYDRATES IN MALARIA: APPROACH FOR POTENTIAL DRUG TARGET DISCOVERY

Glycosaminoglycans (GAGs) are abundant in both host and parasites; they are composed of basic units of carbohydrates that rearrange themselves in various ways, changing function and location (Griffin and Hsieh-Wilson, 2013). Glycobiological approaches investigate the influence of these carbohydrates on host-parasite binding interactions, such as glycolytic enzymes that are adequate in predicting a good understanding of parasite metabolism and glycosylation of malaria proteins. The first evidence about sugars mediatING the parasite-red blood cell invasion was cited by Miller et al. (1977). Experiments determinated that O-linked oligosaccharides, such as NeuNAc and GalNac, were found in high concentration (20 mM) and inhibited the parasite intracellular invasion in RBCs (Pasvol, 1984). Other sugars such as Gal (β1-3) GalNAc disaccharide associated with glycophorin was more inhibitory in the same context (Hermentin et al., 1984).

Thus, these tools give support to studies currently in development in this regard. In addition, some pathogen-associated molecular patterns (PAMPs) consist primarily of carbohydrates structures, although these are not yet well known or understood in malaria parasites (Hoving et al., 2014). However, recently, the most discussed PAMPs are GPI anchores, haemozoin, and immunostimulatory nucleic acid motifs (Gazzinelli et al., 2014).

Other crucial receptor crucial, that requires specific receptor-ligand interactions to RBC invasion and cytoadherence in malaria, is Duffy-binding-like domains (DBLs). In *P. vivax* and *P. knowlesi,* parasites invade RBC exclusively through the DARC receptors (Duffy antigen receptor for chemokines). However, in *P. knowlesi* DBL domain (Pkalpa-DBL) to due a immune pressure they seems development a evasion strategy to run away, mapping to opposite surface of the DBL.

Spitzmuller and Mestres (2013) addressed the design of a generation of new antimalarials drugs. A major challenge is to identify *P. falciparum* proteins, among million possible combinations that can be targeted at the same time by the just one drug. In their studies, they analyzed databases and to identify drugs with multi protein targets, because the drugs until now supported specific protein targets, which in a few time allows the parasite to mutate only at this target reaching. Unlikely, Artemisinin which is regarded as a multi-target drug, maintaining as a new generation drug and which is advocated throughout malaria treatment (Spitzmuller and Mestres, 2013).

Still regarding innate immunity, there are two major families of pattern recognition receptors (PPR) predominantly expressed by cells of innate immune system are TLRs and C-type lectin receptors (CLRs). CLRs are important for the immune response against parasites, and in some studies example has been observed that, for example, CLRs are related with cerebral malaria in mice infected by *Plasmodium berghei*, and CARD9 is upregulated, but CARD9$^{-/-}$ mice were not protected from infection, suggesting that the CARD9 receptor influences infectivity by the plasmodium but in its absence (as demonstrated in knockout animals) does not prevent the disease from occurring (McGuinness et al., 2003). Another study conducted with *P. chabaudi* demonstrated that the mannose receptor C type 2 (MRC2) increased with parasitemia, but toll-like receptors and sialoadhesin decreased in contrast to other MRCs (1 and 2), and that decreased with parasitemia in *P. yoelii*, suggesting the importance of lectin-receptors in the development of mounting of the immune response (Rosanas-Urgell et al., 2012).

Studies regarding parasite sugar supply demand has increased in the last decades, given that *Plasmodium* parasites require a high sugar demand to replicate. These parasites also show the ability to manipulate vector behavior to ensure survival, including increased sugar seeking, although it is unclear how this manipulation affects vector-plant interactions and sugar uptake (Nyasembe et al., 2014). Parasite manipulation in search of sugar supplies has been described as established at the moment of vector infection. *Plasmodium* present in the bloodstream require glucose, which crosses the plasma membranes and enters the parasite cytosol (Coppi et al., 2005; von Itzstein et al., 2008; Bertolino and Bowen, 2015; Swearingen et al., 2016). The parasites are able expose the RBC hexose transporter to facilitate sugar nucleotide uptake, allowing *Plasmodium* to biosynthesize certain glycans for maintenance (Cova et al., 2015). After the release of the sequence in the PlasmoDB, database facilitated the search for tools in interventions in this receptor for therapeutic

purposes. The hexoses receptor (PfHT) has been widely studied, because its decrease implies in the lower supply of glucose to parasites and causing the plasmodium elimination (Bahl et al., 2003).

The surface of infected red blood cells (RBCs) are rich in glycophorins and *Plasmodium* possesses some proteins like erythrocytes binding-like (EBL) and reticulocyte binding-like (Rh) protein families that recognize them, playing a critical role in attachment in invasion (Davidson and Gowda, 2001; Salinas et al., 2014), Studies demonstrated that glycophorins as play crucial role in *Plasmodium* invasion, in absence of glycophorins A relatively resistant to the invasion in red blood cells (Pasvol, 1984). Other portion of glycophorin A has sialic acid residues, which is known as EBA-175 (175 kDa), and it mediates binding of *P. falciparum* to RBCs. A part of this, EBA-175 is highly conserved and rich in cysteine, is referred to as F2 (PfF2) and it has receptor binding sites that have been studied as a possible recombinant protein in malaria vaccines trials (Pattnaik et al., 2007).

In Tham et al. (2015) the cytoplasmatic tails of these proteins were phosphorylated *in vitro* and blocked RBC invasion, evidencing the importance of these proteins for invasion (Tham et al., 2015).

In addition to studies on the interactions between the parasite and carbohydrates in RBCs, it has been demonstrated that blood type (ABO, Lewis, Duffy, and others surface antigens) influences erythrocyte parasitism, with certain types more susceptible to *Plasmodium* infection (Cooling, 2015). Studies indicate that individuals of blood group A are highly susceptible to *P. falciparum* induced-malaria, while blood group O has been shown to be protective against complicated cases (Fischer and Boone, 1998; Lell et al., 1999). The CSP and TRAP domains on the sporozoite that mediate the adhesive contact with the sulphated glycoconjugates on the surface of hepatocytes allow plasmodium invasion to the bloodstream. Thus, these proteins are extremely important for the parasite, since it is from the entry in the hepatocytes that the cycle begins. Taking this into account, these same proteins have been studied extensively, including in the manufacturing of anti-malarial vaccines, such as RTS,S (Coppi et al., 2005; Swearingen et al., 2016). Despite having obtained good results in treating mice with anti-CSP, it has been verified that, in the absence of this protein, the cycle of hepatocyte invasion continues normally, since after invasion CSPs are less expressed, while other proteins become highly expressed (Bertolino and Bowen, 2015).

As the *Plasmodium* parasite uses sugar-requirements to ensure survival, approaches with drug-targeting carbohydrates have increasingly been proposed as possible treatments. Regarding *in vitro* studies, Plaimas et al. (2013) investigated a database of genetic information from *Plasmodium* to try to decipher which points of the proteins expressed could be future of therapeutic targets. 22 potential targets, refined the search by removing false positives, leaving only 5 targets, among them, glutamyl-tRNA (gln) aminotransferase and with a known inhibitor of this transferase 6-diazo-5-oxonorleucine (Don). The tests were carried out and the growth of the parasite decreased both *in vitro* and *in vivo* in Swiss mice, despite the side effects related to the dosage (Plaimas et al., 2013). Similar results have recently been observed with DON in experimental cerebral malaria mice models, although this compound has shown inhibitory effects by blocking CD8+ T-cell effector function, which is the highest cause of mice death (Gordon et al., 2015). On the other hand, other studies indicate that mice mortality was attenuated due to GPI anchors, not T cells, since several literature reports indicate the importance of glycophosphatidylinositol (GPI) anchors for the success of *Plasmodium* infection (Naik et al., 2000). CSP have also been associated to GPI, which have a canonical domain in the COOH portion, although this has not yet been demonstrated (Coppi et al., 2005).

Of significance, people living in endemic areas are more resistant to malaria, due to the production of antibodies against GPI anchors (Vijaykumar et al., 2001).

In addition to inhibitors that hinder this type of parasite-host interaction, mice immunized with the glycan moiety of GPIs were able to produce anti-GPI antibodies to prevent progression to cerebral malaria (Schofield et al., 2002). It has been reported that a microbial polyssacharide, Gellan Gum (GG), containing a sugar moiety produced by the *Sphingomonas* (*Pseudomonas*) *elodea* bacterium, strongly inhibited parasite invasion; this also inhibits growth (strains 3D7 and Dd2), demonstrating that "natural" sugars can also contain *Plasmodium* effects (Recuenco et al., 2014). In human malaria, successive pregnancies contribute to resistance against *Plasmodium,* which mediates binding to chondroitin sulfate A (CSA) in the placenta through the VAR2CSA protein (Salanti et al., 2004; Gamain et al., 2005); this leads to accumulation of *Plasmodium* parasites in the placenta, resulting in severe clinical consequences for both mother and child (Resende et al., 2008), such that the investigation of this interaction is a viable target for vaccines (Clausen et al., 2012; Fried and Duffy, 2017). Through all this information about the various receivers composed by sugar and the interactions required these reinforce the importance of deciphering the nature of glycan functions in malaria in order to improve approaches for predicting drug-target interactions for this complex.

AUTHOR CONTRIBUTIONS

PSG, DFF, AM, and CGF-de-L wrote the paper. All authors read and approved the final version of the manuscript.

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REFERENCES

Achan, J., Talisuna, A. O., Erhart, A., Yeka, A., Tibenderana, J. K., Baliraine, F. N., et al. (2011). Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malar. J.* 10:144. doi: 10.1186/1475-2875-10-144

Arnot, D. E., and Jensen, A. T. (2011). Antigenic variation and the genetics and epigenetics of the PfEMP1 erythrocyte surface antigens in *Plasmodium falciparum* Malaria. *Adv. Appl. Microbiol.* 74, 77–96. doi: 10.1016/B978-0-12-387022-3.00007-0

Bahl, A., Brunk, B., Crabtree, J., Fraunholz, M. J., Gajria, B., Grant, G. R., et al. (2003). PlasmoDB: the Plasmodium genome resource. A database integrating experimental and computational data. *Nucleic Acids Res.* 31, 212–215. doi: 10.1093/nar/gkg081

Bertolino, P., and Bowen, D. G. (2015). Malaria and the liver: immunological hide-and-seek or subversion of immunity from within? *Front. Microbiol.* 6:41. doi: 10.3389/fmicb.2015.00041

Binns, C., and Low, W. Y. (2015). Malaria continues as a major public health problem. *Asia Pac. J. Public Health* 27, 261–262. doi: 10.1177/1010539515583388

Brito, C. F., and Ferreira, M. U. (2011). Molecular markers and genetic diversity of *Plasmodium vivax*. *Mem. Inst. Oswaldo Cruz* 106 (Suppl. 1), 12–26. doi: 10.1590/S0074-02762011000900003

Clausen, T. M., Christoffersen, S., Dahlback, M., Langkilde, A. E., Jensen, K. E., Resende, M., et al. (2012). Structural and functional insight into how the *Plasmodium falciparum* VAR2CSA protein mediates binding to chondroitin sulfate A in placental malaria. *J. Biol. Chem.* 287, 23332–23345. doi: 10.1074/jbc.M112.348839

Cooling, L. (2015). Blood Groups in Infection and Host Susceptibility. *Clin. Microbiol. Rev.* 28, 801–870. doi: 10.1128/CMR.00109-14

Coppi, A., Pinzon-Ortiz, C., Hutter, C., and Sinnis, P. (2005). The Plasmodium circumsporozoite protein is proteolytically processed during cell invasion. *J. Exp. Med.* 201, 27–33. doi: 10.1084/jem.20040989

Cova, M., Rodrigues, J. A., Smith, T. K., and Izquierdo, L. (2015). Sugar activation and glycosylation in Plasmodium. *Malar. J.* 14, 427. doi: 10.1186/s12936-015-0949-z

Cui, L., Mascorro, C. N., Fan, Q., Rzomp, K. A., Khuntirat, B., Zhou, G., et al. (2003). Genetic diversity and multiple infections of *Plasmodium vivax* malaria in Western Thailand. *Am. J. Trop. Med. Hyg.* 68, 613–619. doi: 10.4269/ajtmh.2003.68.613

Davidson, E. A., and Gowda, D. C. (2001). Glycobiology of *Plasmodium falciparum*. *Biochimie* 83, 601–604. doi: 10.1016/S0300-9084(01)01316-5

Feng, J., Liu, J., Feng, X., Zhang, L., Xiao, H., and Xia, Z. (2016). Towards Malaria Elimination: Monitoring and Evaluation of the "1-3-7" Approach at the China-Myanmar Border. *Am. J. Trop. Med. Hyg.* 95, 806–810. doi: 10.4269/ajtmh.15-0888

Fischer, P. R., and Boone, P. (1998). Short report: severe malaria associated with blood group. *Am. J. Trop. Med. Hyg.* 58, 122–123.

Fried, M., and Duffy, P. E. (2017). Malaria during pregnancy. *Cold Spring Harb. Perspect. Med.* 7:a025551. doi: 10.1101/cshperspect.a025551

Gamain, B., Trimnell, A. R., Scheidig, C., Scherf, A., Miller, L. H., and Smith, J. D. (2005). Identification of multiple chondroitin sulfate A (CSA)-binding domains in the var2CSA gene transcribed in CSA-binding parasites. *J. Infect. Dis.* 191, 1010–1013. doi: 10.1086/428137

Gazzinelli, R. T., Kalantari, P., Fitzgerald, K. A., and Golenbock, D. T. (2014). Innate sensing of malaria parasites. *Nat. Rev. Immunol.* 14, 744–757. doi: 10.1038/nri3742

Gordon, E. B., Hart, G. T., Tran, T. M., Waisberg, M., Akkaya, M., Kim, A. S., et al. (2015). Targeting glutamine metabolism rescues mice from late-stage cerebral malaria. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13075–13080. doi: 10.1073/pnas.1516544112

Griffin, M. E., and Hsieh-Wilson, L. C. (2013). Synthetic probes of glycosaminoglycan function. *Curr. Opin. Chem. Biol.* 17, 1014–1022. doi: 10.1016/j.cbpa.2013.09.015

Hermentin, P., Paulsen, H., Kolar, C., and Enders, B. (1984). *Plasmodium falciparum*: carbohydrates as receptor sites of invasion. *Exp. Parasitol.* 58, 290–306.

Hoving, J. C., Wilson, G. J., and Brown, G. D. (2014). Signalling C-type lectin receptors, microbial recognition and immunity. *Cell. Microbiol.* 16, 185–194. doi: 10.1111/cmi.12249

Imrie, H., Fowkes, F. J., Michon, P., Tavul, L., Reeder, J. C., and Day, K. P. (2007). Low prevalence of an acute phase response in asymptomatic children from a malaria-endemic area of Papua New Guinea. *Am. J. Trop. Med. Hyg.* 76, 280–284. doi: 10.4269/ajtmh.2007.76.280

Kishore, S. P., Stiller, J. W., and Deitsch, K. W. (2013). Horizontal gene transfer of epigenetic machinery and evolution of parasitism in the malaria parasite *Plasmodium falciparum* and other apicomplexans. *BMC Evol. Biol.* 13:37. doi: 10.1186/1471-2148-13-37

Kofoed, P. E., Poulsen, A., Co, F., Hedegaard, K., Aaby, P., and Rombo, L. (2003). No benefits from combining chloroquine with artesunate for three days for treatment of *Plasmodium falciparum* in Guinea-Bissau. *Trans. R. Soc. Trop. Med. Hyg.* 97, 429–433. doi: 10.1016/S0035-9203(03)90078-2

Lell, B., May, J., Schmidt-Ott, R. J., Lehman, L. G., Luckner, D., Greve, B., et al. (1999). The role of red blood cell polymorphisms in resistance and susceptibility to malaria. *Clin. Infect. Dis.* 28, 794–799. doi: 10.1086/515193

Loff, B., and Cordner, S. (1999). World War II malaria trials revisited. *Lancet* 353, 1597.

McGuinness, D. H., Dehal, P. K., and Pleass, R. J. (2003). Pattern recognition molecules and innate immunity to parasites. *Trends Parasitol.* 19, 312–319. doi: 10.1016/S1471-4922(03)00123-5

Miller, L. H., Haynes, J. D., McAuliffe, F. M., Shiroishi, T., Durocher, J. R., and McGinniss, M. H. (1977). Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. *J. Exp. Med.* 146, 277–281.

Naik, R. S., Davidson, E. A., and Gowda, D. C. (2000). Developmental stage-specific biosynthesis of glycosylphosphatidylinositol anchors in intraerythrocytic *Plasmodium falciparum* and its inhibition in a novel manner by mannosamine. *J. Biol. Chem.* 275, 24506–24511. doi: 10.1074/jbc.M002151200

Nyasembe, V. O., Teal, P. E., Sawa, P., Tumlinson, J. H., Borgemeister, C., and Torto, B. (2014). *Plasmodium falciparum* infection increases *Anopheles gambiae* attraction to nectar sources and sugar uptake. *Curr. Biol.* 24, 217–221. doi: 10.1016/j.cub.2013.12.022

Parola, P., and Miller, R. S. (2002). Quinine in the modern treatment of falciparum malaria. *Lancet Infect. Dis.* 2, 206–207. doi: 10.1016/S1473-3099(02)00237-2

Pasvol, G. (1984). Receptors on red cells for *Plasmodium falciparum* and their interaction with merozoites. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 307, 189–200.

Pattnaik, P., Shakri, A. R., Singh, S., Goel, S., Mukherjee, P., and Chitnis, C. E. (2007). Immunogenicity of a recombinant malaria vaccine based on receptor binding domain of *Plasmodium falciparum* EBA-175. *Vaccine* 25, 806–813. doi: 10.1016/j.vaccine.2006.09.048

Pichyangkul, S., Kum-Arb, U., Yongvanitchit, K., Limsalakpetch, A., Gettayacamin, M., Lanar, D. E., et al. (2008). Preclinical evaluation of the safety and immunogenicity of a vaccine consisting of *Plasmodium falciparum* liver-stage antigen 1 with adjuvant AS01B administered alone or concurrently with the RTS,S/AS01B vaccine in rhesus primates. *Infect. Immun.* 76, 229–238. doi: 10.1128/IAI.00977-07

Plaimas, K., Wang, Y., Rotimi, S. O., Olasehinde, G., Fatumo, S., Lanzer, M., et al. (2013). Computational and experimental analysis identified 6-diazo-5-oxonorleucine as a potential agent for treating infection by *Plasmodium falciparum*. *Infect. Genet. Evol.* 20, 389–395. doi: 10.1016/j.meegid.2013.09.019

Pousibet-Puerto, J., Salas-Coronas, J., Sanchez-Crespo, A., Molina-Arrebola, M. A., Soriano-Perez, M. J., Gimenez-Lopez, M. J., et al. (2016). Impact of using artemisinin-based combination therapy (ACT) in the treatment of uncomplicated malaria from *Plasmodium falciparum* in a non-endemic zone. *Malar. J.* 15, 339. doi: 10.1186/s12936-016-1408-1

Recuenco, F. C., Kobayashi, K., Ishiwa, A., Enomoto-Rogers, Y., Fundador, N. G., Sugi, T., et al. (2014). Gellan sulfate inhibits *Plasmodium falciparum* growth and invasion of red blood cells *in vitro*. *Sci. Rep.* 4:4723. doi: 10.1038/srep04723

Resende, M., Nielsen, M. A., Dahlback, M., Ditlev, S. B., Andersen, P., Sander, A. F., et al. (2008). Identification of glycosaminoglycan binding regions in the *Plasmodium falciparum* encoded placental sequestration ligand, VAR2CSA. *Malar. J.* 7:104. doi: 10.1186/1475-2875-7-104

Rice, B. L., Acosta, M. M., Pacheco, M. A., Carlton, J. M., Barnwell, J. W., and Escalante, A. A. (2014). The origin and diversification of the merozoite surface

protein 3 (msp3) multi-gene family in *Plasmodium vivax* and related parasites. *Mol. Phylogenet. Evol.* 78, 172–184. doi: 10.1016/j.ympev.2014.05.013

Rocha, M. V., Francoso, K. S., Lima, L. C., Camargo, T. M., Machado, R. L., Costa, F. T., et al. (2017). Generation, characterization and immunogenicity of a novel chimeric recombinant protein based on *Plasmodium vivax* AMA-1 and MSP119. *Vaccine* 35, 2463–2472. doi: 10.1016/j.vaccine.2017.03.023

Rosanas-Urgell, A., Martin-Jaular, L., Ricarte-Filho, J., Ferrer, M., Kalko, S., Kimura, E., et al. (2012). Expression of non-TLR pattern recognition receptors in the spleen of BALB/c mice infected with *Plasmodium yoelii* and *Plasmodium chabaudi* chabaudi AS. *Mem. Inst. Oswaldo Cruz* 107, 410–415. doi: 10.1590/S0074-02762012000300017

Salanti, A., Dahlback, M., Turner, L., Nielsen, M. A., Barfod, L., Magistrado, P., et al. (2004). Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J. Exp. Med.* 200, 1197–1203. doi: 10.1084/jem.200break41579

Salinas, N. D., Paing, M. M., and Tolia, N. H. (2014). Critical glycosylated residues in exon three of erythrocyte glycophorin A engage *Plasmodium falciparum* EBA-175 and define receptor specificity. *MBio* 5, e01606–e01614. doi: 10.1128/mBio.01606-14

Savarino, A., Lucia, M. B., Giordano, F., and Cauda, R. (2006). Risks and benefits of chloroquine use in anticancer strategies. *Lancet Oncol.* 7, 792–793. doi: 10.1016/S1470-204570875-0

Schofield, L., Hewitt, M. C., Evans, K., Siomos, M. A., and Seeberger, P. H. (2002). Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* 418, 785–789. doi: 10.1038/nature00937

Skvara, F. C. (2004). World War II: exhibit pages (The Red Cross society, blood transfusions, discovery of penicillin, malaria). *Scalpel Tongs* 48, 60–64.

Spitzmuller, A., and Mestres, J. (2013). Prediction of the *P. falciparum* target space relevant to malaria drug discovery. *PLoS Comput. Biol.* 9:e1003257. doi: 10.1371/journal.pcbi.1003257

Swearingen, K. E., Lindner, S. E., Shi, L., Shears, M. J., Harupa, A., Hopp, C. S., et al. (2016). Interrogating the Plasmodium Sporozoite Surface: Identification of Surface-Exposed Proteins and Demonstration of Glycosylation on CSP and TRAP by Mass Spectrometry-Based Proteomics. *PLoS Pathog.* 12:e10 05606. doi: 10.1371/journal.ppat.1005606

Tanner, M., and Hommel, M. (2010). Towards malaria elimination–a new thematic series. *Malar. J.* 9:24. doi: 10.1186/1475-2875-9-24

Tham, W. H., Lim, N. T., Weiss, G. E., Lopaticki, S., Ansell, B. R., Bird, M., et al. (2015). *Plasmodium falciparum* adhesins play an essential role in signalling and activation of invasion into human erythrocytes. *PLoS Pathog.* 11:e1005343. doi: 10.1371/journal.ppat.1005343

Vijaykumar, M., Naik, R. S., and Gowda, D. C. (2001). *Plasmodium falciparum* glycosylphosphatidylinositol-induced TNF-α secretion by macrophages is mediated without membrane insertion or endocytosis. *J. Biol. Chem.* 276, 6909–6912. doi: 10.1074/jbc.C100007200

Visser, B. J., Wieten, R. W., Kroon, D., Nagel, I. M., Belard, S., van Vugt, M., et al. (2014). Efficacy and safety of artemisinin combination therapy (ACT) for non-falciparum malaria: a systematic review. *Malar. J.* 13:463. doi: 10.1186/1475-2875-13-463

von Itzstein, M., Plebanski, M., Cooke, B. M., and Coppel, R. L. (2008). Hot, sweet and sticky: the glycobiology of *Plasmodium falciparum*. *Trends Parasitol.* 24, 210–218. doi: 10.1016/j.pt.2008.02.007

Watsierah, C. A., and Ouma, C. (2014). Access to artemisinin-based combination therapy (ACT) and quinine in malaria holoendemic regions of western Kenya. *Malar. J.* 13:290. doi: 10.1186/1475-2875-13-290

WHO (2015). *World Malaria Report 2015.* Geneva: WHO.

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Transcriptional Profiling of Midguts Prepared from *Trypanosoma/ T. congolense*-Positive *Glossina palpalis palpalis* Collected from Two Distinct Cameroonian Foci: Coordinated Signatures of the Midguts' Remodeling As *T. congolense*-Supportive Niches

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Our previous transcriptomic analysis of *Glossina palpalis gambiensis* experimentally infected or not with *Trypanosoma brucei gambiense* aimed to detect differentially expressed genes (DEGs) associated with infection. Specifically, we selected candidate genes governing tsetse fly vector competence that could be used in the context of an anti-vector strategy, to control human and/or animal trypanosomiasis. The present study aimed to verify whether gene expression in field tsetse flies (*G. p. palpalis*) is modified in response to natural infection by trypanosomes (*T. congolense*), as reported when insectary-raised flies (*G. p. gambiensis*) are experimentally infected with *T. b. gambiense*. This was achieved using the RNA-seq approach, which identified 524 DEGs in infected vs. non-infected tsetse flies, including 285 downregulated genes and 239 upregulated genes (identified using DESeq2). Several of these genes were highly differentially expressed, with log2 fold change values in the vicinity of either +40 or −40. Downregulated genes were primarily involved in transcription/translation processes, whereas encoded upregulated genes governed amino acid and nucleotide biosynthesis pathways. The BioCyc metabolic pathways associated with infection also revealed that downregulated genes were mainly involved in fly immunity processes. Importantly, our study demonstrates that data on the molecular cross-talk between the host and the parasite (as well as the always present fly microbiome) recorded from an experimental biological model has a counterpart in field flies, which in turn validates the use of experimental host/parasite couples.

Keywords: field tsetse fly, Cameroonian foci, RNAseq, vector control, trypanosomiasis

INTRODUCTION

Human African trypanosomiasis [HAT or sleeping sickness; (1)] and animal African trypanosomiasis [AAT or nagana; (2)] are two vector-borne diseases that inflict heavy social and economic burdens on sub-Saharan African populations. Although the number of newly diagnosed HAT cases is decreasing (<10,000 per year) (3), more than 60 million people living in endemic areas are at risk of infection (4). In addition, AAT causes a large amount of livestock loss, which has been estimated as high as US$ 4.5 billion per year (5). HAT is due to either *Trypanosoma brucei gambiense* (Tbg; the chronic form of the disease in West and Central Africa) or *T. b. rhodesiense* (the acute form of the disease in East Africa), which are, respectively, transmitted by *Glossina palpalis* and *G. morsitans*. In contrast, AAT is caused by *T. b. brucei*, *T. congolense* (Tc; the forest or savannah type), or *T. vivax*, and is transmitted by *G. palpalis* or *G. morsitans*.

Despite differences between Tc and Tbg [reviewed in Ref. (6)], the parasites share several important characteristics. In particular, they are digenetic, meaning that they need to successively infect two different hosts to achieve their life cycle. One of these hosts, a *Glossina* fly, is the vector, whereas the other host is a vertebrate, typically a mammal. These parasites must accomplish a crucial part of their life cycle within their vector, namely their multiplication and maturation into the infectious form that can be transmitted to the vertebrate host while the tsetse fly ingests its blood meal. More specifically, Tc and Tbg undergo sequential differentiations after their ingestion by the fly, from the ingested blood stream form to the vertebrate-infective metacyclic form. The latter differentiation occurs either in the proboscis (for Tc) or in the salivary glands (for Tbg) (7), which is the basis for their respective classification into two different subgenera, *Nannomonas* and *Trypanozoon* (8). They also share the ability to excrete/secrete a number of proteins, some of which are considered to be involved in their establishment in the tsetse midgut and/or in the pathogenic process developed within the vertebrate host (9–12). Finally, the establishment of both Tc and Tbg in the *G. palpalis* vector is reported to be favored by *Sodalis glossinidius*, the secondary symbiont inhabitant of the tsetse gut (13). This finding demonstrates the occurrence in naturally infected field tsetse flies of a tripartite interaction (fly/trypanosome/gut bacteria) already reported to occur in experimentally infected insectary flies (14–18).

Another similarity between the parasites is that their mantle, which consists of a variant surface glycoprotein, allows them to evade the host's immune system by means of antigenic variations (19–21), thus rendering ineffective any vaccine approaches to fight HAT or AAT. Nevertheless, progress has been made in rapid diagnosis (22) and therapy that uses a nifurtimox–elfornitine combination in the treatment of the second phase of HAT (23). Furthermore, besides the use of trypanocidal drugs, the incidence of AAT can be lowered by introducing trypanotolerant cattle into AAT-infected area or through the antibody-mediated inhibition of trypanosome-secreted proteins involved in the parasite pathogenic process (24, 25).

Another approach to fight HAT or AAT is by vector control. Diverse strategies are available, including the application of pesticides, the use of sterile males, and the development of paratransgenic approaches (26–32).

The normal status of tsetse flies is considered to be refractory to trypanosome infection, given that artificial or natural infection rates are always low (28, 33–36). Recently, a global transcriptomic analysis was performed (15–17) in the context of an anti-vector strategy, aimed at deciphering the molecular cross-talk occurring between the different participants involved in tsetse infection: the fly, the trypanosome, and the fly gut bacteria, especially the primary (*Wigglesworthia glossinidia*) and secondary (*S. glossinidius*) symbionts. The authors also focused on identifying differentially expressed genes (DEGs) associated with fly susceptibility or refractoriness as a result of fly infection by the trypanosome. These investigations were performed on insectary-raised *G. p. gambiensis* (Gpg) flies that were artificially infected (or not) by Tbg. This study raised the question of whether the results recorded under these experimental conditions could be transposed to what actually occurs under natural conditions in HAT and AAT foci.

To address this question we have conducted similar transcriptomic analyses on *G. p. palpalis* (Gpp) flies infected or not with Tc, collected in two HAT foci in southern Cameroon. Our experimental design involved a different host vector/parasite couple (Gpp/Tc) from what was used in the previous insectary-raised approach. However, as shown above and in support of this approach, several notable characteristics are shared between the Gpp/Tc couple and the previously used Gpg/Tbg couple, including life cycle, sequential differentiation within the vector, transmission modalities, host immune response escape, and pathological effects on susceptible vertebrate hosts, among others. Thus, the objectives of this study were to determine whether or not field-collected tsetse flies react to trypanosome infection under natural conditions similar to insectary flies under experimental conditions, and whether or not Tc induces molecular disruptions in Gpp similar to those provoked by Tbg in Gpg. Importantly, our approach provides novel evidence that validates the use of experimental host/parasite couples in the context of investigating anti-vector strategies.

MATERIALS AND METHODS

Sampling Areas

Tsetse flies were sampled in May and June 2015 in two active HAT foci (Campo and Bipindi), located in the Ocean Division of the southern region of Cameroon. The Campo focus (2°20′N, 9°52′E) is located on the Atlantic coast and extends along the Ntem river. The HAT National Control Program that visits Campo once per year diagnosed 61 novel HAT cases between 2001 and 2011. The passive identification of two cases in 2012 (37) indicates that HAT is still present. The Bipindi focus (3°2′N, 10°22′E) has a typical forest bioecological environment, including equatorial forest and farmland along roads and around villages. This focus has been recognized since 1920 (38) and includes several villages. Sleeping sickness is still present, since approximately 83 HAT cases were identified by the National Control Program in this focus between 1998 and 2011 (Ebo'o Eyenga, personal communication). In addition to HAT cases that involve *G. palpalis* and Tbg, regular global

surveys have identified the presence of several other *Glossina* (including Gpp) and *Trypanosoma* species (including Tc) in both foci. Surveys have also identified a variety of domestic and wild animals that serve as reservoirs for diverse *Trypanosoma* species (39–42). As described below, flies were trapped in these areas in order to select non-infected and Tc-infected individuals.

Fly Sampling, Dissection, and Subsequent RNA Preservation

The May 2015 tsetse fly trapping campaign was conducted in three Campo villages (Ipono, Mabiogo, and Campo-Beach), and the June 2015 campaign was conducted in three Bipindi villages (Lambi, Bidjouka, and Ebiminbang). The geographical positions of the sampling sites were determined by GPS. Tsetse flies were captured using pyramidal traps (43) placed in suitable tsetse fly biotopes. Each trap was installed for four consecutive days, and the flies were collected twice per day.

Prior to handling samples, work stations and dissecting instruments were cleaned with RNase away (Ambion) in order to eliminate any RNases that could degrade sample RNA. Furthermore, tsetse flies were dissected alive to prevent RNA degradation by normal *post mortem* degradation processes. The first step in sample processing consisted in identifying the collected tsetse flies to the species level on the basis of morphological criteria and adapted taxonomic keys (44). Next, the samples were separated into two groups of teneral and non-teneral flies. The non-teneral Gpp flies were dissected in a drop of sterile 0.9% saline solution, according to the midgut dissection protocol developed by Penchenier and Itard (45). The organs were immediately transferred to tubes containing RNAlater (Ambion) for DNA and RNA extraction. These samples were then used for parasite identification by specific PCR amplification, and ultimately for transcriptomic analysis. All tools were carefully cleaned after the dissection of each fly to prevent cross-contamination. During field manipulations, the tubes containing the organs were stored at −20°C for 5 days; subsequently, they were stored in the laboratory at −80°C until use.

DNA and RNA Extraction

To prepare for extraction, samples stored at −80°C were thawed and RNAlater was removed. The midguts were treated with the NucleoSpin TriPrep extraction kit (Macherey-Nagel) according to the manufacturer's instructions, which allow the separate extraction of DNA and RNA. RNase free water (40 µl) was added to elute the RNA, and 100 µl of DNA elute solution was added to recover the DNA. RNA quality and the absence of any DNA contamination were checked on an Agilent RNA 6000 Bioanalyzer and quantified using the Agilent RNA 6000 Nano kit (Agilent Technologies, France).

PCR Amplification

To identify which trypanosome species had infected the sampled tsetse flies, the isolated DNA samples stored at −80°C were thawed and used as a template for PCR amplification with specific primers (**Table 1**). PCR amplification of parasites was performed as described by Herder et al. (46) and consisted of a denaturing step at 94°C (5 min) followed by 44 amplification cycles, each comprising a denaturing step at 94°C (30 s), annealing at 55°C (30 s), and an extension step at 72°C (1 min). A final extension was performed at 72°C for 10 min. The amplified products were separated on a 2% agarose gel containing ethidium bromide and visualized under UV illumination. Positive (2 ng of reference DNA) and negative controls were included in each PCR amplification experiment. PCR amplifications that gave a positive result were repeated once for confirmation.

RNA-Seq Processing

Preparation of cDNA Libraries

Total RNA from 10 Gpp flies (5 non-infected flies and 5 flies infected by Tc s.l.) was assayed using the TruSeq mRNA-seq Stranded v2 Kit (Illumina), according to the manufacturer's instructions. Briefly, 4 µg of total RNA were used for poly(A)-selection to generate 120–210 bp cDNA fragments (mean size: 155 bp) after an 8-min elution-fragmentation incubation. Each library was barcoded using TruSeq Single Index (Illumina), according to the manufacturer's instructions. After library preparation, Agencourt AMPure XP (Beckman Coulter, Inc.) was used to select 200- to 400-bp size libraries. Each library size distribution was examined using the Bioanalyzer with a High Sensitivity DNA chip (Agilent) to ensure that the samples had the proper size and that they were devoid of any adaptor contamination. The sample concentration was quantified on Qubit with the Qubit® dsDNA HS Assay Kit (Life Technologies). Each library was then diluted to 4 nM and pooled at an equimolar ratio.

NextSeq-500 Sequencing

For sequencing, 5 µL of pooled libraries (4 nM) were denatured with 5 µl NaOH (0.2 N) according to the manufacturer's instructions. Following a 5-min incubation, 5 µl of Tris–HCl (200 mM; pH 7) were added, and 20 pM of the pooled libraries were diluted with HT1 to a 1.6-pM final concentration. As a sequencing

TABLE 1 | Primers used for PCR amplification of trypanosomes.

Species	Primer sequence	Amplified product (bp)	Reference
T. brucei s.l.	5′-CGAATGAATATTAAACAATGCGCAG-3′ 5′-AGAACCATTTATTAGCTTTGTTGC-3′	164	Masiga et al. (47)
T. congolense ("forest" type)	5′-CGAATGAATATTAAACAATGCGCAG-3′ 5′-AGAACCATTTATTAGCTTTGTTGC-3′	350	Masiga et al. (47)
T. congolense ("savannah" type)	5′-CGAATGAATATTAAACAATGCGCAG-3′ 5′-AGAACCATTTATTAGCTTTGTTGC-3′	341	Moser et al. (48)

T, Trypanosoma; s.l., sensu lato.

control, a PhiX library was denatured and diluted according to the manufacturer's instructions, and 1.2 µl were added to the sample of denatured and diluted pooled libraries before loading. Finally, the libraries were sequenced on a high-output flow cell (400M clusters) using the NextSeq® 500/550 High Output Kit v2 (150 cycles; Illumina) in paired-end 75/75 nt mode, according to the manufacturer's instructions.

Datasets for the reads are available from the NCBI, GEO submission, accession number GSE98989.

Bioinformatics Analysis

Workflow

The successive tasks of the bioinformatics analysis were managed using a Snakemake workflow (49). This workflow enables the reproduction of all analyses from the raw read files and is available from the supporting Web site.[1]

Reference Genomes

The *G. palpalis* genomic sequences (Glossina-palpalis-IAEA_SCAFFOLDS_GpapI1.fa) and annotations (Glossina-palpalis-IAEA_BASEFEATURES_GpapI1.1.gff3) were downloaded from VectorBase (50). For the annotation of Tc genes, the reference genome (TriTrypDB-9.0_TcongolenseIL3000.gff) was downloaded from TriTrypDB (51), whereas the *Drosophila melanogaster* reference genome (Drosophila_melanogaster.BDGP6.30.gff3) was downloaded from Flybase (52).

Lane Merging

Since each sample was sequenced on four lanes, the original fastq-formatted read files were merged to produce two files per sample (one for each paired-end extremity).

Read Quality Control

FastQC[2] was run on the raw reads in order to check their quality.

Read Mapping

Raw reads were mapped onto the genome with the local alignment algorithm Subread-align (53) in paired-ends mode with at most 10 mismatches. Read mapping statistics were computed using samtools flagstats (54) and are summarized in Table S1 in Supplementary Material.

Read Counts per Gene

The number of read pairs (fragments) per gene was counted using the featureCounts tool from the Subread package (55), including the option "feature type" to only count reads overlapping transcripts.

Visualization

Genome maps were generated using the Integrative Genomic Viewer (56).

Detection of DEGs

Differential gene expression analysis was performed using the SARTools R package (57), which separately runs DESeq2 (58) and egdeR (59) as well as generates readable reports.

Identification of Orthologs between Gpp and *D. melanogaster*

Because the *G. palpalis* genome is inadequately assembled and annotated, our functional interpretation of the DEGs relied on a comparative genomics approach. This was based on the identification of bidirectional best hits (BBH) between all sequences of *G. palpalis* and *D. melanogaster* (assembly BDGP6). BBH were identified using blastp (60) and the BLOSUM45 substitution matrix, and by setting a threshold of 10^{-5} on the expected score.

Functional Enrichment of DEGs

Identification of functions associated with the DEGs was based on *Drosophila* orthologs of the DEGs (ortho-DEG). Functional enrichment was separately performed using the DAVID (61) and g:Profiler (62) tools. The Bonferroni correction was used to obtain the enrichments of these functions, with a threshold set at 10^{-3}.

Pathway Mapping of DEGs

Drosophila orthologs of the DEGs were loaded into the metabolic cellular overview of BioCyc (63) in order to highlight the pathways affected by the infection.

Statistical Treatment of Entomological Data

Entomological data, as well as all other calculations, were evaluated using the statistical package SPSS version 2.0. Spreadsheets were made using Microsoft Office Excel 2007.

RESULTS

Entomological Data

A total of 1,991 tsetse flies were collected during the entomological survey (775 flies from Campo and 1,216 flies from Bipindi). The Campo fly population was composed of Gpp (95.61%), *Glossina caliginea* (2.06%), *Glossina palicera* (1.87%), and *Glossina nigrofusca* (0.52%). Two tsetse fly species were identified at the Bipindi focus: Gpp (99.34%) and *G. palicera* (0.66%). The mean apparent density was 4.24 flies per trap per day; however, this parameter was highly variable between the different villages and was higher in Bipindi (8.1) than in Campo (3.52) (**Table 2**). The frequency of teneral flies was typically low in both Bipindi (0.08%) and Campo (1.16%). These data are roughly in line with data recorded in 2007/2008 (13), although the rate of teneral flies was much lower in the present study. Only 1,245 of the trapped 1,991 tsetse flies were dissected, since 10 flies were teneral and 736 flies were desiccated.

PCR Identification of Trypanosome Species in the Tsetse Midgut

The number of flies carrying single or mixed trypanosome infections is presented in **Table 3**. Of the 337 Campo flies analyzed, 25 (7.41%) were infected by the Tc "forest" type, 16 (4.74%) by the

[1] https://github.com/rioualen/gene-regulation.

[2] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

TABLE 2 | Entomological field data from the Bipindi and Campo foci.

Focus	Village	Number of traps	Number of tsetse flies captured	ADT	Number of teneral tsetse flies (%)	Number of tsetse flies dissected
Campo	Ipono	15	161	2.68	4 (2.48)	110
	Beach	15	341	4.55	3 (0.87)	264
	Mabiogo	18	273	3.21	2 (0.73)	228
	Total Campo	48	775	3.52	9 (1.16)	602
Bipindi	Bidjouka	23	608	5.28	1 (0.16)	278
	Lambi	12	486	8.1	0 (0)	303
	Ebimimbang	15	122	1.74	0 (0)	72
	Total Bipindi	50	1,216	4.96	1 (0.50)	653
Total		98	1,991	4.24	10 (0.50)	1,255

ADT, apparent density per trap per day.

TABLE 3 | Number of *Trypanosoma congolense* s.l. simple and mixed infections by village.

Focus	Village	Number of tsetse flies analyzed	Number of flies infected with TcF	Number of flies infected with TcS	Number of flies carrying a mixed infection
Campo	Ipono	63	2	1	1
	Beach	170	15	8	7
	Mabiogo	104	8	7	6
	Total Campo	337	25	16	14
Bipindi	Bidjouka	40	1	0	0
	Lambi	33	5	0	0
	Ebimimbang	11	1	0	0
	Total Bipindi	84	7	0	0
Total		421	32	16	14

TcF, T. congolense "forest" type; TcS, T. congolense "savannah" type.

Tc "savanah" type, and 14 (4.15%) by both parasites. In contrast, Bipindi flies only carried the Tc "forest" type (8.33%). Table S1 in Supplementary Material details the characteristics of the different samples including those used for transcriptomic analyses.

Raw Data

The sequencing of libraries produced a total of 400 million reads (theoretically 40 million reads per sample), which represent a satisfactory sequencing depth for subsequent differential gene expression analysis (**Figure 1**). Out of the 328 raw clusters generated, 77.6% were successfully filtered, with each sample producing 50–72 million clusters (mean: 61 million clusters) (**Figure 1**). Sequencing also revealed a total of 31,320 contigs distributed in 3,926 scaffolds with a mean size of 96,817 bp (varying in size from 545 bp to 3.6 Mb).

Mapping on Gpp

RNA-seq sequencing produced an average of 124 million reads per sample. From this, 111.45 million reads (83.6%) were mapped onto the genome of Gpp, 103.68 M (73.7%) of which were properly paired (**Table 4**).

Mapping on Tc

Reads were also mapped onto the trypanosome genome in order to validate the infection status of the samples, as well as to investigate the role played by the trypanosome in this molecular dialog. Since trypanosome cells represented a small fraction of the analyzed material, only a smaller fraction of the reads could be mapped. Specifically, 300,302.5 (0.24%) reads were mapped from an average of 124 million reads per sample. This resulted in 137,305.4 (0.11%) properly paired reads and 157,712.5 (0.12%) singletons (**Table 4**).

DEGs between Infected and Non-Infected Flies

We used DESeq2 (**Table 5**) to detect genes that were differentially expressed between the five infected and five non-infected samples. When a Benjamini–Hochberg corrected *p*-value lower than 0.05 was applied, 524 genes were observed to be significantly differentially expressed in infected vs. non-infected flies, among which 285 genes were downregulated and 239 were upregulated. A similar DEG analysis was performed using edgeR (**Table 5**), which identified only 20 downregulated genes and 53 upregulated genes. **Figure 2** presents the volcano plots produced by DESeq2 (**Figure 2A**) and edgeR (**Figure 2B**); genes that were significantly (p-value <0.05) differentially expressed and with a fold change of log2 (fold change) >2 (i.e., upregulated genes) or log2 (fold change) <-2 (i.e., downregulated genes) were considered relevant.

Functional Annotation

To understand the roles of DEGs associated with tsetse fly infection by Tc, identifiers of the *Drosophila* orthologs of the *Glossina* DEGs were examined using the DAVID-functional enrichment tool. Separately, we analyzed the 290 downregulated and 213 upregulated genes reported by DESeq2 and identified 207 *Drosophila* best hits (121 downregulated and 86 upregulated genes). The same analysis by edgeR only resulted in 25 DEG orthologs (6 downregulated and 19 upregulated genes). This reduced number of orthologs could possibly be due to the incomplete assembly and annotation of the *Glossina* genome and/or the high stringency of the BBH criterion (which discards the case where several tsetse fly proteins have the same closest hit in *Drosophila*). These DEGs were examined using DAVID, which compares the list of input genes with a variety of functional annotations. This analysis was focused on the three primary categories of the Gene

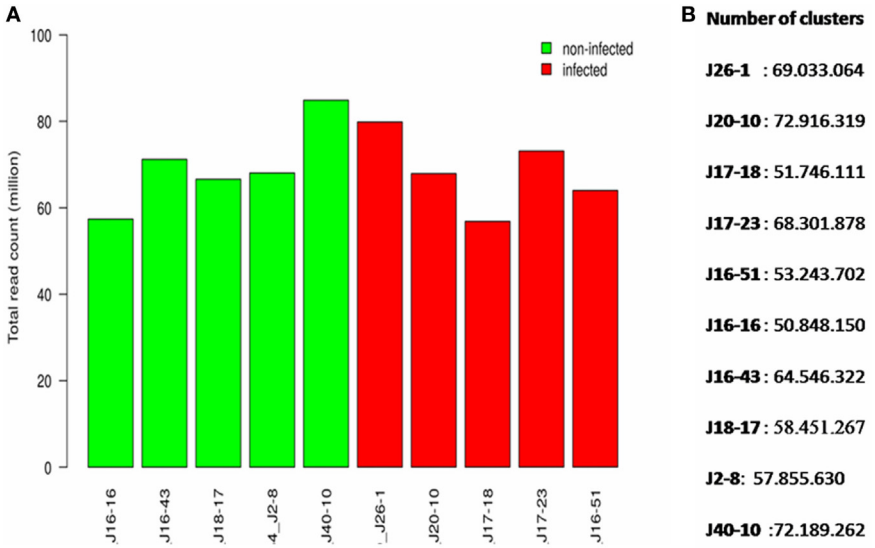

FIGURE 1 | The score quality of different sequences of clusters, presented base by base. Green indicates a good quality. **(A)** Total read count per sample (millions). **(B)** Number of clusters per sample.

TABLE 4 | Read mapping statistics.

Mapping on	Mapped reads (%)	Properly paired reads (%)	Singleton reads (%)	QC-passed reads
Glossina	111.45 M (89.87)	103.68 M (83.61)	3.62 M (2.92)	123.8 M
Trypanosoma	0.3 M (0.24)	0.13 M (0.11)	0.15 M (0.12)	123.8 M

M, millions of reads.

TABLE 5 | Differentially expressed genes.

	Downregulated	Upregulated	Total
DESeq2	285	239	524
edgeR	20	53	73
Common	20	46	66
Total	285	246	531

Ontology annotation: biological process (BP), molecular function (MF), and cellular component (CC). The list of different features is provided in **Table 6**.

The 285 downregulated genes identified by DESeq2 in flies infected with Tc mainly belonged to the BP category, in which the major functional classes were RNA processing (58 genes; 23.48%), ribosome biogenesis (24 genes; 9.71%), and translation (13 genes; 5.26%); the other genes corresponded to several poorly represented classes. The MF category included the functional RNA binding classes (20 genes; 8.09%) and catalytic activity (25 genes; 10.12%). Finally, the CC category included the intracellular lumen (48 genes; 19.43%), non-membrane-bound organelle (34 genes; 13.75%), and ribonucleoprotein complex (25 genes; 10.12%) functional classes (**Figure 3**).

In addition, 239 DEGs were overexpressed in Tc-infected tsetse flies. These DEGs encoded proteins corresponding to the same three primary ontology categories. The BP category include the neuron morphogenesis functional class (83 genes; 38.96%), amino acid biosynthesis (24 genes; 11.26%), and carboxylic acid biosynthesis (10 genes; 4.7%). The MF category included the iron binding (64 genes, 30%) and catalytic activity (24 genes; 11.26%) functional classes. Finally, the CC category only included the mitochondrion functional class (8 genes; 3.75%).

As already shown for DEGs, edgeR provides a much lower number of functional annotations than DESeq2. Here, using edgeR, fly genes that displayed an increased expression in response to Tc infection were found to belong to the MF category, with only three functional classes: phospholipase activity (2 genes; 10.5%), lipase activity (2 genes; 10.5%), and carboxylesterase activity (2 genes; 10.5%). Finally, expression was decreased for only two genes belonging to the one-carbon metabolic process term (BP category).

Functional Enrichment of DEGs

To make our analysis more focused and efficient, a functional enrichment was performed to refine the list of tsetse fly DEGs in which expression was influenced by Tc infection. We therefore combined fold enrichment and the *p*-value at a 5% threshold, which allowed applying a Bonferroni correction to eliminate false positives. The Bonferroni correction threshold was fixed at $\alpha = 10^{-2}$, and all functionality with a Bonferroni value below this threshold was considered to be due to trypanosome infection.

Following this correction, 16 functional classes were found to be selectively altered by trypanosome infection. These classes are mainly involved in the transcription process, including (a) RNA related processes (rRNA processing, rRNA metabolic process, ncRNA processing, ncRNA metabolic processes, and RNA processing), involving 63 DEGs; (b) monitoring processes related to the synthesis of the ribonucleoprotein complex (ribonucleoprotein complex biogenesis and ribonucleoprotein

FIGURE 2 | Comparisons of significantly differentially expressed genes. DESeq2 **(A)** and edgeR **(B)** results are illustrated by volcano plots, in which the differentially expressed features are shown in red. Upregulated genes are thus observed as positive values, and downregulated genes as negative values. Triangles correspond to features where the log of the adjusted *p*-value is too low or too high to be displayed on the plot.

complex), involving 29 DEGs; (c) RNA binding, involving 24 DEGs; (d) nucleolus biogenesis (nucleolus and nuclear lumen), involving 23 DEGs; (e) ribosome synthesis, involving 17 DEGs; and (f) eukaryotic translation factor 3 complex, involving 4 DEGs. In contrast to the transcription process, which involved 160 DEGs, the BPs of organic acid synthesis (amine biosynthetic process, cellular amino acid biosynthetic process, carboxylic acid biosynthetic process, and organic acid biosynthetic process) that were found to be activated by trypanosome infection only involved 21 DEGs (**Table 7**).

Associated Metabolic Pathways

The BioCyc metabolic map (**Figure 4**) and **Table 8** illustrate the different pathways that the DEGs are involved in. Among these, the amino acid biosynthesis pathway (which includes the biosynthesis of L-glutamine, L-glutamate, L-serine, L-asparagine, L-aspartate, etc.) is controlled by genes that were shown to be overexpressed following trypanosome infection in the flies. Similarly, DEGs associated with the nucleotide biosynthesis pathway were overexpressed following trypanosome infection, especially uridine monophosphate, an RNA monomer. In contrast, genes involved in the pentose phosphate pathway, namely those implicated in the synthesis of D-ribose 5-phosphate, were downregulated. Phosphorylated pentose is converted by ribose phosphate diphosphokinase into phosphoribosylpyrophosphate, a precursor of nucleotide synthesis. Finally, regarding the carbohydrate biosynthesis pathway, we observed an overexpression of genes encoding malate dehydrogenase, which converts malate into pyruvate.

Interestingly, a large number of up- and downregulated DEGs were related to a given biosynthetic process meaning that upregulated DEGs encoding amino acids (such as tyrosine, serine, glutamine, and several others) were found in the amino acid synthesis pathway. Other overexpressed genes that were identified are involved in the biosynthesis of galactosyltransferase, *N*-acetylglucosamine, and beta-1,4-manosylglycolipide, which are all molecules that interact with the immune system of the tsetse fly (64–66). In contrast, genes involved in the biosynthesis of cytokines were downregulated in trypanosome-infected flies, as compared to non-infected flies. Similarly, genes involved in folate metabolism (e.g., the biosynthesis of formyltetrahydrofolate dehydrogenase), the main source of energy in flies, were downregulated.

Finally, DEGs involved in the transport of several molecules from the extracellular space toward the cytosol compartment were upregulated. This transport includes molecules with a role in cell nutrition, and nutrients such as lipids, but also ATP, succinate, and L-carnitine (which participates in the degradation of fats).

DISCUSSION

Understanding the mechanisms involved in tsetse fly susceptibility or refractoriness to trypanosome infection is crucial for developing a novel anti-vector based strategy to control the spread of sleeping sickness and nagana. One recent study was performed within this context to identify genes in Gpg associated with its susceptibility or refractoriness to Tbg infection, using an RNA-seq approach (17). The underlying hypothesis was that some of the genes involved in controlling fly susceptibility/resistance to trypanosome infection could be targeted in order to increase the refractoriness of the fly, thereby decreasing its vector competence while enabling the development of an anti-vector strategy against

TABLE 6 | Functional annotation of differentially expressed genes (DEGs).

Category	Term	Number of DEG	*p*-value	Log2 (fold enrichment)
DEGs identified with DESeq2				
GOTERM_BP_FAT	Amine biosynthetic process	6	2.80E−05	15.70
SP_PIR_KEYWORDS	Amino acid biosynthesis	3	2.00E−03	42.80
GOTERM_MF_FAT	ATPase activity, uncoupled	5	9.10E−02	−2.90
GOTERM_MF_FAT	ATP-dependent helicase activity	4	5.80E−02	−4.50
GOTERM_MF_FAT	ATP-dependent RNA helicase activity	4	1.10E−02	−8.40
GOTERM_BP_FAT	Axon guidance	6	2.90E−03	5.90
GOTERM_BP_FAT	Axonal defasciculation	2	8.70E−02	21.50
GOTERM_BP_FAT	Axonogenesis	6	1.20E−02	4.20
GOTERM_BP_FAT	Carboxylic acid biosynthetic process	5	9.90E−04	10.80
GOTERM_MF_FAT	Cation binding	18	6.10E−02	1.50
GOTERM_BP_FAT	Cell morphogenesis	7	8.10E−02	2.20
GOTERM_BP_FAT	Cell morphogenesis involved in differentiation	6	5.70E−02	2.80
GOTERM_BP_FAT	Cell morphogenesis involved in neuron differentiation	6	5.00E−02	2.90
GOTERM_BP_FAT	Cell motion	6	5.20E−02	2.90
GOTERM_BP_FAT	Cell part morphogenesis	6	7.60E−02	2.60
GOTERM_BP_FAT	Cell projection morphogenesis	6	7.20E−02	2.60
GOTERM_BP_FAT	Cell recognition	3	4.40E−02	8.70
GOTERM_BP_FAT	Cellular amino acid biosynthetic process	5	5.40E−05	22.40
GOTERM_BP_FAT	Chemical homeostasis	3	7.70E−02	6.30
GOTERM_MF_FAT	Coenzyme binding	4	6.40E−02	4.30
GOTERM_CC_FAT	Cytosol	6	9.30E−02	−2.40
INTERPRO	DEAD-like helicase, N-terminal	4	6.20E−02	−4.30
GOTERM_BP_FAT	Defasciculation of motor neuron axon	2	6.20E−02	30.70
SMART	DEXDc	4	4.30E−02	−4.90
GOTERM_BP_FAT	Di-, tri-valent inorganic cation transport	3	3.40E−02	10.10
INTERPRO	DNA/RNA helicase, C-terminal	4	6.20E−02	−4.30
INTERPRO	DNA/RNA helicase, DEAD/DEAH box type, N-terminal	4	1.60E−02	−7.40
GOTERM_MF_FAT	Electron carrier activity	5	2.50E−02	4.40
GOTERM_MF_FAT	Enzyme inhibitor activity	3	9.90E−02	5.50
GOTERM_CC_FAT	Eukaryotic translation initiation factor 3 complex	4	4.40E−04	−24.50
GOTERM_MF_FAT	Glutamate synthase activity	2	2.10E−02	93.60
GOTERM_BP_FAT	Glutamine family amino acid biosynthetic process	2	9.50E−02	19.60
GOTERM_BP_FAT	Glutamine family amino acid metabolic process	3	1.50E−02	15.40
GOTERM_BP_FAT	Glutamine metabolic process	2	6.20E−02	30.70
SP_PIR_KEYWORDS	Helicase	4	6.80E−02	−4.20
INTERPRO	Helicase, superfamily 1 and 2, ATP-binding	4	6.00E−02	−4.40
SMART	HELICc	4	4.30E−02	−4.90
SP_PIR_KEYWORDS	Heme	3	8.40E−02	6.10
GOTERM_MF_FAT	Heme binding	4	2.90E−02	5.90
GOTERM_BP_FAT	Homeostatic process	4	8.90E−02	3.70
SP_PIR_KEYWORDS	Hydrolase	14	6.20E−02	1.70
SP_PIR_KEYWORDS	Initiation factor	3	3.40E−02	−10.10
GOTERM_CC_FAT	Intracellular non-membrane-bounded organelle	17	3.60E−03	−2.00
GOTERM_CC_FAT	Intracellular organelle lumen	12	1.00E−02	−2.30
GOTERM_MF_FAT	Ion binding	18	6.40E−02	1.50
GOTERM_MF_FAT	Iron ion binding	6	1.10E−02	4.30
KEGG_PATHWAY	Limonene and pinene degradation	3	6.60E−02	6.60
GOTERM_MF_FAT	Lipase activity	3	9.30E−02	5.70
SP_PIR_KEYWORDS	Lipid-binding	2	4.80E−02	39.90
GOTERM_CC_FAT	Membrane-enclosed lumen	12	1.20E−02	−2.20
GOTERM_MF_FAT	Metal ion binding	18	5.10E−02	1.50
INTERPRO	Mitochondrial substrate carrier	3	3.10E−02	10.60
INTERPRO	Mitochondrial substrate/solute carrier	3	3.30E−02	10.20
GOTERM_CC_FAT	Mitochondrion	8	6.00E−02	2.10
GOTERM_BP_FAT	Mitotic spindle elongation	4	6.50E−02	−4.20
GOTERM_MF_FAT	mRNA binding	6	3.70E−02	−3.20
GOTERM_BP_FAT	ncRNA metabolic process	12	3.10E−07	−7.50
GOTERM_BP_FAT	ncRNA processing	12	6.00E−09	−10.80
GOTERM_BP_FAT	Neuron development	6	9.30E−02	2.40
GOTERM_BP_FAT	Neuron projection development	6	5.00E−02	2.90
GOTERM_BP_FAT	Neuron projection morphogenesis	6	4.90E−02	2.90
GOTERM_BP_FAT	Neuron recognition	3	4.40E−02	8.70
GOTERM_BP_FAT	Nitrogen compound biosynthetic process	6	1.10E−02	4.20

(*Continued*)

TABLE 6 | Continued

Category	Term	Number of DEG	*p*-value	Log2 (fold enrichment)
GOTERM_CC_FAT	Non-membrane-bounded organelle	17	3.60E−03	−2.00
GOTERM_CC_FAT	Nuclear lumen	12	4.10E−04	−3.30
GOTERM_CC_FAT	Nucleolus	11	2.20E−09	−13.50
SP_PIR_KEYWORDS	Nucleus	18	1.90E−02	−1.80
GOTERM_CC_FAT	Organelle lumen	12	1.00E−02	−2.30
GOTERM_BP_FAT	Organic acid biosynthetic process	5	9.90E−04	10.80
GOTERM_BP_FAT	Oxidation reduction	8	3.10E−02	2.50
GOTERM_MF_FAT	Phospholipase activity	3	3.60E−02	9.70
SP_PIR_KEYWORDS	Phosphoprotein	16	4.90E−02	−1.70
GOTERM_BP_FAT	Positive regulation of protein kinase cascade	2	8.50E−02	−22.30
GOTERM_CC_FAT	Preribosome	3	3.40E−03	−31.50
SP_PIR_KEYWORDS	Protein biosynthesis	5	1.70E−02	−5.00
GOTERM_BP_FAT	Pseudouridine synthesis	2	9.90E−02	−19.10
GOTERM_MF_FAT	Purine NTP-dependent helicase activity	4	5.80E−02	−4.50
GOTERM_BP_FAT	Regulation of translational initiation	3	1.10E−02	−18.20
SP_PIR_KEYWORDS	Ribonucleoprotein	6	1.30E−02	−4.20
GOTERM_CC_FAT	Ribonucleoprotein complex	13	6.50E−05	−3.70
GOTERM_BP_FAT	Ribonucleoprotein complex biogenesis	12	2.80E−09	−11.60
GOTERM_BP_FAT	Ribosome biogenesis	12	7.10E−11	−16.00
SP_PIR_KEYWORDS	Ribosome biogenesis	5	1.20E−04	−18.60
GOTERM_MF_FAT	RNA binding	14	9.00E−05	−3.60
GOTERM_MF_FAT	RNA helicase activity	4	1.50E−02	−7.50
INTERPRO	RNA helicase, ATP-dependent, DEAD-box, conserved site	3	4.20E−02	−9.00
INTERPRO	RNA helicase, DEAD-box type, Q motif	4	6.50E−03	−10.20
GOTERM_BP_FAT	RNA modification	3	7.10E−02	−6.70
GOTERM_BP_FAT	RNA processing	14	1.40E−05	−4.30
SP_PIR_KEYWORDS	RNA-binding	10	2.20E−05	−6.40
GOTERM_MF_FAT	RNA-dependent ATPase activity	4	1.10E−02	−8.40
GOTERM_BP_FAT	rRNA metabolic process	10	2.10E−10	−22.30
GOTERM_BP_FAT	rRNA modification	2	9.90E−02	−19.10
GOTERM_BP_FAT	rRNA processing	10	2.10E−10	−22.30
SP_PIR_KEYWORDS	rRNA processing	5	1.50E−04	−17.70
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, and catabolism	3	3.40E−02	8.60
GOTERM_CC_FAT	Small nuclear ribonucleoprotein complex	4	1.80E−02	−6.80
GOTERM_CC_FAT	Small nucleolar ribonucleoprotein complex	4	4.20E−05	−49.00
GOTERM_CC_FAT	Small-subunit processome	3	1.70E−03	−44.10
GOTERM_BP_FAT	Spindle elongation	4	6.70E−02	−4.20
GOTERM_MF_FAT	Tetrapyrrole binding	4	2.90E−02	5.90
GOTERM_BP_FAT	Translation	9	2.10E−02	−2.60
GOTERM_BP_FAT	Translational initiation	4	1.80E−02	−7.00
SP_PIR_KEYWORDS	Transport	7	5.60E−02	2.50
SP_PIR_KEYWORDS	WD repeat	7	1.00E−02	−3.80
SMART	WD40	7	8.30E−03	−3.80
INTERPRO	WD40 repeat	7	1.80E−02	−3.30
INTERPRO	WD40 repeat, conserved site	5	5.30E−02	−3.50
INTERPRO	WD40 repeat, region	6	2.60E−02	−3.60
INTERPRO	WD40 repeat, subgroup	7	7.80E−03	−4.00
INTERPRO	WD40/YVTN repeat-like	8	5.90E−03	−3.60
DEGs identified with DESeq2				
GOTERM_BP_FAT	One-carbon metabolic process	2	6.00E−02	−27.2
GOTERM_MF_FAT	Carboxylesterase activity	2	8.40E−02	20.4
GOTERM_MF_FAT	Lipase activity	2	7.90E−02	21.7
GOTERM_MF_FAT	Phospholipase activity	2	4.80E−02	36.6

Categories: GOTERM_BP_FAT, biological process; GOTERM_CC_FAT, cellular component; GOTERM_MF_FAT, molecular function.
SMART & INTERPRO, protein domains; SPIR_KEYWORD, protein information resource provided by SWISSPROT and UniProt.
CDG_ONTOLOGY, cluster orthology group.
Black fonts: downregulated DEGs; red fonts: upregulated DEGs.

the disease. As this analysis was performed with insectary flies artificially infected with trypanosomes, it was necessary to verify that similar molecular events occur in field flies naturally infected by trypanosome vs. non-infected flies. To accomplish this, we have chosen the Gpp/Tc couple, whose prevalence (even in HAT foci) is often higher than observed with the Gpp/Tbg couple.

As in the previous study (17), we employed an RNA-seq approach. This provided satisfactory results regarding the

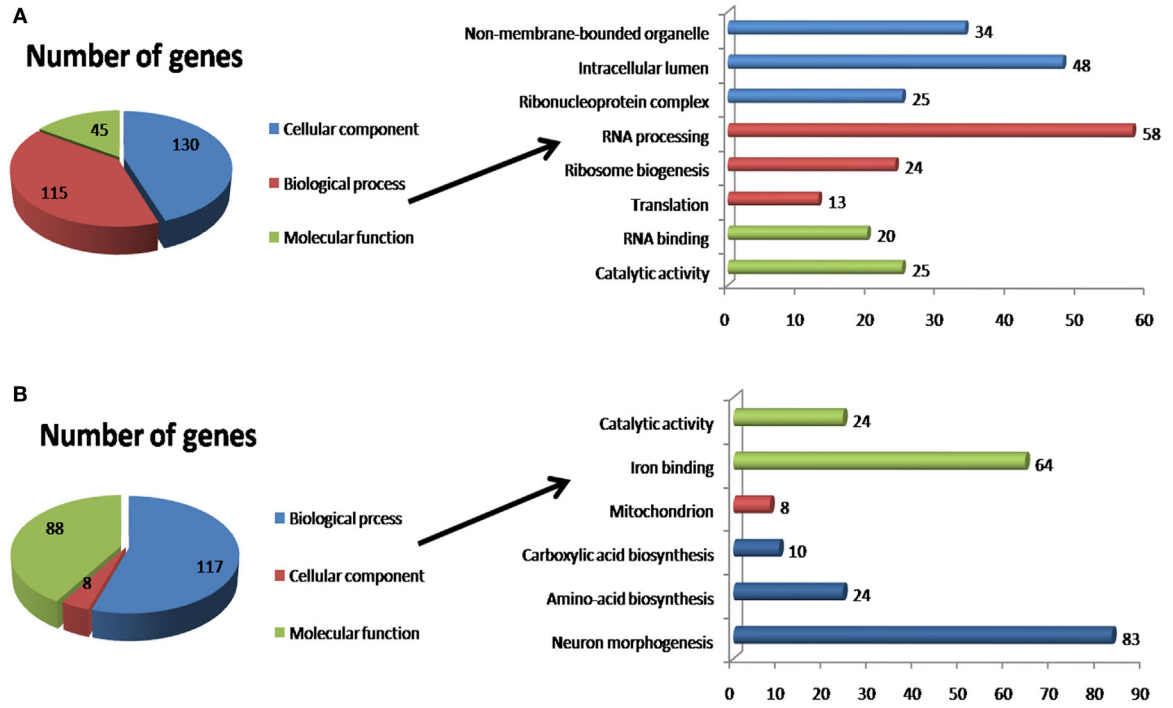

FIGURE 3 | Functional annotation of differentially expressed genes using DAVID. **(A)** Downregulated genes identified by DESeq2. **(B)** Upregulated genes identified by DESeq2. The *x*-axis indicates the number of genes enriched for the term, and the *y*-axis indicates the functional classes that were differentially expressed.

TABLE 7 | Bonferroni correction for differentially expressed genes (DEGs) enriched functionalities.

Category	Term	Number of DEGs	Bonferroni
	Downregulated DESEQ2		
GOTERM_BP_FAT	Ribosome biogenesis	12	3.90E−08
GOTERM_BP_FAT	rRNA processing	10	5.80E−08
GOTERM_BP_FAT	rRNA metabolic process	10	5.80E−08
GOTERM_BP_FAT	Ribonucleoprotein complex biogenesis	12	5.10E−07
GOTERM_BP_FAT	ncRNA processing	12	8.30E−07
GOTERM_BP_FAT	ncRNA metabolic process	12	3.40E−05
GOTERM_BP_FAT	RNA processing	14	1.20E−03
GOTERM_CC_FAT	Nucleolus	11	2.00E−07
GOTERM_CC_FAT	Small nucleolar ribonucleoprotein complex	4	1.90E−03
GOTERM_CC_FAT	Ribonucleoprotein complex	13	2.00E−03
GOTERM_CC_FAT	Nuclear lumen	12	9.40E−03
GOTERM_CC_FAT	Eukaryotic transl. initiation factor 3 complex	4	8.00E−03
GOTERM_MF_FAT	RNA binding	14	1.40E−02
SP_PIR_KEYWORDS	RNA-binding	10	1.80E−03
SP_PIR_KEYWORDS	Ribosome biogenesis	5	4.70E−03
SP_PIR_KEYWORDS	rRNA processing	5	4.00E−03
	Upregulated DESEQ2		
GOTERM_BP_FAT	Amine biosynthetic process	6	8.80E−03
GOTERM_BP_FAT	Cellular amino acid biosynthetic process	5	8.50E−03
GOTERM_BP_FAT	Carboxylic acid biosynthetic process	5	9.90E−02
GOTERM_BP_FAT	Organic acid biosynthetic process	5	9.90E−02

Categories: GOTERM_BP_FAT, biological process; GOTERM_CC_FAT, cellular component; GOTERM_MF_FAT, molecular function. SMART & INTERPRO, protein domains; SPIR_KEYWORD, protein information resource provided by SWISSPROT and UniProt.

mapping of reads on the fly genome, since nearly 75% of the 124 million reads (mean number per sample) were properly paired. However, this was not the case for the Tc genome, which displayed an average of less than 1% of properly mapped reads. This result is not surprising, given that our pre-sequencing manipulations did not target the trypanosome genome. Other contributing factors include the preparation of libraries, which was based on poly(a) selection using Oligo(dT) beads (67) and

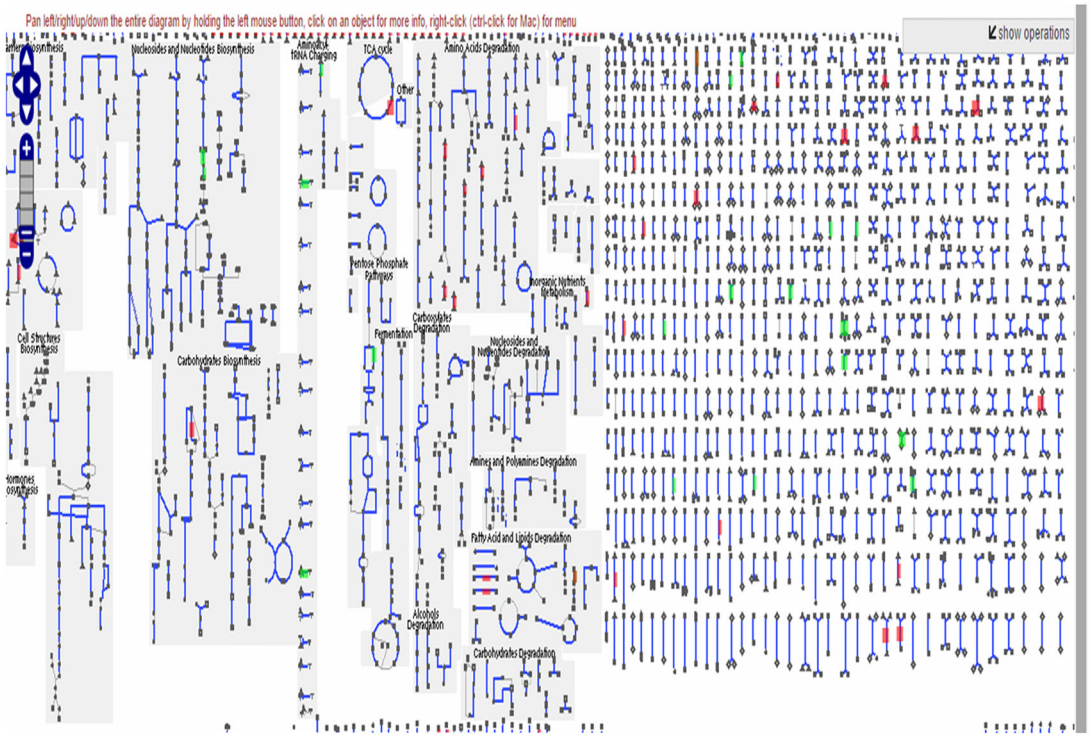

FIGURE 4 | BioCyc metabolic map illustrating the different differentially expressed genes involved in tsetse fly metabolic pathways. The genes activated by the infection are displayed in red, whereas the repressed genes are displayed in green.

the *Trypanosoma* genome, which is organized in polycistron units (68).

The infection duration in artificial infection experiments was monitored in the previous report, revealing that the levels of over- or under-expression in DEGs at 3, 10, or 20 days after infection can vary largely (17). In contrast, the present study was performed on tsetse flies sampled in the field, thus neither their age- nor the time-elapsed post-fly infection could be measured. Consequently, the recorded results represent an average level of DEG expression in Gpp flies that may have been infected by trypanosomes (Tc) recently or in the past several days. Similarly, it is possible that non-infected samples could group together flies that were truly never infected with flies that have eliminated their ingested trypanosomes (i.e., "self-cured" or "refractory" flies).

Despite this uncertainty, the results clearly demonstrate a very strong interaction between the parasite and its host/vector, resulting in major transcriptomic changes in the fly. For instance, the level of the "rRNA processing" function in infected vs. non-infected flies was as low as $\log2 = -22.3$. In other words, when the infected flies were captured and dissected, the "rRNA processing" function was $2^{22.3} = 5.16 \times 10^6$ fold lower than the value recorded in non-infected flies sampled at the same time and in the same areas. This indicates that the "rRNA processing" function was not effective at that time, and that at least 1 of the 10 DEGs shown to be involved in this function was essentially no longer expressed; however, this does not mean that it could not be reactivated at a later point in a fly's life.

In this study, we reported that 290 fly genes were down-regulated and 213 were upregulated. This type of imbalance is expected to be induced either by a parasite or a symbiont, and to result in disturbing the host metabolism in such a way as to facilitate microorganism establishment (69, 70). In agreement with this, we observed the repression or non-activation of transcription genes that may allow the trypanosome to alter its host's transcription steps. Furthermore, certain metabolic pathways were downregulated that can prevent the host from synthesizing factors (proteins or metabolites) needed to fight infection (71). In this context and concerning the "Biological Process," "Cellular Component," and "Molecular Function" categories, most of the functional classes were associated with the host transcription/translation machinery (translation, RNA binding, ribonucleoprotein complex, ribosome processing helicase, etc.). Only 10% of the DEGs were related to "Catalytic activities." In contrast, overexpressed DEGs were involved with catalytic activities, cellular activities (morphogenesis, motion, and cell recognition) and, surprisingly, neuron activities (neuron development and neuron recognition). This is coherently illustrated in **Table 6**, where those "terms" that were over- or under-represented in DEGs (equal to or higher than a fourfold change) and that were identified through functional annotation on the *D. melanogaster* database have been alphabetically classified.

Our identification of the metabolic pathways associated with infection (**Table 8**) highlights the importance of the amino acid

TABLE 8 | Metabolic pathways associated with fly infection.

Pathways	Functions
Amino acid biosynthesis pathway	Synthesis of L-glutamine
	Synthesis of L-glutamate
	Synthesis of L-serine
	Synthesis of L-asparagine
	Synthesis of L-aspartate
	Synthesis of L-valine
	Synthesis of L-proline
	Synthesis of L-isoleucine
Nucleotide biosynthesis pathway	Biosynthesis of uridine monophosphate
Pentose phosphate pathway	Repression of D-ribose-5-phosphate
	Synthesis of orotidine
	Synthesis of pyruvate
Carbohydrate biosynthesis pathway	Biosynthesis of pyruvate
Isolated reactions	Synthesis of tyrosine
	Synthesis of serine
	Synthesis of L-glutamine
	Synthesis of L-glutamate
	Synthesis of L-serine
	Synthesis of L-asparagine
	Synthesis of L-methionine
	Synthesis of L-aspartate
	Synthesis of L-valine
	Synthesis of L-isoleucine
	Synthesis of *N*-acetylglucosamine
	Synthesis of galactosyltransferase
	Synthesis of beta-1,4-manosylglycolipid
	Synthesis of S-adenosyl-L-homocysteine
	Repression of immune cytokines
	Repression of formyltetrahydrofolate DH
Transport	Transport of lipids
	Transport of ATP
	Transport of succinate
	Transport of L-carnitine
	Transport of GTP
	Transport of acid dicarboxylic
	Transport of acid monocarboxylic
	Transport of L-tyrosine
	Transport of L-serine
	Transport of Ca^{2+}
	Transport of nucleotide
	Transport of Cyclic GMP
	Transport of proteinogenic amino acid
	Transport of NAD^{+}
	Transport of L-fructose
	Transport of GDP
	Transport of fatty acid
	Transporter activity
	Calcium ion binding

biosynthesis pathway. This provides the parasite with a broad range of amino acids that serve as a valuable source of energy, as previously reported for *T. cruzi*, the parasite causing Chagas disease (72), and microsporidia, a parasite of fishes (73). One such amino acid that we identified is proline, whose synthesis was overexpressed in Gpg infected with Tbg in comparison to non-infected flies (17). We also observed an increase in the biosynthesis of *N*-acetyl-glucosamine, a molecule that can affix itself to lectins that possess a sugar recognition area (74). This process inactivates tsetse fly lectins that are otherwise lethal to procyclic forms of trypanosomes (65), which consequently favor trypanosome installation in the fly vector. Interestingly, this mechanism has also been reported in Gpg infected with Tbg.

As reported in experimental Gpg insectary flies infected with Tbg, we have shown that field-collected Gpp naturally infected with Tc exhibit a strong cytokine repression in comparison to uninfected tsetse flies. This result indicates that strong alteration of the immune system occurred in infected flies, favoring parasite installation. In addition, *Trypanosoma* infection repressed 34 DEGs encoding non-membrane-bound organelles and 48 DEGs encoding expression of the intracellular lumen (an organelle consisting of chromatin). This type of scenario has also been described for the herpes simplex virus type 1, which can modify the structure and dynamics of chromatin through posttranscriptional modification of histone or other chromatin-forming proteins, contributing to their establishment within the host (75).

This is the first study to evaluate the transcriptomic events associated with infection by the Tc trypanosome in field Gpp tsetse flies. Our results establish that field flies naturally infected by trypanosomes display disruptions in their gene expression that result in either overexpression or under-expression of certain fly genes, as similarly observed in experimentally infected insectary flies. Furthermore, molecular disruptions occur in Gpp when infected with Tc, just as in Gpg that have been artificially infected with Tbg. Importantly, these findings indicate that different *Glossina* species infected with different trypanosome species under different conditions display comparable molecular reactions, which validate the use of experimental host/parasite couples for future research programs.

AUTHOR CONTRIBUTIONS

AG conceived and designed the experiments. JMTN, FN, BL, GK-N, and NF-N performed the experiments. JMTN, CR, JH, and AG analyzed the data. BL and AG contributed reagents/materials/analysis tools. JMTN, FN, JH, and AG wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00876/full#supplementary-material.

REFERENCES

1. Buguet A, Bert J, Tapie P, Bogui P, Doua F, Mouanga G, et al. The distribution of sleep and wakefulness in human African trypanosomiasis. *Bull Soc Pathol Exot* (1994) 87:362–7.
2. Mehlitz D, Brinkmann U, Haller L. Epidemiological studies on the animal reservoir of gambiense sleeping sickness. Part I. Review of literature and description of the study areas. *Tropenmed Parasitol* (1981) 32:129–33.
3. Simarro PP, Diarra A, Postigo JAR, Franco JR, Jannin JG. The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000–2009: the way forward. *PLoS Negl Trop Dis* (2011) 5:e1007. doi:10.1371/journal.pntd.0001007
4. Louis FJ, Simarro PP, Lucas P. Sleeping sickness: one hundred years of control strategy evolution. *Bull Soc Pathol Exot* (2002) 95:331–6.
5. Reinhardt E. *Travailler ensemble: la mouche tsé-tsé et la pauvreté rurale*. Chronique ONU (2002). Available from: www.un.org/french/pubs/chronique/2002/numero2/0202p17_la_mouche_tsetse.html
6. Gibson W, Kay C, Peacock L. *Trypanosoma congolense*: molecular toolkit and resources for studying a major livestock pathogen and model trypanosome. *Advances in Parasitology*. (Vol. 98) (2017). doi:10.1016/bs.apar.2017.03.002
7. Van den Abbeele J, Claes Y, Bockstaele D, Ray D, Coosemans M. *Trypanosoma brucei* spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. *Parasitology* (1999) 118:469–78. doi:10.1017/S0031182099004217
8. Peacock L, Cook S, Ferris V, Bailey M, Gibson W. The life cycle of *Trypanosoma (Nannomonas) congolense* in the tsetse fly. *Parasit Vectors* (2012) 5:109. doi:10.1186/1756-3305-5-109
9. Holzmuller P, Grébaut P, Peltier JB, Brizard JP, Perrone T, Gonzatti M, et al. Secretome of animal trypanosomes. *Ann N Y Acad Sci* (2008) 1149:337–42. doi:10.1196/annals.1428.097
10. Grébaut P, Chuchana P, Brizard JP, Demettre E, Seveno M, Bossard G, et al. Identification of total and differentially expressed excreted-secreted proteins from *Trypanosoma congolense* strains exhibiting different virulence and pathogenicity. *Int J Parasitol* (2009) 39:1137–50. doi:10.1016/j.ijpara.2009.02.018
11. Atyame Nten CM, Sommerer N, Rofidal V, Hirtz C, Rossignol M, Cuny G, et al. Excreted/secreted proteins from trypanosome procyclic strains. *J Biomed Biotechnol* (2010) 2010:212817. doi:10.1155/2010/212817
12. Geiger A, Hirtz C, Bécue T, Bellard E, Centeno D, Gargani D, et al. Exocytosis and protein secretion in *Trypanosoma*. *BMC Microbiol* (2010) 10:20. doi:10.1186/1471-2180-10-20
13. Farikou O, Njiokou F, Mbida Mbida JA, Njitchouang GR, Djeunga HN, Asonganyi T, et al. Tripartite interactions between tsetse flies, *Sodalis glossinidius* and trypanosomes—An epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. *Infect Genet Evol* (2010) 10:115–21. doi:10.1016/j.meegid.2009.10.008
14. Geiger A, Ravel S, Mateille T, Janelle J, Patrel D, Cuny G, et al. Vector competence of *Glossina palpalis gambiensis* for *Trypanosoma brucei* s.l. and genetic diversity of the symbiont *Sodalis glossinidius*. *Mol Biol Evol* (2007) 24:102–9. doi:10.1093/molbev/msl135
15. Hamidou Soumana I, Tchicaya B, Simo G, Geiger A. Comparative gene expression of *Wigglesworthia* inhabiting non-infected and *Trypanosoma brucei gambiense*-infected *Glossina palpalis gambiensis* flies. *Front Microbiol* (2014) 5:620. doi:10.3389/fmicb.2014.00620
16. Hamidou Soumana I, Loriod B, Ravel S, Tchicaya B, Simo G, Rihet P, et al. The transcriptional signatures of *Sodalis glossinidius* in the *Glossina palpalis gambiensis* flies negative for *Trypanosoma brucei gambiense* contrast with those of this symbiont in tsetse flies positive for the parasite: possible involvement of a *Sodalis*-hosted prophage in fly *Trypanosoma* refractoriness? *Infect Genet Evol* (2014) 24:41–56.
17. Hamidou Soumana I, Klopp C, Ravel S, Nabihoudine I, Tchicaya B, Parrinello H, et al. RNA-seq de novo assembly reveals differential gene expression in *Glossina palpalis gambiensis* infected with *Trypanosoma brucei gambiense* vs. non-infected and self-cured flies. *Front Microbiol* (2015) 6:1259. doi:10.3389/fmicb.2015.01259
18. Hamidou Soumana I, Tchicaya B, Rialle S, Parinello H, Geiger A. Comparative genomics of *Glossina palpalis gambiensis* and *G. morsitans morsitans* to reveal gene orthologs involved in infection by *Trypanosoma brucei gambiense*. *Front Microbiol* (2017) 8:540. doi:10.3389/fmicb.2017.00540
19. Matthews KR. 25 years of African trypanosome research: from description to molecular dissection and new drug discovery. *Mol Biochem Parasitol* (2015) 200:30–40. doi:10.1016/j.molbiopara.2015.01.006
20. Mugnier MR, Stebbins CE, Papavasiliou FN. Masters of disguise: antigenic variation and the VSG coat in *Trypanosoma brucei*. *PLoS Pathog* (2016) 12:e1005784. doi:10.1371/journal.ppat.1005784
21. Tiengwe C, Muratore KA, Bangs JD. Surface proteins, ERAD and antigenic variation in *Trypanosoma brucei*. *Cell Microbiol* (2016) 18:1673–88. doi:10.1111/cmi.12605
22. Odongo S, Sterckx YGJ, Stijlemans B, Pillay D, Baltz T, Muyldermans S, et al. An anti-proteome nanobody library approach yields a specific immunoassay for *Trypanosoma congolense* diagnosis targeting glycosomal aldolase. *PLoS Negl Trop Dis* (2016) 10:e0004420. doi:10.1371/journal.pntd.0004420
23. WHO. *The World Health Report – Working Together for Health*. (2006). Available from: http://www.who.int/whr/2006/en/
24. Authié E, Boulangé A, Muteti D, Lalmanach G, Gauthier F, Musoke AJ. Immunisation of cattle with cysteine proteinases of *Trypanosoma congolense:* targetting the disease rather than the parasite. *Int J Parasitol* (2001) 31:1429–33. doi:10.1016/S0020-7519(01)00266-1
25. Huson LE, Authié E, Boulangé AF, Goldring JP, Coetzer TH. Modulation of the immunogenicity of the *Trypanosoma congolense* cysteine protease, congopain, through complexation with alpha(2)-macroglobulin. *Vet Res* (2009) 40:52. doi:10.1051/vetres/2009036
26. Aksoy S, Weiss B, Attardo G. Paratransgenesis applied for control of tsetse transmitted sleeping sickness. In: Aksoy S, editor. *Transgenesis and the Management of Vector-Borne Disease, Advances in Experimental Medicine and Biology*. New York: Springer (2008). p. 35–48.
27. Matetovici I, Caljon G, Van Den Abbeele J. Tsetse fly tolerance to *T. brucei* infection: transcriptome analysis of trypanosome-associated changes in the tsetse fly salivary gland. *BMC Genomics* (2016) 17:971. doi:10.1186/s12864-016-3283-0
28. Rio RVM, Hu Y, Aksoy S. Strategies of the home-team: symbioses exploited for vector-borne disease control. *Trends Microbiol* (2004) 12:325–36. doi:10.1016/j.tim.2004.05.001
29. Abd-Alla AMM, Bergoin M, Parker AG, Maniania NK, Vlak JM, Bourtzis K, et al. Improving Sterile Insect Technique (SIT) for tsetse flies through research on their symbionts and pathogens. *J Invertebr Pathol* (2013) 112:S2–10. doi:10.1016/j.jip.2012.07.009
30. Vreysen MJB, Saleh KM, Ali MY, Abdulla AM, Zhu Z-R, Juma KG, et al. *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *J Econ Entomol* (2000) 93:123–35. doi:10.1603/0022-0493-93.1.123
31. Geiger A, Ponton F, Simo G. Adult blood-feeding tsetse flies, trypanosomes, microbiota and the fluctuating environment in sub-Saharan Africa. *ISME J* (2015) 9:1496–507. doi:10.1038/ismej.2014.236
32. Geiger A, Bossard G, Sereno D, Pissarra J, Lemesre JL, Vincendeau P, et al. Escaping deleterious immune response in their hosts: lessons from trypanosomatids. *Front Immunol* (2016) 7:212. doi:10.3389/fimmu.2016.00212
33. Frezil J-L, Cuisance D. Trypanosomiases, maladies d'avenir: leurs perspectives et leurs inconues. Trypanosomiasis, diseases with future: prospects and uncertainty. *Bull Soc Pathol Exot* (1994) 87:391–3.
34. Aksoy S, Gibson WC, Lehane MJ. Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. *Adv Parasitol* (2003) 53:1–83. doi:10.1016/S0065-308X(03)53002-0
35. Lehane MJ, Aksoy S, Gibson W, Kerhornou A, Berriman M, Hamilton J, et al. Adult midgut expressed sequence tags from the tsetse fly *Glossina morsitans morsitans* and expression analysis of putative immune response genes. *Genome Biol* (2003) 4:R63. doi:10.1186/gb-2003-4-10-r63
36. Lehane MJ, Gibson W, Lehane SM. Differential expression of fat body genes in *Glossina morsitans morsitans* following infection with *Trypanosoma brucei brucei*. *Int J Parasitol* (2008) 38:93–101. doi:10.1016/j.ijpara.2007.06.004
37. Grébaut P, Melachio T, Nyangmang S, Eyenga VE, Njitchouang GR, Ofon E, et al. Xenomonitoring of sleeping sickness transmission in Campo (Cameroon). *Parasit Vectors* (2016) 9:201. doi:10.1186/s13071-016-1479-4
38. Grebaut P, Bodo JM, Assona A, Foumane Ngane V, Njiokou F, Ollivier G, et al. Risk factors for human African trypanosomiasis in the Bipindi region of Cameroon. *Med Trop Rev Corps Sante Colon* (2001) 61:377–83.

39. Farikou O, Njiokou F, Simo G, Asonganyi T, Cuny G, Geiger A. Tsetse fly blood meal modification and trypanosome identification in two sleeping sickness foci in the forest of southern Cameroon. *Acta Trop* (2010) 116:81–8. doi:10.1016/j.actatropica.2010.06.002
40. Njiokou F, Simo G, Nkinin SW, Laveissière C, Herder S. Infection rate of *Trypanosoma brucei* s.l., *T. vivax*, *T. congolense* "forest type", and *T. simiae* in small wild vertebrates in south Cameroon. *Acta Trop* (2004) 92:139–46. doi:10.1016/j.actatropica.2004.04.011
41. Njiokou F, Laveissière C, Simo G, Nkinin S, Grébaut P, Cuny G, et al. Wild fauna as a probable animal reservoir for *Trypanosoma brucei gambiense* in Cameroon. *Infect Genet Evol* (2006) 6:147–53. doi:10.1016/j.meegid.2005.04.003
42. Simo G, Njiokou F, Mbida Mbida JA, Njitchouang GR, Herder S, Asonganyi T, et al. Tsetse fly host preference from sleeping sickness foci in Cameroon: epidemiological implications. *Infect Genet Evol* (2008) 8:34–9. doi:10.1016/j.meegid.2007.09.005
43. Lancien J. Description du piège monoconique utilisé pour l'élimination des glossines en République Populaire du Congo. *Ent Méd Parasitol* (1981) 19:235–8.
44. Grébaut P, Mbida JAM, Kondjio CA, Njiokou F, Penchenier L, Laveissiére C. Spatial and temporal patterns of human African trypanosomosis (HAT) transmission risk in the bipindi focus, in the forest zone of southern Cameroon. *Vector Borne Zoonotic Dis* (2004) 4:230–8. doi:10.1089/vbz.2004.4.230
45. Penchenier L, Itard J. Une nouvelle technique de dissection rapide des glandes salivaires et de l'intestin de glossines. *Ent Méd Parasitol* (1981) 19:55–7.
46. Herder S, Simo G, Nkinin S, Njiokou F. Identification of trypanosomes in wild animals from southern Cameroon using the polymerase chain reaction (PCR). *Parasite* (2002) 9:345–9. doi:10.1051/parasite/2002094345
47. Masiga DK, Smyth AJ, Ayes P, Bromidge TJ, Gibson WC. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *Int J Parasitol* (1992) 22:909–18. doi:10.1016/0020-7519(92)90047-O
48. Moser DR, Cook GA, Ochs DE, Bailey CP, McKane MR, Donelson JE. Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* (1989) 99:57–66. doi:10.1017/S0031182000061023
49. Köster J, Rahmann S. Snakemake – a scalable bioinformatics workflow engine. *Bioinformatics* (2012) 28:2520–2. doi:10.1093/bioinformatics/bts480
50. Giraldo-Calderón GI, Emrich SJ, MacCallum RM, Maslen G, Dialynas E, Topalis P, et al. VectorBase: an updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. *Nucleic Acids Res* (2015) 43:D707–13. doi:10.1093/nar/gku1117
51. Aslett M, Aurrecoechea C, Berriman M, Brestelli J, Brunk BP, Carrington M, et al. TriTrypDB: a functional genomic resource for the *Trypanosomatidae*. *Nucleic Acids Res* (2010) 38:D457–62. doi:10.1093/nar/gkp851
52. Attrill H, Falls K, Goodman JL, Millburn GH, Antonazzo G, Rey AJ, et al. FlyBase: establishing a gene group resource for *Drosophila melanogaster*. *Nucleic Acids Res* (2016) 44:D786–92. doi:10.1093/nar/gkv1046
53. Liao Y, Smyth GK, Shi W. The subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* (2013) 41:e108. doi:10.1093/nar/gkt214
54. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. *Bioinformatics* (2009) 25:2078–9. doi:10.1093/bioinformatics/btp352
55. Liao Y, Smyth GK, Shi W. FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* (2014) 30:923–30. doi:10.1093/bioinformatics/btt656
56. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. *Nat Biotechnol* (2011) 29:24–6. doi:10.1038/nbt.1754
57. Varet H, Brillet-Guéguen L, Coppée J-Y, Dillies M-A. SARTools: a DESeq2- and EdgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data. *PLoS One* (2016) 11:e0157022. doi:10.1371/journal.pone.0157022
58. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) 15:550. doi:10.1186/s13059-014-0550-8
59. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* (2010) 26:139–40. doi:10.1093/bioinformatics/btp616
60. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* (1997) 25:3389–402. doi:10.1093/nar/25.17.3389
61. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* (2009) 4:44–57. doi:10.1038/nprot.2008.211
62. Reimand J, Kull M, Peterson H, Hansen J, Vilo J. g:Profiler—a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res* (2007) 35:W193–200. doi:10.1093/nar/gkm226
63. Caspi R, Billington R, Ferrer L, Foerster H, Fulcher CA, Keseler IM, et al. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* (2016) 44:D471–80. doi:10.1093/nar/gkv1164
64. Maudlin I, Ellis DS. Association between intracellular *Rickettsia*-like infections of midgut cells and susceptibility to trypanosome infection in *Glossina* spp. *Z Parasitenkd* (1985) 71:683–7. doi:10.1007/BF00925601
65. Welburn SC, Maudlin I. Tsetse–trypanosome interactions: rites of passage. *Parasitol Today* (1999) 15:399–403. doi:10.1016/S0169-4758(99)01512-4
66. Dale C, Welburn SC. The endosymbionts of tsetse flies: manipulating host-parasite interactions. *Int J Parasitol* (2001) 31:628–31. doi:10.1016/S0020-7519(01)00151-5
67. Koch H, Raabe M, Urlaub H, Bindereif A, Preußer C. The polyadenylation complex of *Trypanosoma brucei*: characterization of the functional poly(A) polymerase. *RNA Biol* (2016) 13:221–31. doi:10.1080/15476286.2015.1130208
68. Kolev NG, Franklin JB, Carmi S, Shi H, Michaeli S, Tschudi C. The transcriptome of the human pathogen *Trypanosoma brucei* at single-nucleotide resolution. *PLoS Pathog* (2010) 6:e1001090. doi:10.1371/journal.ppat.1001090
69. Martinson EO, Wheeler D, Wright J, Mrinalini Siebert AL, Werren JH. *Nasonia vitripennis* venom causes targeted gene expression changes in its fly host. *Mol Ecol* (2014) 23:5918–30. doi:10.1111/mec.12967
70. Snyder AK, Rio RVM. "Wigglesworthia morsitans" folate (vitamin b9) biosynthesis contributes to tsetse host fitness. *Appl Environ Microbiol* (2015) 81:5375–86. doi:10.1128/AEM.00553-15
71. Zhang Q, Lai M-M, Lou Y-Y, Guo B-H, Wang H-Y, Zheng X-Q. Transcriptome altered by latent human cytomegalovirus infection on THP-1 cells using RNA-seq. *Gene* (2016) 594:144–50. doi:10.1016/j.gene.2016.09.014
72. Barisón MJ, Damasceno FS, Mantilla BS, Silber AM. The active transport of histidine and its role in ATP production in *Trypanosoma cruzi*. *J Bioenerg Biomembr* (2016) 48:437–49. doi:10.1007/s10863-016-9665-9
73. Dean P, Hirt RP, Embley TM. *Microsporidia*: why make nucleotides if you can steal them? *PLoS Pathog* (2016) 12:e1005870. doi:10.1371/journal.ppat.1005870
74. Kanost MR, Jiang H, Yu X-Q. Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol Rev* (2004) 198:97–105. doi:10.1111/j.0105-2896.2004.0121.x
75. Kent JR, Zeng P-Y, Atanasiu D, Gardner J, Fraser NW, Berger SL. During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. *J Virol* (2004) 78:10178–86. doi:10.1128/JVI.78.18.10178-10186.2004

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Intestinal Bacterial Communities of Trypanosome-Infected and Uninfected *Glossina palpalis* palpalis from Three Human African Trypanomiasis Foci in Cameroon

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Glossina sp. the tsetse fly that transmits trypanosomes causing the Human or the Animal African Trypanosomiasis (HAT or AAT) can harbor symbiotic bacteria that are known to play a crucial role in the fly's vector competence. We hypothesized that other bacteria could be present, and that some of them could also influence the fly's vector competence. In this context the objectives of our work were: (a) to characterize the bacteria that compose the *G. palpalis palpalis* midgut bacteriome, (b) to evidence possible bacterial community differences between trypanosome-infected and non-infected fly individuals from a given AAT and HAT focus or from different foci using barcoded Illumina sequencing of the hypervariable V3-V4 region of the *16S rRNA gene*. Forty *G. p. palpalis* flies, either infected by *Trypanosoma congolense* or uninfected were sampled from three trypanosomiasis foci in Cameroon. A total of 143 OTUs were detected in the midgut samples. Most taxa were identified at the genus level, nearly 50% at the species level; they belonged to 83 genera principally within the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Prominent representatives included *Wigglesworthia* (the fly's obligate symbiont), *Serratia,* and *Enterobacter hormaechei*. *Wolbachia* was identified for the first time in *G. p. palpalis*. The average number of bacterial species per tsetse sample was not significantly different regarding the fly infection status, and the hierarchical analysis based on the differences in bacterial community structure did not provide a clear clustering between infected and non-infected flies. Finally, the most important result was the evidence of the overall very large diversity of intestinal bacteria which, except for *Wigglesworthia*, were unevenly distributed over the sampled flies regardless of their geographic origin and their trypanosome infection status.

Keywords: *Glossina*, trypanosome, microbiome, meta-taxonomics, sleeping sickness, nagana

INTRODUCTION

Animal African Trypanosomiasis (AAT), or Nagana, is primarily caused by *Trypanosoma brucei brucei* (Tbb), *Trypanosoma vivax,* and *Trypanosoma congolense*, which are transmitted to their mammalian hosts by tsetse flies belonging to the species *Glossina palpalis* and *Glossina morsitans*. This disease is responsible for significant economic losses in areas where cattle are exposed to parasites (Shaw et al., 2013). Human African Trypanosomiasis (HAT), or sleeping sickness, is endemic to Sub-Saharan Africa, where it causes significant health and economic impacts (Welburn and Maudlin, 2012). The chronic form of HAT is caused by *Trypanosoma brucei gambiense* (Tbg), whereas *Trypanosoma brucei rhodesiense* (Tbr) causes the acute form; the two forms are transmitted to humans by *G. palpalis* and *G. morsitans*, respectively.

Currently, about 60 million people continue to be at risk for HAT in 36 Sub-Saharan African countries. Several factors complicate the treatment of HAT. The medications that are currently used to treat human patients can generate many side effects (reviewed in Geiger et al., 2011a). In addition, the emergence of drug-resistant trypanosomes was recently reported (Baker et al., 2013). These factors are pertinent, since epidemiological modeling of the impact of global change on HAT expansion predicts that 46–70 million more people will be at risk by 2090 (Moore et al., 2012). Thus, the search for new control strategies is a priority to impede the spread of the disease. In this context, identifying the factors involved in fly vector competence (as well as understanding how they work) may open new perspectives.

Trypanosomes ingested by the tsetse fly during a blood meal on an infected host passively reach the tsetse intestine, where they differentiate into their procyclic form, mature, and multiply. Then, depending on the species, the trypanosomes migrate toward either the fly's salivary glands (in the case of *T. brucei* sp.) or the mouth parts, where they undergo the maturation step into their infective metacyclic form. The trypanosomes may then be transmitted to a mammalian host during a subsequent blood meal ingested by the fly (Vickerman et al., 1988). This ability of tsetse flies to acquire trypanosomes and to subsequently favor their maturation and transmit them to a vertebrate host, known as the fly's vector competence, depends on several factors including the production of antimicrobial peptides (e.g., cecropin and attacin; Hu and Aksoy, 2006), reactive oxygen species (MacLeod et al., 2007), EP-protein [Glutamic acid (E)—Proline (P) rich protein, an immune responsive protein], (Haines et al., 2010), intestinal lectin (Welburn et al., 1994), as well as the peritrophic matrix (Weiss et al., 2013, 2014; Rose et al., 2014; Aksoy et al., 2016).

As previously reported, tsetse flies can harbor three different symbionts that play a crucial role in the physiology of their host. First, the obligate Enterobacteria *Wigglesworthia glossinidia* is present intracellularly in the bacteriocytes, specialized cells of the intestine that form the bacteriome (Aksoy, 1995). This ancient host association (Chen et al., 1999) is involved in the tsetse fly's fertility, nutrition, and the development of its immune system (Akman et al., 2002; Weiss et al., 2011; Rio et al., 2012). Second, the facultative symbiont *Sodalis glossinidius* is present in the cells of the intestine, but it can also be found extracellularly and in other tissues of the fly (Cheng and Aksoy, 1999; Balmand et al., 2013). There is substantial evidence that certain *S. glossinidius* genotypes favor the establishment of trypanosomes in the fly's gut (Geiger et al., 2007; Farikou et al., 2010a; Hamidou Soumana et al., 2014). *W. glossinidia* and *S. glossinidius* are both transmitted maternally to the progeny *via* milk secretions during the intra-uterine development of the larvae (Aksoy, 1995). Third, the facultative symbiont *Wolbachia* can be detected very early in oocytes, embryos and larvae (Cheng et al., 2000). This symbiont acts on the reproductive process of tsetse flies by inducing cytoplasmic incompatibility (Alam et al., 2011). *Wolbachia* infection has been negatively correlated with the prevalence of trypanosome and of salivary gland hypertrophy virus (Alam et al., 2012). This indicates that *Wolbachia* may have a role in preventing infection of tsetse flies from trypanosomes (Aksoy et al., 2013), which interestingly has not been revealed for plasmodium infection in mosquitoes that harbor *Wolbachia* (Zélé et al., 2014). This symbiont is trans-ovary transmitted to the progeny (Cheng and Aksoy, 1999).

The presence of bacteria (other than symbionts) in the gut of field-collected tsetse flies has been assessed by bacterial isolation and culture approaches (Geiger et al., 2009, 2011b; Lindh and Lehane, 2011), as well as by direct molecular identification methods. Using deep sequencing of the V4 hypervariable region of the *16S rRNA* gene, Aksoy et al. (2014) revealed the limited diversity of gut-specific microbiota in wild tsetse flies from Uganda. Surprisingly, bacteria belonging to the genera *Enterobacter*, *Acinetobacter,* and *Enterococcus* were found in the gut of the blood-sucking fly, despite the fly feeds on animals which blood is normally devoid of any bacteria. The source of these bacteria is unknown, but their presence could be due to the fact that tsetse flies can occasionally feed on nutrients other than blood that may be contaminated by diverse bacteria (Colman et al., 2012). Flies may also ingest bacteria present on the skin surface of the hosts when taking their bloodmeal (Poinar et al., 1979). Finally, differences in their diet that may depend on the environmental conditions could lead to differences in the fly's gut bacteriome as it was shown for other insects (Colman et al., 2012). Finding such bacteria in an organ where trypanosomes are localized raised the question whether these bacteria could be involved, besides the known symbionts, in fly infection by trypanosomes, inasmuch as certain bacteria species have been shown to produce antiparasitic molecules (Lazaro et al., 2002; Moss, 2002). In this context, a novel bacterial species, *Serratia glossinae*, has been identified in the gut of insects reared in insectarium (Geiger et al., 2010). This bacterium is related to *Serratia marcescens* (Enterobacteriaceae) a bacterium involved in the production of pigments that are toxic for *Trypanosoma cruzi* (Azambuja et al., 2004). In *Anopheles*, the abundance of intestinal Enterobacteriaceae has been associated to the presence of the parasite *Plasmodium falciparum* (Boissière et al., 2012). This illustrates the need for a better understanding of the composition of the tsetse fly bacteriome and its potential variations, since differences in the environment could induce differences in the bacteriome

composition which, in turn, could induce differences in the fly vector competence.

Using bar-coded Illumina sequencing of the hypervariable V3-V4 region of the *16S rRNA* gene, we aimed to characterize bacterial communities in the midgut of *G. p. palpalis* sampled from three different sleeping sickness foci in Cameroon, and to verify the hypotheses that the composition of the midgut bacterial communities of trypanosome-infected *G. p. palpalis* might be different from that of non-infected flies across these three HAT foci. Finally we aimed to identify bacteria that could be associated with the infection status of the fly. Those, if any, associated with fly refractoriness would be of particular interest inasmuch if they would be maternally transmitted to offspring. In the case they would not be maternally transferred, their trypanocidal compound(s) should be identified and possibly delivered via the paratransgenic approach described by Rio et al. (2004). Both approaches may offer the possibility to block the fly vector competence, hence the spread of the sleeping disease.

MATERIALS AND METHODS

Sampling Areas

The study was performed in three sleeping sickness foci (Bipindi, Campo, and Fontem) located in the forest region of southern-Cameroon (**Figure 1**).

Bipindi (3°2′N, 10°22′E) is a historical sleeping sickness focus originally described in 1920 (Grébaut et al., 2001). It is situated between Lolodorf and Kribi, 75 km from the Atlantic Ocean. Although, a 2004 survey identified four tsetse fly species (Grébaut et al., 2004), *G. p. palpalis* was the only species found in this focus since 2007 (Farikou et al., 2010a,b; Melachio et al., 2011; Tchouomene-Labou et al., 2014). Bipindi is surrounded by hills and covers several villages, mainly located along the roads. The focus has a typical forest bioecological environment, including equatorial forest and farmland along the roads and surrounding the villages. Bipindi is home to highly diverse wild fauna (Njiokou et al., 2004, 2006) and it has a dense network of rivers crossing cocoa farms, all of which offer suitable habitats for tsetse flies.

Campo (2°20′N, 9°52′E) is a hypo-endemic focus where several cases of sleeping sickness are diagnosed every year. It is located along the Ntem River, which separates Cameroon from Equatorial Guinea. Several tsetse fly species (including *G. p. palpalis*, and to a lesser extent *G. pallicera*, *G. caliginea,* and *G. nigrofusca*) can be encountered in this focus (Simo et al., 2008). Several biotopes are found in the Campo focus, including farmland, marshes, swampy areas, and rainy forest.

Fontem (5°40′N, 9°55′E), in the Southwest Region of Cameroon, has been recognized as a sleeping sickness focus since 1949. Since that time, the prevalence of HAT has decreased considerably. The landscape is characterized by the presence of hills and valleys that are crossed by rivers with fast currents. *G. p. palpalis* is the only tsetse fly species found in this area (Simo et al., 2008). The focus is divided into the North, Center and South sub-foci. The Center sub-focus (comprising the villages of Menji, Fotabong, Nsoko, and Azi), where flies were sampled, has only had a few sleeping sickness cases in the past decade. In the Northern sub-focus (comprising the villages of Bechati, Folepi, and Besali) no new HAT patients have been diagnosed in the last 20 years (Ebo'o Eyenga, personal communication), although 15% of all pigs were infected with *T. b. gambiense* group 1 in 1999 (Nkinin et al., 2002).

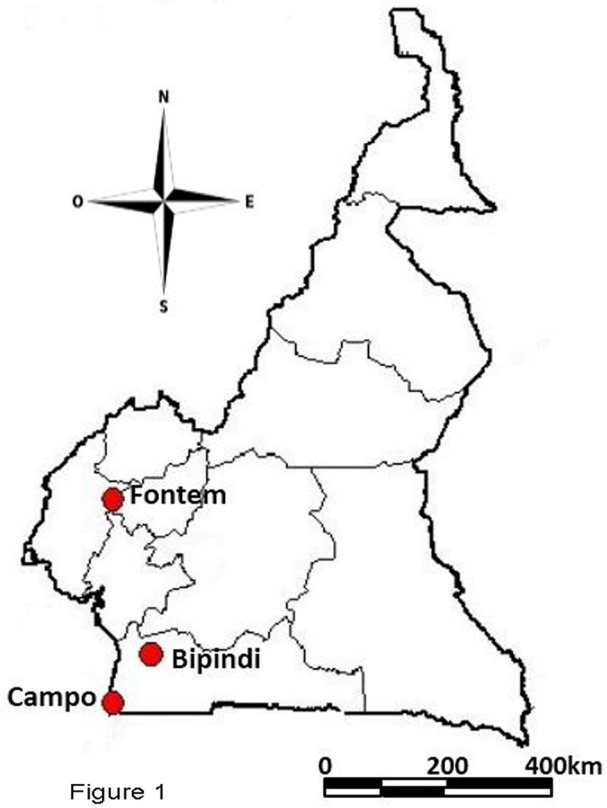

FIGURE 1 | Geographical map showing the three described Cameroonian HAT foci.

Fly Sampling and Experimental Design

Entomological surveys were conducted in the Campo focus in March 2008, and in the Bipindi and Fontem foci in November 2013. Pyramidal traps (Gouteux and Lancien, 1986) were placed for 4 consecutive days in suitable tsetse fly biotopes of each village located within each focus. The geographical position of the sampling sites was determined by GPS. Trapped tsetse flies were harvested twice a day (before 12 a.m. and before 3 p.m.).

The *Glossina* species were first identified according to morphological criteria (Grébaut et al., 2004), and then sorted into two groups of teneral (young flies that had never taken a blood meal) and non-teneral flies, respectively. Among the 599 non-teneral flies that were caught in the Campo focus, 200 were randomly sampled, their midguts dissected (see below) and observed under a light microscope (magnification x100) to detect the presence of motile trypanosomes (infected flies) or the absence of trypanosomes (non-infected flies). The trypanosomes from infected flies were subsequently identified (species or sub-species) by PCR using specific primers (**Table 1**). The absence

TABLE 1 | Primers used for PCR amplification of trypanosome DNA (Farikou et al., 2010a).

Specificity	Primer sequence	Amplified product (bp)	References
T. congolense (forest type)	5′-GGACACGCCAGAAGGTACTT-3′	350	Masiga et al., 1992
	5′-GTTCTCGCACCAAATCCAAC-3′		
T. congolense (savannah type)	5′-TCGAGCGAGAACGGGCACTTTGCGA-3′	341	Moser et al., 1989
	5′-ATTAGGGACAAACAAATCCCGCACA-3′		
T. brucei s.l.	5′-CGAATGAATATTAAACAATGCGCAG-3′	164	Masiga et al., 1992
	5′-AGAACCATTTATTAGCTTTGTTGC-3′		
T. vivax	5′-CGACTCCGGGCGACCGT-3′	600	Majiwa et al., 1994
	5′-CATGCGGCGGACCGTGG-3′		

of any trypanosomes (negative PCR assay) was confirmed in all non-infected flies using the same specific primers. Among the 300 flies caught in Bipindi, 200 were sampled and processed as for the Campo flies. Only 27 non-teneral flies could be caught in Fontem; as shown after dissection, microscopy, and PCR analyses, they all were non-infected by trypanosomes.

The microbiota analysis was performed on *G. p. palpalis* flies that had undergone further selection. The midguts from the 200 sampled flies from Campo were separated into two groups composed, respectively, of trypanosome infected and non-infected midguts. Among the infected midguts, only those harboring *T. congolense* (either the forest or savannah type; mixed infections were discarded) were taken into account. Finally, from the non-infected group and from the *T. congolense* infected group, respectively, eight midguts were randomly selected and identified by numbering. The 200 samples from Bipindi were processed similarly. Among the midguts from the 27 flies from Fontem, eight were randomly selected, which formed a unique group of non-infected samples. In total, five groups were formed that differed from each other regarding the infection status of the flies and/or their geographic origin. The "sex ratio" (12.5, 37.5, 37.5, 75, and 25% male flies in, respectively group 1–5) was recorded but not taken into account in the subsequent analyses. The characteristics of each group, including sample numbering, are the followings: "Group 1/Campo infected flies: number 1–8; Group 2/Campo non-infected flies: number 9–16; Group 3/Bipindi infected flies: number 17–24; Group 4/Bipindi non-infected flies: number 25–32; Group5/Fontem non-infected flies: number 33–40 (**Figure 2**).

Dissection of Tsetse Flies, DNA Extraction, and Trypanosome PCR Amplification

The non-teneral flies were surface-sterilized (once with 5% sodium hypochlorite for 10 min and twice with 70% ethanol, each for 10 min) and the midgut of each fly was dissected under sterile conditions using a Bunsen burner, and in sterile NaCl 0.9%. The instruments used were carefully cleaned after the dissection of each fly to prevent cross-contamination. Midguts were recovered and then separately transferred into tubes containing 95° ethanol for further DNA extraction. During the field surveys (10 days for each mission), tubes containing the samples were maintained at −20°C; thereafter, in the laboratory, the midgut samples were immediately processed and subjected to DNA extraction using the DNeasy kit (Qiagen, Paris, France) according to Geiger et al. (2007). Genomic DNA was quantified using NanoDrop (Thermo Scientific, Paris).

PCR amplification was performed as previously described (Farikou et al., 2010a) using trypanosome-specific primers (**Table 1**; Moser et al., 1989; Masiga et al., 1992; Majiwa et al., 1994; Herder et al., 2002). This procedure allowed detection of *T. brucei s.l.*, *T. congolense* (both forest and savannah types), and *T. vivax*.

Meta-Barcoding Analysis

Amplification of the highly variable V3-V4 region of the *16S rRNA* gene, using the forward primer 5′-CCTACGGGNGGC WGCAG-3′ and the reverse primer 5′-GACTACHVGGGTHTC TAATCC-3′ (N representing A, C, G, or T; V representing A, C, or G; H representing A, C, or T; W representing A or T; IUPAC codes), was performed to evaluate the bacterial communities of tsetse midguts on the Illumina MiSeq (MR DNA Laboratory-http://www.mrdnalab.com/shallowater, USA). The primers were specifically designed by one of the co-authors of the current study. As previously described in the method of Porter et al. (2016), each PCR amplifying medium contained 2 μL DNA template, 17.5 μL molecular biology-grade water, 2.5 μL 10× reaction buffer, 1 μL 50× MgCl2 (50 mM), 0.5 μL dNTPs mix (10 mM), 0.5 μL forward primer (10 mM), 0.5 μL reverse primer (10 mM), and 0.5 μL Invitrogen Platinum *Taq* polymerase (5 U/μL) in a total volume of 25 μL. The amplification program was: 95°C for 5 min, followed by 25 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 30 s, and lasted at 72°C for 5 min. Amplicons were purified using MiniElute PCR purification columns (Qiagen, Paris, France) and were eluted in 50 μL molecular biology-grade water. The purified amplicons from the first PCR round served as templates for the second PCR round, using Illumina adaptor-tailed primers in a 10-cycle amplification regime. All PCR reactions were performed in an Eppendorf Mastercycler ep gradient S thermal cycler. Negative controls (containing all reagents except the midgut DNA that was replaced by water) were included in all experiments. Amplicons were quantified using a Bioanalyzer (Agilent Technologies, Paris) and then the KAPA Quantification Kit (Roche, Paris). Equimolar amounts of the generated libraries were dual-indexed, combined, and sequenced on an Illumina MiSeq platform (MRDNA Laboratory, Shallowater, USA) using

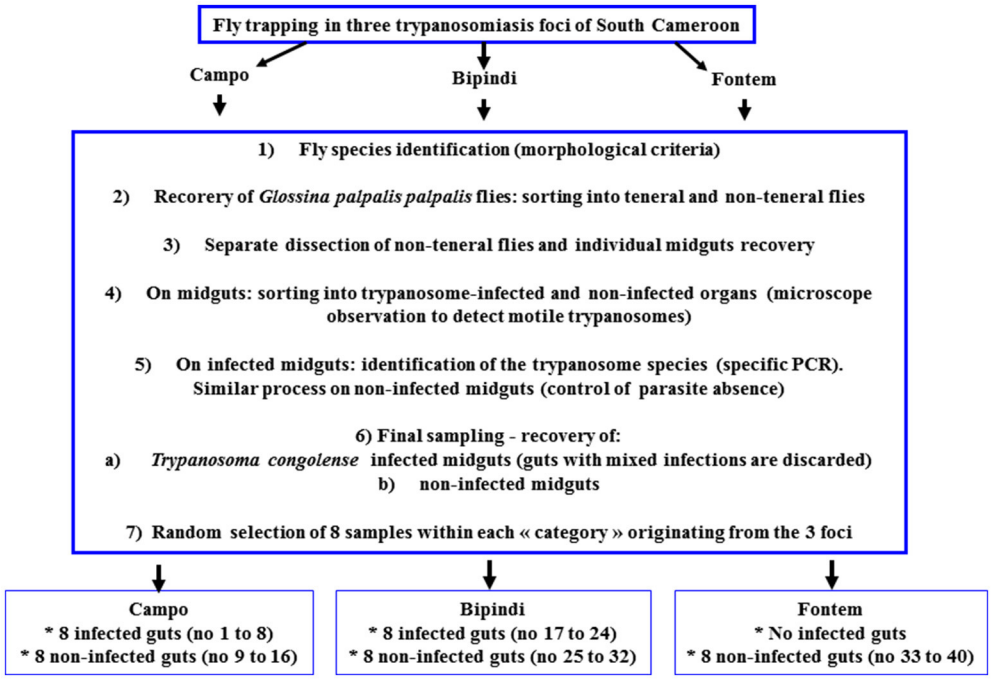

FIGURE 2 | *Glossina* sampling and selection process.

MiSeq Reagent kits (300 cycles), following the 2 × 300-bp paired-end sequencing protocol. The generated sequences have been deposited in the EMBL-EBI (Study accession number PRJEB14010; Secondary study accession number ERP015605 and Locus tag prefix BN6929).

Quality Filtering and Taxonomic Classification of Sequencing Data

Illumina MiSeq reads were analyzed using in-house pipelines (Richard Christen; described in Boissière et al., 2012; Hartmann et al., 2012; Gimonneau et al., 2014; Massana et al., 2015). Briefly, Silva 119 NR (analyses performed in 2014) was used as the reference database for taxonomic identification (Quast et al., 2012). An *in silico* extraction of Silva amplicons using forward: 5′-CCTACGGGNGGCWGCAG-3′ and reverse: 5′-GACTACHVGGGTHTCTAATCC-3′ primers, followed by an analysis by length/number of amplicons, yielded the following results: 1–50/0; 51–100/0; 101–150/2; 151–200/1; 201–250/1; 251–300/11; 301–350/40; 351–400/21,669; 401–450/448,575; 451–500/450; 501–550/192; 551–600/1,198; 601–650/0. This demonstrates that most amplicons were between 350 and 450 nucleotides in length. The extracted database is referenced below as the refseq. Each sequence identifier was reformatted to eight taxonomic fields as in PR2 (Guillou et al., 2012) making it easier to use a pipeline for analyses.

In order to assemble paired-end reads, the software programs PEAR (Zhang et al., 2014) and FLASH (Magoc and Salzberg, 2011) were both tested. PEAR using default parameters merged more pairs, and paired-end reads were therefore assembled and quality filtered (using Illumina quality scores) with PEAR. 94.26% (3,622,233 reads) of the total reads (3,842,672 reads) were assembled; 0.003% were discarded and 5.734% were not assembled.

Reads were then sorted by length using a dedicated python script, yielding the following results: 0–0/0; 1–50/3; 51–100/1,556; 101–150/1,435; 151–200/1,535; 201–250/1,578; 251–300/2,251; 301–350/9,401; 351–400/38,323; 401–450/132,487; 451–500/3,433,239; 501–550/381; 551–600/44; 601–650/0. Reads shorter than 300 nucleotides (0.23% out of the overall number of reads) were discarded, resulting in the extraction of 3,613,875 reads. The fasta file was then demultiplexed using a dedicated C++ program. Primer trimming was performed using CutAdapt v1.8.1 (Martin, 2011).

Each file was dereplicated, sorted by decreasing abundance, chimera checked with UCHIME (Edgar et al., 2011) and then clustered using Crunchclust (Mondani et al., 2011; Gimonneau et al., 2014; Massana et al., 2015; Tchioffo et al., 2016; available from https://github.com/dumaatravaie/crunchclust/blob/master/Documentation; at a Levenstein distance of 5). After this clustering step, clusters that contained <2 reads were discarded as artifacts (see Boissière et al., 2012). All these steps resulted in 2,562,144 high-quality sequences contained in clusters with at least two reads (70.73% of the total assembled reads) that were subsequently used for taxonomic assignment. In each cluster, the most abundant sequence was kept as the representative one, since it was assumed to have the least errors in a cluster. Taxonomic assignment was done as in Pawlowski et al. (2011). Briefly, a Needleman–Wunsch algorithm to search for the 30 most similar sequences to each representative sequence from the refseq was employed. The reference sequences

with the highest percentage were then used, and taxonomy to a given level was obtained. When more than one result emerged, the two highest hits were reported. When similarity was <80%, sequences were not assigned. Abundance matrices were generated for statistical analyses at each taxonomic level. Several abundant Operational Taxonomic Units (OTUs) could not be identified satisfactorily down to the genus or species level. In these cases (rough estimation: 1%), reads sequences and similar refseq sequences were selected and then aligned using ClustalO (Sievers et al., 2011) and SeaView (Gouy et al., 2010). Trees were plotted using TreeDyn (Chevenet et al., 2006) or MetaPhlAn (Segata et al., 2012), and distinct robust subtrees were annotated as distinct species whenever possible.

Data and Statistical Analysis

Statistical analyses were performed using the R package vegan. Rarefaction curves (**Figure S1**) were performed prior to comparative analyses between infected and uninfected flies, and between the sampling sites. Significant differences in bacterial richness between the infected and uninfected flies, and between the three sampling sites were tested using non-parametric Kruskal–Wallis test. We used vegdist and hclust using single, average, and complete linkage methods for hierarchical clustering and then compared them for the presence of sub-trees. Nonmetric multidimensional scaling (nMDS) were generated using the R packages ggplot2 and phyloseq (McMurdie and Holmes, 2013).

RESULTS

Distribution of Tsetse Flies by Infected vs. Non-infected Status

The distribution of trypanosome species and/or subspecies in the midgut of tsetse flies from Campo (200 flies) and Bipindi (200 flies) is displayed in **Figure 3**. Nearly half of all flies, whether from Campo (51.4%) or from Bipindi (48.6%), were infected with at least one of the four studied trypanosome species and/or subspecies. The prevalence of the four trypanosome species was highly dependent on the sampling site. In flies sampled from Campo, *T. congolense* (both forest and savannah types) displayed the highest prevalence, accounting for 83% of all infected flies (including mixed infections), while *T. brucei sensu lato* displayed the lowest prevalence (12%, including mixed infections). In contrast, *T. brucei s.l.* displayed the highest prevalence (43%) in infected flies from Bipindi, with *T. congolense* accounting for 42%. As for the Fontem focus, the low number of collected flies (27 flies) prevented any reliable statistical analysis regarding the prevalence of the infected flies in this focus, inasmuch as all of the 27 flies were uninfected (which does not imply that the overall population of flies is uninfected).

Bacterial Communities in the G. *p. palpalis* Midgut

Illumina sequencing of *16S rDNA* gene generated a total of 2,562,144 high-quality sequence reads across the V3–V4 region in 40 tsetse flies, for the 40 samples randomly selected. The average number of tags/sample for the V3–V4 region was 12,988 (ranging from 6,525 to 19,694 per gut), with read length varying from 300 to 600 nucleotides. After taxonomic assignation, a total of 13 phyla were identified from the 40 gut samples (**Table S1**); 12 phyla belonged to the Bacterial kingdom and one phylum belonged to the Archaea. Proteobacteria were present in all 40 samples and had a relative abundance higher than 68% in each sample (even higher than 97% in 37 out of 40 samples), while the other phyla (as well as their respective relative abundances) were unevenly distributed among the different samples. The Bacteroidetes phylum was detected in 26 samples, Firmicutes was observed in 19 samples, Proteobacteria/Actinobacteria was detected in 14 samples, and Actinobacteria was found in 7 samples. The 7 other phyla could only be detected in 4 samples or less. Samples from groups 1 and 2 (infected and non-infected midguts from Campo; sample numbers 1–16) harbored 1 to 4 different phyla; samples from group 3 (infected midguts from Bipindi; sample numbers 17–24) contained 2–5 different phyla; samples from group 4 (non-infected midguts from Bipindi; sample numbers 25–32) contained 3–7 different phyla; and samples from group 5 (non-infected midguts from Fontem;

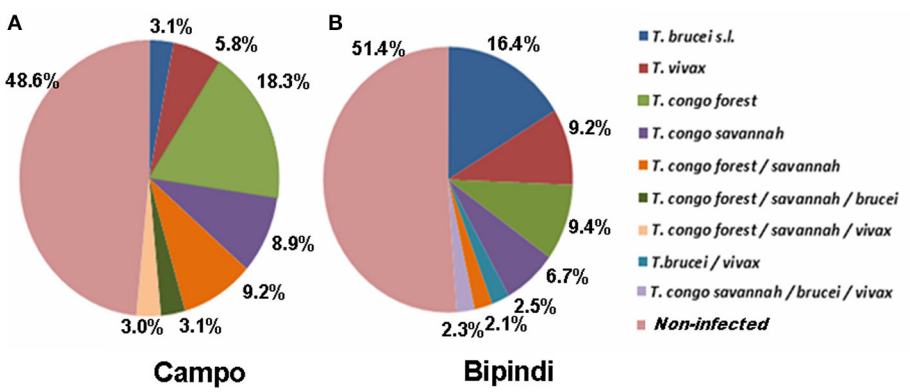

FIGURE 3 | Prevalence of the different trypanosome species or subspecies infecting tsetse flies collected in the Campo **(A)** and Bipindi **(B)** foci. Single as well as mixed infections have been considered.

sample numbers 33–40) harbored 1–4 different phyla (**Table S1**). A heat map analysis of the distribution and abundance of the bacterial phyla showed the sampled midgut did not cluster together, confirming that bacterial communities at the phylum level were unevenly distributed among the samples (**Figure 4**).

The distribution of bacterial taxa among the 40 samples at the class and genus levels and their relative abundance in each sample are reported in **Table S1**. Twenty-four classes and 83 genera could be distinguished. The gut microbiota presented a large inter-individual variability, although only a few taxa were prominent.

Bacteria Classes

As reported above, all 40 samples contained Proteobacteria, accounting for more than 97% of all microbiota in 37 samples. The Gammaproteobacteria, present in all 40 samples and accounting for more than 95% of the total microbiota in 38 samples, was the most prominent class in this phylum. Alpha-proteobacteria (maximal abundance per sample: 11.08%) were found in 32 samples. In contrast, Beta-proteobacteria were present in only 7 samples (maximal abundance per sample: 2.38%). Flavobacteria were present in 26 samples (maximum abundance per sample: 31.09%). Bacteroidia, Mollicutes, Actinobacteria/Gammaproteobacteria, and Bacilli were present in 1, 4, 14, and 19 samples, respectively [maximum abundance (%) of the total microbiota per sample: 0.6, 4.96, 1.88, and 4.11, respectively]. Finally, Actinobacteria were present in 7 samples (maximum abundance per sample: 0.19%). The heat map analysis in **Figure 5** shows the distribution of the most abundant classes (those that were present in at least 7 samples) among the different samples. The other identified classes, such as Negativicutes, Rubrobacteria, Sphingobacteriia, and Clostridia, were present in <7 samples at a frequency of <0.25% per sample. In general, bacterial classes were very unevenly distributed among the samples. For example, whereas the abundance of Gammaproteobacteria was 100% in sample 15 and sample 32, sample 20 harbored representatives of Gammaproteobacteria, Flavobacteria, and Alphaproteobacteria (respective abundance: 57.74, 31.11, and 11.10%).

Bacterial Genera

Out of the 40 samples, 83 different bacteria genera (**Table S1**) were detected. The heat map analysis (**Figure S2**) performed on genera present in at least four samples, displays the distribution of genera, and their respective abundance, among the 40 samples. Midgut bacteria were unevenly distributed among individual tsetse flies and between the different sampled localities. Several genera were found in all (or most of) tsetse flies, possibly representing the midgut core microbiota. This was the case for the genus *Wigglesworthia*, as well as for several genera belonging to the Enterobacteriaceae family. The relative abundance of *Wigglesworthia* ranged from 34.5 to 99.8% across the 40 samples. Other genera widely distributed included two *Enterobacter* that were present, respectively, in 40 (relative abundance: 0.02–61.1%) and in 21 samples (relative abundance: 0.02–1.11%); *Candidatus Sulcia muelleri*, present in 26 samples (relative abundance: 0.01–31.09%); two *Serratia* present in 29 samples (relative abundance: 0.01–14%) and in 20 samples (relative abundance: 0.01–4.95%).

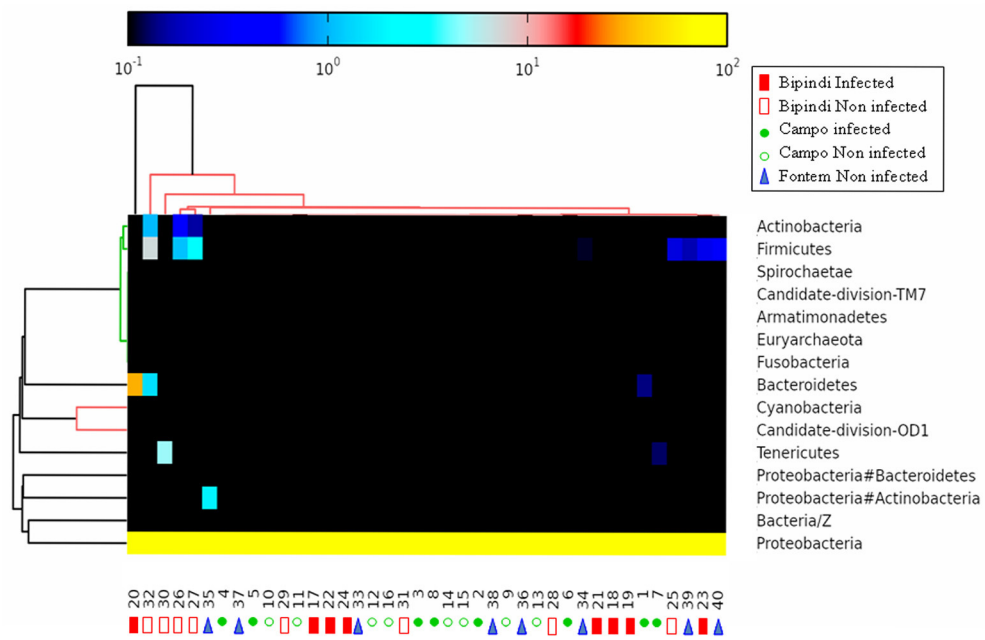

FIGURE 4 | Heat map analysis of the distribution and abundance of the bacterial phyla in midgut samples. The samples did not cluster together, indicating highly variable bacterial communities. Numbers 1–8: infected samples from the Campo focus; Numbers 9–16: non-infected samples from the Campo focus; Numbers 17–24: infected samples from the Bipindi focus; Numbers 25–32: non-infected samples from the Bipindi focus; Numbers 33–40: non infected samples from the Fontem focus.

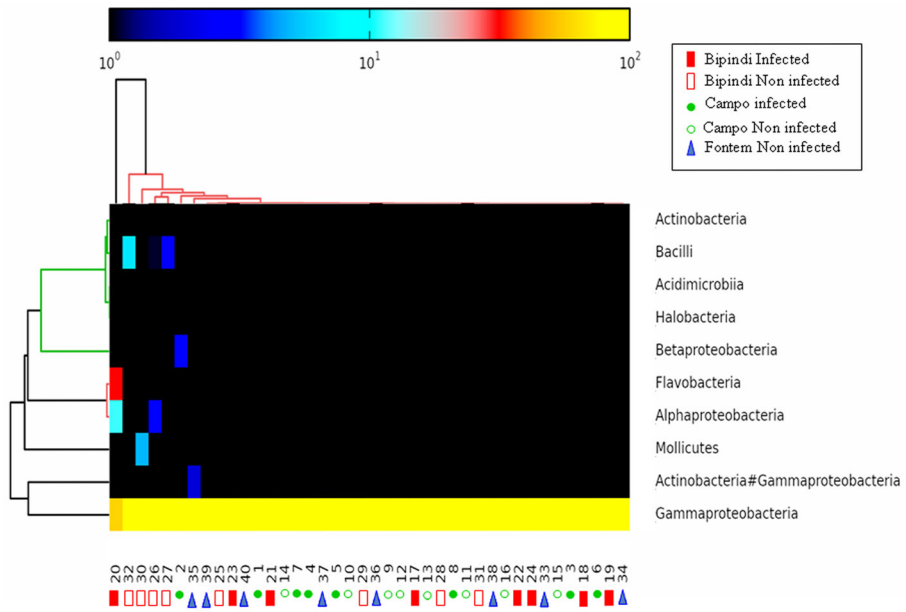

FIGURE 5 | Heat map analysis of the distribution and abundance of the bacterial classes in midgut samples. The samples did not cluster together, indicating highly variable bacterial communities. Sample numbering as in **Figure 4**.

Furthermore, *Wolbachia* was present in 25 samples (relative abundance: 0.01–11.08%), while *Sodalis* was only present in 4 samples, with a maximum relative abundance of 0.06%.

These results indicate that the midgut bacterial flora was mainly composed of gram-negative communities. Gram-positive bacteria belonged to the Bacilli, Mollicutes, Actinobacteria, Rubrobacteria, Clostridia, Fusobacteriia, Acidimicrobiia, and Thermoleophilia classes, and represented 10% of the total bacteria.

Finally, a total of 103 bacterial species (**Table S1**) and one Miscellaneous Euryarchaeotic Group (MEG, archeae) were detected. Only a few genera were shown to include more than one species, including *Bacillus*, *Burkholderia*, *Enterobacter*, *Neisseria*, *Prevoltella*, *Providencia,* and *Serratia*.

Of note, sequences assigned to *W. glossinidia* showed a relative abundance higher than 80% in all except two samples (samples 20 and 35, relative abundance 51.27 and 34.55%, respectively; **Figure S3**). A high number of sequences assigned to the *Pseudomonas*, *Sphingomonas,* and Hyphomicrobiaceae were detected in sample 26, and 74 species were found in sample 32.

Bacterial Communities between Infected and Uninfected Flies from the Different Geographic Located HAT

The average number of bacterial species per tsetse sample was not significantly different regarding their group (Kruskal–Wallis *p-value* = 0.342), their geographical origin (Kruskal–Wallis *p-value* = 0.167), or their status (infected vs. non-infected; Kruskal–Wallis *p-value* = 0.836).

We investigated potential relationships between the structure of the gut microbial communities of tsetse flies from Campo, Bipindi, and Fontem foci and the fly's trypanosome infection status. A hierarchical clustering using the Bray–Curtis indice did not discriminate unambiguously the different groups (**Figure 6**). It however showed a trend of infected flies from a given focus to be separated from non-infected flies of the same focus. One can also notice that infected flies from Bipindi are closely related to non-infected flies from Campo and vice versa. In contrast, the non-infected samples from Fontem were distributed over the different clusters. These results were reinforced by the nonmetric multidimensional scaling plots shown in **Figures S4A,B**.

Novel Bacteria Taxa

A phylogenetic tree was inferred to further investigate the potential dissimilarities between the bacteria sequences identified in this study and the sequences currently available in public databases, thereby illustrating the taxonomic relationships between the representative OTU sequences (**Figures S5A–D**). The most abundant OTU present in each of the 40 samples showed sequence similarities with *Wigglesworthia* sp. We identified 60 different genotypes of *Wigglesworthia*, similar to those from *G. morsitans* or *G. brevipalpis*. Depending on the sample, there were one to four genotypes per sample. However, most samples harbored one genotype, except those from Fontem that harbored two to four different genotypes in the same sample.

Some OTU-representative sequences did not match known targets in the NCBI database (analyses performed in 2014) and had to be annotated manually. Sample 20 (**Figure 7**) was shown to harbor four different genotypes of several bacteria species, including *Candidatus Nasuia* sp., *Wigglesworthia* sp., and *Wolbachia* sp. Similarly, four different genotypes of

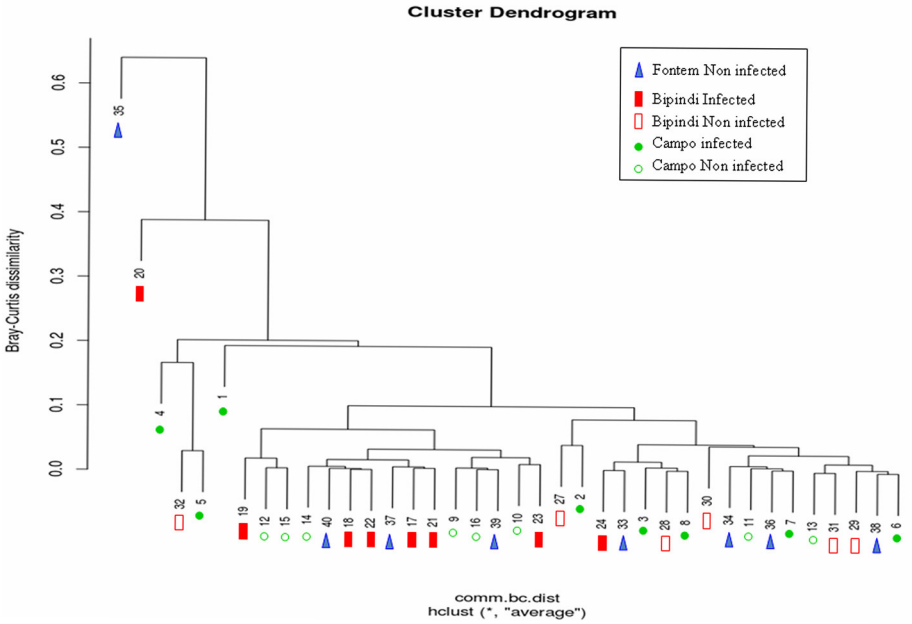

FIGURE 6 | Hierarchical cluster dendrogram based on Bray-Curtis Index values, showing the relationship between different samples (represented by numbers) and foci. Sample numbering as in **Figure 4**.

Enterobacteriaceae sp., *Wigglesworthia* sp., *Wolbachia* sp., and *Spiroplasma* sp. were detected in sample 30 (**Figure 8**).

Presence of Symbionts

Special attention was paid to symbionts, due to their role in tsetse physiology and their interaction with trypanosomes. *Wigglesworthia glossinidia* was once again characterized by its high density in most samples and groups; this was assessed by the high percentages (more than 80% in each sample), except in two samples from groups 4 and 5 (Bipindi and Fontem, non-infected flies). *Wolbachia* sp. sequences were also present in all groups, but in smaller amounts. Nevertheless, a very high number of *Wolbachia* sequences were observed in sample 20 (11.08%). In addition, we detected, at low relative abundance, the presence of the symbiont *S. glossinidius* in four samples distributed over all groups, except Bipindi-uninfected (group 4).

DISCUSSION

The main objective of this study was to characterize the bacterial communities inhabiting the midgut of field-collected tsetse flies from sleeping sickness foci in Cameroon using deep sequencing. Trypanosome-infected and non-infected flies were sampled in three sleeping sickness foci in Cameroon (Bipindi, Campo, and Fontem) that differ in their natural environment, thus offering the possibility to investigate the potential association between specific bacterial communities composition and the environment and/or the ability of the flies to be infected by trypanosomes. In this study the targeted trypanosome species was *T. congolense* (causing AAT) since, as shown in **Figure 3**, its prevalence was large enough in both Campo and Bipindi sampled flies. In contrast, *T. brucei* (*sensu lato*, Tb *sl* hereafter) had a much lower prevalence. Indeed, only 6 Tb *sl* infected flies were recorded in the sampled sites which, furthermore, included the two sub-species, *T. brucei brucei* and *T. brucei gambiense*—the former causing AAT and the second being responsible for the chronic form of HAT.

The Campo sampling campaign was performed in 2008, while the ones regarding Bipindi and Fontem were performed in 2013. A reduced but significant gene flow, due to fly migration, was previously reported between Campo and Bipindi (Farikou et al., 2011b), raising the question whether the 5 years delay between the sampling periods could affect the fly infection status and the microbiota profiles. One may consider that in the case of an important and/or continuous migration flow for years, differences in fly population diversity between the two foci would have been reduced or even suppressed. This was clearly not the case, and was never recorded (Farikou et al., 2010a,b). Although, the possibility that the 5 years delay could marginally affect the comparison between the 3 foci cannot be totally excluded, it could not affect the intra-foci comparison between trypanosome infected and non-infected flies.

Diversity of the Bacterial Communities and Its Origin

We detected a very large, unexpected number of different bacterial taxa (103 species), among which 26 were found in more than one sample. The number of different species identified in the present study was clearly much higher than in previous reports. For instance, three species were identified in *G. p. palpalis* in Angola (Geiger et al., 2009); one, three and eight species were, respectively, found in *G. nigrofusca*, *G. pallicera,* and *G. p. palpalis*

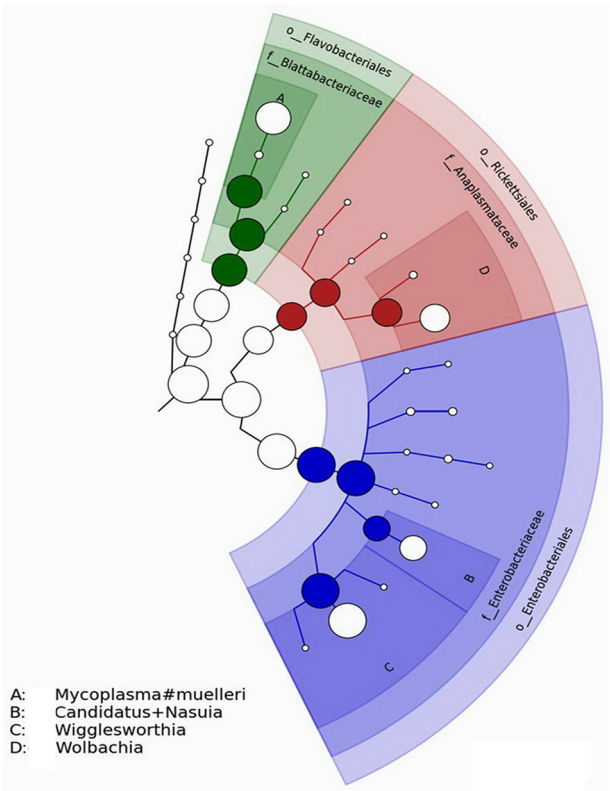

FIGURE 7 | Phylogenetic diversity of bacterial *16S rRNA* sequences found in sample 20 (infected tsetse fly midguts from Bipindi).

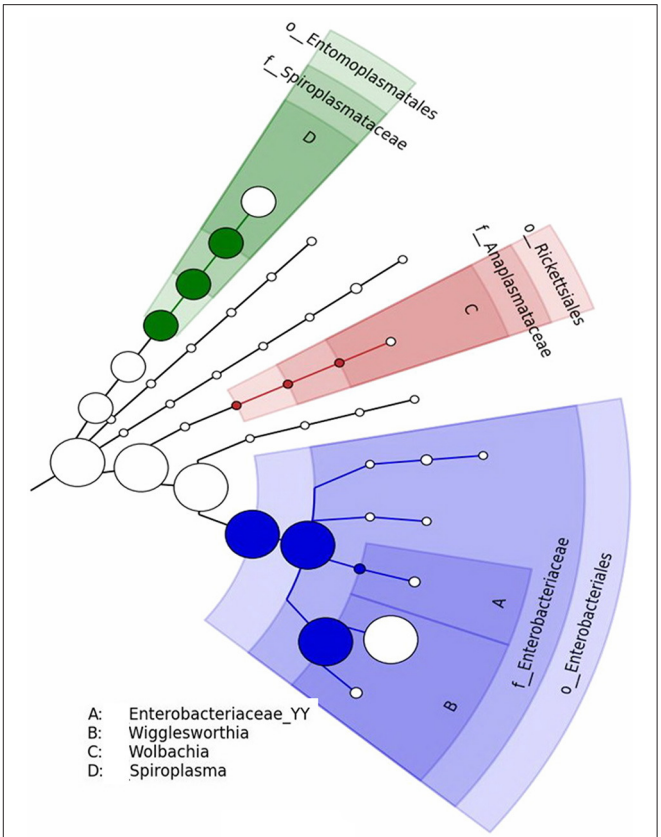

FIGURE 8 | Phylogenetic diversity of bacterial *16S rRNA* sequences found in sample 30 (uninfected tsetse fly midguts from Bipindi).

in Cameroon (Geiger et al., 2011b); one species was identified in *G. p. gambiensis*; and 24 species were found in *G. f. fuscipes* in Kenya (Lindh and Lehane, 2011). With the exception of two bacterial species, all species detected in these previous studies were identified using culture-dependent methods. Many bacteria species are difficult to culture and the density of some species may be too low for proper isolation. This could explain the low number of bacteria identified in other studies as compared to the high number of species we characterized in the gut of tsetse flies using molecular approach.

A more recent study (Aksoy et al., 2014) identified a high number of bacteria species in the gut microbiota of several tsetse fly species collected in Uganda using molecular approaches, although the bacteriome of Uganda flies was less diverse than the one of flies from Cameroon.

Compared to the bacterial communities of flies from Uganda, flies from Cameroon revealed both similarities (namely Enterobacteriaceae and an unexpected high abundance of *Wigglesworthia*) and differences (bacteria belonging to the genus *Delftia*, *Stenotrophomonas*, *Thermoanaerobacterium*, *Proteus*, and *Ochrobactrum*, among others, were not found in flies from Cameroon). These differences may be due to differences in the respective investigated fly species, *G. f. fuscipes*, *G. m. morsitans*, *G pallidipes* vs. *G. p. palpalis*, and/or the respective environmental factors and the geographic locations of the HAT/AAT foci in Uganda vs. Cameroon.

Aside from the question of what effect these gut bacteria may have on tsetse fly physiology and/or on their ability to become trypanosomes infected (i.e., their vector competence), there is also the question of how tsetse flies can acquire such a large diversity of bacterial species. In their report, Aksoy et al. (2014) mentioned the possibility that some of the low abundant bacteria "may have arisen from contamination from environmental sources, such as during field-based dissection processes,...." Although, the occurrence of contamination can never be totally excluded, our data do not support this comment since it would be expected, in such case, that several bacteria species from the environment would be detected, at low abundance, in all or most of the samples, inasmuch the 40 fly were processed at the same time. As shown in **Table S1**, this is not the case; most of the low abundant bacteria (74 out of 103) are present in only one sample and, in contrast, one of the samples (number 32) was shown to harbor 59 species. Nevertheless, further independent studies should be undertaken to confirm the finding. An alternative explanation could account for the complexity of the microbiota as it was recently reported, that is, tsetse flies are not strictly hematophagous; they can occasionally feed on nectar from a broad range of plants growing within their different ecological environments, and, thus, may ingest bacteria colonizing this nutriment (Colman et al., 2012; Solano et al., 2015). Such effect

of diet differences and their links to environmental conditions, on bacteriome community structure of insects has been documented by Colman et al. (2012). Finally, as reported by Poinar et al. (1979), adult flies may be contaminated with bacteria present on the epidermis of humans or animals they bite during blood meal. This could lead to a large diversity of the fly bacteriome since tsetse flies are known to ingest their blood meals from a variety of vertebrates (Moloo et al., 1988; Simo et al., 2008; Farikou et al., 2010b). Thus, despite the viviparous reproduction of tsetse flies (in contrast to other dipterans) that prevent fly contamination at the larval stage (except for the symbionts that are vertically transmitted from the mother to the larva *via* milk gland secretions), the modes of acquiring "exogenous" bacteria by tsetse flies seems to be more diverse than previously believed and could explain the recorded large microbiota diversity herein.

One however has to keep in mind that in addition to true tsetse gut inhabitants, it is likely that some—or many—of the 143 OTUs are solely opportunistic, simply in transit or possibly present only as DNA fragments. Since dead bacteria provide the same DNA signature as do living bacteria, further investigations should aim to isolate bacteria of interest; only such approach will provide best evidence of their presence as a living organism in the tsetse gut.

Bacterial Species Richness of the Samples (Alpha Diversity)

Although, the analyzed midgut bacteriome of adult tsetse flies included 103 species belonging to 13 phyla, only 4 of them were dominant (Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes), showing some similarities with gut microbial communities from other invertebrates such as cattle tick (Andreotti et al., 2011), several species of *Anopheles* (Rani et al., 2009; Boissière et al., 2012) and of *Aedes* (Zouache et al., 2011; Terenius et al., 2012); Acridid (Dillon et al., 2008); Asian longhorned beetle (Geib et al., 2009); *Lutzomyia longipalpis* (Gouveia et al., 2008); honey and bumble bees (Martinson et al., 2011). However, there is a major difference between the bacteriome of all these invertebrates and those of tsetse flies, namely the presence, in the latter, of *W. glossinidia* in all samples. *W. glossinidia*'s presence is not surprising as it is the tsetse fly's obligate symbiont; its abundance in the different samples was however remarkable as the mean relative abundance over the 40 samples was as high as 95.3% (even higher than 99% in all but five samples—see below), while that of *Enterobacter homaechei*, also present in all samples, was only 1.8%. Such an "overabundance" of *Wigglesworthia* was previously reported by Aksoy et al. (2014) who estimate that it may disrupt proper identification of low abundant bacteria thus leading to an underestimation of the alpha diversity (species richness). In order to decrease the abundance of this bacterium—and thus overcoming the difficulty related to its overabundance—the group of Aksoy et al. (2014) excised part of the bacteriome organ harboring *Wigglesworthia* and was able to discover some bacteria previously non-identified when analyzing the whole bacteriome. In our study, five samples showed smaller *Wigglesworthia* relative abundance than the 35 others; these are [sample number and (relative *Wigglesworthia* abundance)] sample 2 (88.83%), 4 (80.52%), 20 (51.27%), 32 (86.96%), and 35 (34.55%) while the numbers of hosted bacteria were, respectively, 16, 9, 8, 73, 10 (*Wigglesworthia* excluded). Thus, concerning our study, it seems that there was no relationship between the *Wigglesworthia* relative abundance and the species richness of the samples [for example, sample 32 hosted the largest number of bacteria although the relative abundance of *Wigglesworthia* was rather high (86.96%)]. Thus, as suggested by Aksoy et al. (2014), further investigations on the tsetse fly bacteriome should be performed using genomic/complementary functional approaches.

Microbiome Composition and the Possible Role of Identified Bacteria

Most of the sequence tags corresponded to few taxa. Only 16 species displayed a maximum relative abundance per sample higher than 1%, whereas 78 species had a maximum relative abundance lower than 0.2%. Nevertheless, some members of the latter group of bacteria are potentially of great interest.

The Symbionts

This group of bacteria includes in particular three species *W. glossinidia*, *S. glossinidius,* and *Wolbacchia*. Besides its prominent role as an obligate symbiont of the tsetse fly, *Wigglesworthia* differs from the other bacteria by the fact that 60 genotypes of this species have been identified, whereas most other bacteria were represented by only one genotype, or at least by a limited number of genotypes. In samples from Uganda, differences were also detected in the sequences of the *Wigglesworthia* V4 region (Aksoy et al., 2014) that confirmed the existence of several genotypes, although the authors reported small amounts of *Wigglesworthia* carryover between the different tsetse species sampled; thus they do not exclude completely the occurrence of a cross contamination. Nevertheless, the occurrence of multiple genotypes of a given species is not a surprise and may not be considered as an artifact generated along the technical process. For example, we have previously identified number of *S. glossinidius* genotypes when using other approaches than amplicon sequencing (Illumina MiSeq) such as AFLP (Geiger et al., 2005, 2007) or microsatellite (Farikou et al., 2011a,b) genotyping on either insectary-reared or field sampled tsetse flies. This finding could be of great interest, as until now only the density of *Wigglesworthia*, not its potential genetic diversity, was considered to be involved in the modulation of tsetse fly infection by trypanosomes (Wang et al., 2009). In fact, the genetic diversity within populations of *W. glossinidia* that infect tsetse flies could play a prominent role, as previously demonstrated for the facultative symbiont *S. glossinidius* (Geiger et al., 2007). However, investigating a possible association between trypanosome infection and specific *W. glossinidia* genotypes (60 have been identified) will require a much larger sampling size.

Attention was paid to *Sodalis* that was previously shown to be involved in modulating the tsetse fly's ability to acquire trypanosomes (Farikou et al., 2010a; Hamidou Soumana et al., 2014). In the present study this symbiont was identified in 10% of the samples, a finding markedly different from the 30% average prevalence we found previously in nearly the

same sampling sites (Farikou et al., 2010a), and from that recorded for example by Aksoy et al. (2014) who suggest the variations could be a matter of sensitivity of the analytical approach. However, this could be only part of an explanation since we used in our study similar analytical approaches and instruments (including the Illumina MiSeq platform). Overall, these data confirm the existence of a very high symbiont variability in natural populations of flies, as previously reported by Farikou et al. (2010a). These variations could be linked to a multitude of factors including differences in tsetse individual flying times, field environment modifications or, as suggested by Aksoy et al. (2014) the competition between *Sodalis* and other intestinal bacteria which diversity and respective abundance may vary.

Wolbachia sp. was previously characterized in field populations of *G. morsitans morsitans*, *G. morsitans centralis*, *G. austeni* flies, *G. brevipalpis*, *G. pallidipes,* and *G. p. gambiensis* (Doudoumis et al., 2012). Interestingly, *Wolbachia* has not been characterized in *G. fuscipes fuscipes* or *G. tachinoides* and, until now in *G. p. palpalis*. The bacterium was detected in 25 out of 40 flies (62.5%) and was distributed over the 5 groups (thus across the three sampling sites, and the fly trypanosome infection status): 5, 4, 7, 5, and 4 *Wolbachia* positive flies in groups 1–5, respectively. At this stage of the investigation we cannot suggest any explanation for its presence; we can only report its relatively high prevalence. The fact that until now *Wolbacchia* was never characterized in *G. palpalis palpalis* does not mean such infection does not occur in other fly individuals; further studies should focus on surveys of larger *G. p. palpalis* populations from the studied HAT foci, or on populations from HAT foci located elsewhere than in Cameroon (for example flies from HAT foci located in RDC—République Démocratique du Congo—or in Angola). In a recent report, Ji et al. (2015) highlight the extreme variability (from 1.54 to 66.67%) of the prevalence of *Wolbacchia* across 25 *Bemisia tabaci* populations from China. The frequency of *Bemisia* infection was shown to be affected by a large panel of factors (putative *B. tabaci* species, geographic location, sex of the host ...). Finally, a cystoplasmic incompatibility occurs when a *Wolbacchia* negative (W−) female fly mates with a *Wolbacchia* positive (W+) male, and the embryos degenerate (Alam et al., 2011). In contrast, when a (W+) female mates with a (W+) or a (W−) male, the offspring is not only fertile but also more numerous than in the case of a (W−) female with (W−) male mating. As *Wolbacchia* is transmitted by the female fly to its offspring, the presence of *Wolbacchia* in a female gives them a reproductive advantage. Could this explain the high prevalence of *Wolbacchia* in the studied Gpp populations?

In contrast to *Anopheles gambiae* whose susceptibility to *Plasmodium* infection increased with the presence of *Wolbachia* under natural conditions (Zélé et al., 2014); the presence of this symbiont was not found to be associated with the infection status of the tsetse flies.

We also identified in tsetse fly midguts the presence of bacteria that are known to be symbionts when infecting other organisms. This is the case of *Candidatus Sulcia muelleri* (Flavobacteriaceae), which displayed an ancient symbiotic relationship with the hemipteran *Auchenorrhyncha* (Moran et al., 2005). The identified bacterium *Candidatus Zinderia* is also a symbiont of the tick *Haemaphysalis longicornis* (McCutcheon and Moran, 2010). Finally, we observed the cohabitation of four symbionts in some of our analyzed samples, although it is unknown if they develop a synergistic effect on the fly's physiology. Given the current state of our investigation, large-scale analyses (including attempts to isolate the two *Candidatus* species) deserve to be undertaken, as until now only three symbionts have been described in tsetse flies.

Other Bacteria of Interest

S. marcescens and *Serratia odorifera* (Enterobacteriaceae) were present in more than 50% of the samples. Both species are considered to be opportunistic pathogens for humans (Chmel, 1988; Hejazi and Falkiner, 1997; Grimont and Grimont, 2015). *Serratia marcescens* is also responsible for the increased mortality in tsetse flies (Poinar et al., 1979) such as *Glossina pallidipes* (Gonzalez-Ceron et al., 2003); it secretes trypanolytic compounds and reduces the establishment of *T. cruzi* in the midgut of its vector, *Rhodnius prolixus* (Triatominae), vector of *T. cruzi* causing the Chagas disease; Azambuja et al., 2004). One previous work reported the presence of the novel species *S. glossinae* in the gut of insectary-reared *G. palpalis gambiensis* flies (Geiger et al., 2010), although this species has never been identified in field-collected *Glossina* flies. Surprisingly, the same situation was observed for mosquitoes where the bacterium *Elizabethkingia* was found only in insectary-reared *Anopheles* sp. (Boissière et al., 2012; Gimonneau et al., 2014). However, this phenomenon cannot be currently explained. The presence of *Serratia* in the guts of *G. f. fuscipes* flies from western Kenya has also been reported (Lindh and Lehane, 2011). In *Anopheles gambiae* mosquito midguts, multiple strains of *Serratia* have been identified, some of which are able to reduce mosquito infections by *Plasmodium* (Bando et al., 2013). *Serratia* has also been identified as a newly acquired symbiotic partner in aphids (Burke and Moran, 2011). Currently, we did not evidence a clear association between the presence of some bacterial and trypanosome infection. However, as suggested previously by Aksoy et al. (2014), understanding the association of *Serratia* with tsetse flies and its functional role in tsetse physiology could provide critical knowledge about trypanosome transmission dynamics in tsetse flies. Similar investigations could be performed with other bacteria.

In addition to *Serratia*, other identified bacteria have the ability to produce antiparasitic compounds, including members of *Acinetobacter*. This genus was previously characterized in the gut of tsetse flies from Cameroon (Geiger et al., 2009). The presence of *Acinetobacter* (*guillouiae* and spp.) was observed in 6 samples in the present study.

Association between the Composition of the Intestinal Communities and the Fly Infection Status and/or the Environmental Differences

The characterization of the composition of the intestinal bacterial communities was an important objective of the work. However,

the statistical analyses of the identified bacteria in this study did not reveal any significant differences in the sample bacterial species richness between the different groups (Campo and Bipindi infected and uninfected flies as well as Fontem uninfected flies), or the status of the flies (infected vs. uninfected). A hierarchical clustering neither discriminate properly the different groups, nor the infected flies from the non-infected flies of the same focus. At the level of individual bacteria, some of them seem to display an association with a given group or a sampling site. However, most often they concern species that are poorly represented. The possibly that environmental differences between the three sampling sites (especially the climate, wildlife, and flora) were too weak to induce significant differences in the flies' microbiome composition may be considered. These data suggest future fly microbiome research should aim for greater numbers of flies and target more ecologically contrasted sites, in order to identify bacteria involved in (or at least associated with) the fly vector competence.

In conclusion the meta-barcoding analysis that we performed on DNA extracted from *G. p. palpalis* collected from three different HAT foci in Cameroon resulted in the detection of 143 OTUs belonging to 83 genera and 13 phyla. Part of these bacteria could be identified at the species level (103 annotated OTUs). However, some species were not identifiable since the corresponding sequences did not match any available sequence libraries; they may correspond to novel species, and need to undergo further identification process. The presence of such a variety of bacteria was unexpected and raises many questions regarding how they are acquired by tsetse flies, and the role they may play regarding the fly's physiology and vector competence. In addition, it would be of interest, in a following investigation campaign, to enlarge the sampling scheme and include teneral flies as a supplementary control besides non-infected non-teneral flies. The former are expected to host a more limited microbiota and to have a less developed immunity compared to the latter. On the other hand, it should be noted that, when teneral flies undergo artificial infection process, about 70% do not develop trypanosome infection (Geiger et al., 2007). This suggests that within a collection of teneral flies most of them, even considered as "naïve," are physiologically and genetically "programmed" to be/become refractory to trypanosome infection.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FJ, AG. Performed the experiments: FJ, TM, GN, FN, AG. Analyzed the data: FJ, RC, AG. Contributed reagents/materials/analysis tools: AG. Wrote the paper: GG, FN, LA, JR, AG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01464/full#supplementary-material

Table S1 | Classification of the total bacteria detected in the 40 tsetse samples from the Campo, Bipindi, and Fontem foci in Cameroon. The table provides, for each sample, the relative abundance (in %) of each bacterium present in this sample. Several of the detected bacteria could not be identified at the species level. The suffix "X or Y" refers to "unclassified" at that rank.

Figure S1 | Rarefaction analysis on the studied samples (Renyi diversity plots); **(A)** Samples 1–24 and 27–40; **(B)** Samples 25 and 26 that were processed apart.

Figure S2 | Heat map analysis of the distribution and abundance of the bacterial genera in midgut samples. The samples did not cluster together, indicating that the bacterial community among them is highly variable. Sample numbering as in **Figure 4**.

Figure S3 | Heat map analysis of the repartition and abundance of bacterial species in midgut samples. The samples did not cluster together, indicating that the bacterial community among them is highly variable. Sample numbering as in **Figure 4**.

Figure S4 | (A) NMDS plot comparing bacterial OTUs in tsetse fly midguts of different foci (Bipindi, Campo, Fontem) using Bray-Curtis dissimilarity indice. **(B)** NMDS plot comparing bacterial OTUs of infected vs. non infected tsetse fly midguts using Bray-Curtis dissimilarity indice.

Figure S5 | Phylogenetic diversity of bacterial *16S rRNA* sequences in tsetse fly midguts. NJ tree clustering of OTU representatives based on homology with known sequences is shown. The width of the branch is proportional to the number of samples exhibiting a given OTU. Colors correspond to different phyla. **(A–D)** Expended scale of four regions from this figure in order to make it easier to read (the corresponding regions are indicated in this figure).

REFERENCES

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., et al. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nat. Genet.* 32, 402–407. doi: 10.1038/ng986

Aksoy, E., Telleria, E. L., Echodu, R., Wu, Y., Okedi, L. M., Weiss, B. L., et al. (2014). Analysis of multiple tsetse fly populations in Uganda reveals limited diversity and species-specific gut microbiota. *Appl. Environ. Microbiol.* 80, 4301–4312. doi: 10.1128/AEM.00079-14

Aksoy, E., Vigneron, A., Bing, X., Zhao, X., O'Neill, M., Wu, Y. N., et al. (2016). Mammalian African trypanosome VSG coat enhances tsetse's vector competence. *Proc. Natl. Acad. Sci. U.S.A.* 113, 6961–6966. doi: 10.1073/pnas.1600304113

Aksoy, S. (1995). *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *Int. J. Syst. Bacteriol.* 45, 848–851. doi: 10.1099/00207713-45-4-848

Aksoy, S., Caccone, A., Galvani, A. P., and Okedi, L. M. (2013). Glossina fuscipes populations provide insights for human African trypanosomiasis transmission in Uganda. *Trends Parasitol.* 29, 394–406. doi: 10.1016/j.pt.2013.06.005

Alam, U., Hyseni, C., Symula, R. E., Brelsfoard, C., Wu, Y., Kruglov, O., et al. (2012). Implications of microfauna-host interactions for trypanosome transmission dynamics in Glossina fuscipes fuscipes in Uganda. *Appl. Environ. Microbiol.* 78, 4627–4637. doi: 10.1128/AEM.00806-12

Alam, U., Medlock, J., Brelsfoard, C., Pais, R., Lohs, C., Balmand, S., et al. (2011). Wolbachia symbiont infections induce strong cytoplasmic incompatibility in the tsetse fly *Glossina morsitans*. *PLoS Pathog.* 7:e1002415. doi: 10.1371/journal.ppat.1002415

Andreotti, R., Pérez de León, A. A., Dowd, S. E., Guerrero, F. D., Bendele, K. G., and Scoles, G. A. (2011). Assessment of bacterial diversity in the cattle tick Rhipicephalus (Boophilus) microplus through tag-encoded pyrosequencing. *BMC Microbiol.* 11:6. doi: 10.1186/1471-2180-11-6

Azambuja, P., Feder, D., and Garcia, E. S. (2004). Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector. *Exp. Parasitol.* 107, 89–96. doi: 10.1016/j.exppara.2004.04.007

Baker, N., de Koning, H. P., Mäser, P., and Horn, D. (2013). Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. *Trends Parasitol.* 29, 110–118. doi: 10.1016/j.pt.2012.12.005

Balmand, S., Lohs, C., Aksoy, S., and Heddi, A. (2013). Tissue distribution and transmission routes for the tsetse fly endosymbionts. *J. Invertebr. Pathol.* 112, S116–S122. doi: 10.1016/j.jip.2012.04.002

Bando, H., Okado, K., Guelbeogo, W. M., Badolo, A., Aonuma, H., Nelson, B., et al. (2013). Intra-specific diversity of *Serratia marcescens* in Anopheles mosquito midgut defines Plasmodium transmission capacity. *Sci. Rep.* 3:1641. doi: 10.1038/srep01641

Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., et al. (2012). Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with *Plasmodium falciparum* infection. *PLoS Pathog.* 8:e1002742. doi: 10.1371/journal.ppat.1002742

Burke, G. R., and Moran, N. A. (2011). Massive genomic decay in Serratia symbiotica, a recently evolved symbiont of aphids. *Genome Biol. Evol.* 3, 195–208. doi: 10.1093/gbe/evr002

Chen, X., Song, L., and Aksoy, S. (1999). Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus Glossina and its bacteriome associated endosymbiont, *Wigglesworthia glossinidia*. *J. Mol. Evol.* 48, 49–58. doi: 10.1007/PL00006444

Cheng, Q., and Aksoy, S. (1999). Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Mol. Biol.* 8, 125–132. doi: 10.1046/j.1365-2583.1999.810125.x

Cheng, Q., Ruel, T. D., Zhou, W., Moloo, S. K., Majiwa, P., O'Neill, S. L., et al. (2000). Tissue distribution and prevalence of Wolbachia infections in tsetse flies, *Glossina* spp. *Med. Vet. Entomol.* 14, 44–50. doi: 10.1046/j.1365-2915.2000.00202.x

Chevenet, F., Brun, C., Ba-uls, A. L., Jacq, B., and Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 7:439. doi: 10.1186/1471-2105-7-439

Chmel, H. (1988). Serratia odorifera biogroup 1 causing an invasive human infection. *J. Clin. Microbiol.* 26, 1244–1245.

Colman, D. R., Toolson, E. C., and Takacs-Vesbach, C. D. (2012). Do diet and taxonomy influence insect gut bacterial communities? *Mol. Ecol.* 21, 5124–5137. doi: 10.1111/j.1365-294X.2012.05752.x

Dillon, R. J., Webster, G., Weightman, A. J., Dillon, V. M., Blanford, S., and Charnley, A. K. (2008). Composition of Acridid gut bacterial communities as revealed by 16S rRNA gene analysis. *J. Invertebr. Pathol.* 97, 265–272. doi: 10.1016/j.jip.2007.09.010

Doudoumis, V., Tsiamis, G., Wamwiri, F., Brelsfoard, C., Alam, U., Aksoy, E., et al. (2012). Detection and characterization of Wolbachia infections in laboratory and natural populations of different species of tsetse flies (genus Glossina). *BMC Microbiol.* 12(Suppl. 1):S3. doi: 10.1186/1471-2180-12-S1-S3

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Farikou, O., Njiokou, F., Cuny, G., and Geiger, A. (2011a). Microsatellite genotyping reveals diversity within populations of *Sodalis glossinidius*, the secondary symbiont of tsetse flies. *Vet. Microbiol.* 150, 207–210. doi: 10.1016/j.vetmic.2011.01.021

Farikou, O., Njiokou, F., Mbida Mbida, J. A., Njitchouang, G. R., Djeunga, H. N., Asonganyi, T., et al. (2010a). Tripartite interactions between tsetse flies, *Sodalis glossinidius* and trypanosomes–an epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. *Infect. Genet. Evol.* 10, 115–121. doi: 10.1016/j.meegid.2009.10.008

Farikou, O., Njiokou, F., Simo, G., Asonganyi, T., Cuny, G., and Geiger, A. (2010b). Tsetse fly blood meal modification and trypanosome identification in two sleeping sickness foci in the forest of southern Cameroon. *Acta Trop.* 116, 81–88. doi: 10.1016/j.actatropica.2010.06.002

Farikou, O., Thevenon, S., Njiokou, F., Allal, F., Cuny, G., and Geiger, A. (2011b). Genetic diversity and population structure of the secondary symbiont of tsetse flies, *Sodalis glossinidius*, in sleeping sickness foci in Cameroon. *PLoS Negl. Trop. Dis.* 5:e1281. doi: 10.1371/journal.pntd.0001281

Geib, S. M., Jimenez-Gasco Mdel, M., Carlson, J. E., Tien, M., and Hoover, K. (2009). Effect of host tree species on cellulase activity and bacterial community composition in the gut of larval Asian longhorned beetle. *Environ. Entomol.* 38, 686–699. doi: 10.1603/022.038.0320

Geiger, A., Cuny, G., and Frutos, R. (2005). Two Tsetse fly species, *Glossina palpalis* gambiensis and *Glossina morsitans* morsitans, carry genetically distinct populations of the secondary symbiont *Sodalis glossinidius*. *Appl. Environ. Microbiol.* 71, 8941–8943. doi: 10.1128/AEM.71.12.8941-8943.2005

Geiger, A., Fardeau, M. L., Falsen, E., Ollivier, B., and Cuny, G. (2010). *Serratia glossinae* sp. nov., isolated from the midgut of the tsetse fly *Glossina palpalis* gambiensis. *Int. J. Syst. Evol. Microbiol.* 60, 1261–1265. doi: 10.1099/ijs.0.013441-0

Geiger, A., Fardeau, M. L., Grebaut, P., Vatunga, G., Josénando, T., Herder, S., et al. (2009). First isolation of *Enterobacter*, *Enterococcus*, and *Acinetobacter* spp. as inhabitants of the tsetse fly (*Glossina palpalis* palpalis) midgut. *Infect. Genet. Evol.* 9, 1364–1370. doi: 10.1016/j.meegid.2009.09.013

Geiger, A., Fardeau, M. L., Njiokou, F., Joseph, M., Asonganyi, T., Ollivier, B., et al. (2011b). Bacterial diversity associated with populations of *Glossina* spp. from Cameroon and distribution within the Campo sleeping sickness focus. *Microb. Ecol.* 62, 632–643. doi: 10.1007/s00248-011-9830-y

Geiger, A., Ravel, S., Mateille, T., Janelle, J., Patrel, D., Cuny, G., et al. (2007). Vector competence of *Glossina palpalis* gambiensis for *Trypanosoma brucei* s.l. and genetic diversity of the symbiont *Sodalis glossinidius*. *Mol. Biol. Evol.* 24, 102–109. doi: 10.1093/molbev/msl135

Geiger, A., Simo, G., Grébaut, P., Peltier, J. B., Cuny, G., and Holzmuller, P. (2011a). Transcriptomics and proteomics in human African trypanosomiasis: current status and perspectives. *J. Proteomics* 74, 1625–1643. doi: 10.1016/j.jprot.2011.01.016

Gimonneau, G., Tchioffo, M. T., Abate, L., Boissière, A., Awono-Ambéné, P. H., Nsango, S. E., et al. (2014). Composition of Anopheles coluzzii and Anopheles gambiae microbiota from larval to adult stages. *Infect. Genet. Evol.* 28, 715–724. doi: 10.1016/j.meegid.2014.09.029

Gonzalez-Ceron, L., Santillan, F., Rodriguez, M. H., Mendez, D., and Hernandez-Avila, J. E. (2003). Bacteria in midguts of field-collected Anopheles albimanus block Plasmodium vivax sporogonic development. *J. Med. Entomol.* 40, 371–374. doi: 10.1603/0022-2585-40.3.371

Gouteux, J. P., and Lancien, J. (1986). Le piège pyramidal à tsé-tsé (Diptera: Glossinidae) pour la capture et la lutte. Essais comparatifs et description de nouveaux systèmes de capture. *Trop. Med. Parasitol.* 37, 61–66.

Gouveia, C., Asensi, M. D., Zahner, V., Rangel, E. F., and Oliveira, S. M. (2008). Study on the bacterial midgut microbiota associated to different Brazilian populations of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae). *Neotrop. Entomol.* 37, 597–601. doi: 10.1590/S1519-566X2008000500016

Gouy, M., Guindon, S., and Gascuel, O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* 27, 221–224. doi: 10.1093/molbev/msp259

Grébaut, P., Mbida, J. A., Kondjio, C. A., Njiokou, F., Penchenier, L., and Laveissiere, C. (2004). Spatial and temporal patterns of human African trypanosomiasis (HAT) transmission risk in the Bipindi focus, in the forest zone of southern Cameroon. *Vector Borne Zoonotic Dis.* 4, 230–238. doi: 10.1089/vbz.2004.4.230

Grébaut, P., Wang, S., Bodo, J. M., Ebo'o Eyenga, V., Binzouli, J. J., Ndong Ngoe, C., et al. (2001). Aspects épidémiologiques d'un foyer de la maladie du sommeil mal connu: le foyer de Bipindi au Cameroun. *Bull. Liaison Document. de l'OCEAC.* 33, 16.

Grimont, F., and Grimont, P. A. (2015). *Serratia. Bergey's Manual of Systematics of Archaea and Bacteria*. (Oxford: John Wiley & Sons, Inc., in association with Bergey's Manual Trust).

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., et al. (2012). The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Res.* 41, D597–D604. doi: 10.1093/nar/gks1160

Haines, L. R., Lehane, S. M., Pearson, T. W., and Lehane, M. J. (2010). Tsetse EP protein protects the fly midgut from trypanosome establishment. *PLoS Pathog.* 6:e1000793. doi: 10.1371/journal.ppat.1000793

Hamidou Soumana, I., Loriod, B., Ravel, S., Tchicaya, B., Simo, G., Rihet, P., et al. (2014). The transcriptional signatures of *Sodalis glossinidius* in the *Glossina palpalis* gambiensis flies negative for *Trypanosoma brucei* gambiense contrast with those of this symbiont in tsetse flies positive for the parasite: possible involvement of a Sodalis-hosted prophage in fly Trypanosoma refractoriness? *Infect. Genet. Evol.* 24, 41–56. doi: 10.1016/j.meegid.2014.03.005

Hartmann, M., Howes, C. G., VanInsberghe, D., Yu, H., Bachar, D., Christen, R., et al. (2012). Significant and persistent impact of timber harvesting on soil microbial communities in Northern coniferous forests. *ISME J.* 6, 2199–2218. doi: 10.1038/ismej.2012.84

Hejazi, A., and Falkiner, F. R. (1997). *Serratia marcescens*. *J. Med. Microbiol.* 46, 903–912. doi: 10.1099/00222615-46-11-903

Herder, S., Simo, G., Nkinin, S., and Njiokou, F. (2002). Identification of trypanosomes in wild animals from southern Cameroon using the polymerase chain reaction (PCR). *Parasite* 9, 345–349. doi: 10.1051/parasite/2002094345

Hu, C., and Aksoy, S. (2006). Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans* morsitans. *Mol. Microbiol.* 60, 1194–1204. doi: 10.1111/j.1365-2958.2006.05180.x

Ji, H. L., Qi, L. D., Hong, X. Y., Xie, H. F., and Li, Y. X. (2015). Effects of host sex, plant species, and putative host species on the prevalence of wolbachia in natural populations of *Bemisia tabaci* (Hemiptera: Aleyrodidae): a modified nested PCR study. *J. Econ. Entomol.* 108, 210–218. doi: 10.1093/jee/tou004

Lazaro, J. E., Nitcheu, J., Predicala, R. Z., Mangalindan, G. C., Nesslany, F., Marzin, D., et al. (2002). Heptyl prodigiosin, a bacterial metabolite, is antimalarial *in vivo* and non-mutagenic *in vitro*. *J. Nat. Toxins* 11, 367–377.

Lindh, J. M., and Lehane, M. J. (2011). The tsetse fly Glossina fuscipes fuscipes (Diptera: Glossina) harbours a surprising diversity of bacteria other than symbionts. *Antonie Van Leeuwenhoek* 99, 711–720. doi: 10.1007/s10482-010-9546-x

MacLeod, E. T., Maudlin, I., Darby, A. C., and Welburn, S. C. (2007). Antioxidants promote establishment of trypanosome infections in tsetse. *Parasitology* 134, 827–831. doi: 10.1017/S0031182007002247

Magoč T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Majiwa, P. A. O., Thatthi, R., Moloo, S. K., Nyeko, J. H. P., Otieno, L. H., and Maloo, S. (1994). Detection of trypanosome infections in the saliva of tsetse flies and buffy-coat samples from antigenaemic but aparasitaemic cattle. *Parasitology* 108, 313–322. doi: 10.1017/S0031182000076150

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17.1, 1–3. doi: 10.14806/ej.17.1.200

Martinson, V. G., Danforth, B. N., Minckley, R. L., Rueppell, O., Tingek, S., and Moran, N. A. (2011). A simple and distinctive microbiota associated with honey bees and bumble bees. *Mol. Ecol.* 20, 619–628. doi: 10.1111/j.1365-294X.2010.04959.x

Masiga, D. K., Smyth, A. J., Ayes, P., Bromidge, T. J., and Gibson, W. C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *Int. J. Parasitol.* 22, 909–918. doi: 10.1016/0020-7519(92)90047-O

Massana, R., Gobet, A., Audic, S., Bass, D., Bittner, L., Boutte, C., et al. (2015). Marine protist diversity in European coastal waters and sediments as revealed by high-throughput sequencing. *Environ. Microbiol.* 17, 4035–4049. doi: 10.1111/1462-2920.12955

McCutcheon, J. P., and Moran, N. A. (2010). Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. *Genome Biol. Evol.* 2, 708–718. doi: 10.1093/gbe/evq055

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Melachio, T. T., Simo, G., Ravel, S., De Meeûs, T., Causse, S., Solano, P., et al. (2011). Population genetics of *Glossina palpalis* palpalis from central African sleeping sickness foci. *Parasit. Vectors* 4:140. doi: 10.1186/1756-3305-4-140

Moloo, S. K., Grootenhuis, J. G., Kar, S. K., and Karstad, L. (1988). Survival and reproductive performance of female *Glossina morsitans* morsitans when maintained on the blood of different species of wild mammals. *Med. Vet. Entomol.* 2, 347–350. doi: 10.1111/j.1365-2915.1988.tb00207.x

Mondani, L., Benzerara, K., Carrière, M., Christen, R., Mamindy-Pajany, Y., Février, L., et al. (2011). Influence of uranium on bacterial communities: a comparison of natural uranium-rich soils with controls. *PLoS ONE* 6:e25771. doi: 10.1371/journal.pone.0025771

Moore, S., Shrestha, S., Tomlinson, K. W., and Vuong, H. (2012). Predicting the effect of climate change on African trypanosomiasis: integrating epidemiology with parasite and vector biology. *J. R. Soc. Interface* 9, 817–830. doi: 10.1098/rsif.2011.0654

Moran, N. A., Tran, P., and Gerardo, N. M. (2005). Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. *Appl. Environ. Microbiol.* 71, 8802–8810. doi: 10.1128/AEM.71.12.8802-8810.2005

Moser, D. R., Cook, G. A., Ochs, D. E., Bailey, C. P., McKane, M. R., and Donelson, J. E. (1989). Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* 99, 57–66. doi: 10.1017/S0031182000061023

Moss, M. (2002). Bacterial pigments. *Microbiologist* 3, 10–12.

Njiokou, F., Laveissière, C., Simo, G., Nkinin, S., Grébaut, P., Cuny, G., et al. (2006). Wild fauna as a probable animal reservoir for *Trypanosoma brucei* gambiense in Cameroon. *Infect. Genet. Evol.* 6, 147–153. doi: 10.1016/j.meegid.2005.04.003

Njiokou, F., Simo, G., Nkinin, S. W., Laveissière, C., and Herder, S. (2004). Infection rate of *Trypanosoma brucei* s.l., *T. vivax, T. congolense* "forest type," and *T. simiae* in small wild vertebrates in south Cameroon. *Acta Trop.* 92, 139–146. doi: 10.1016/j.actatropica.2004.04.011

Nkinin, S. W., Njiokou, F., Penchenier, L., Grébaut, P., Simo, G., and Herder, S. (2002). Characterization of *Trypanosoma brucei s.l* subspecies by izoenzymes in domestic pigs from the Fontem sleeping sickness focus of Cameroon. *Acta Trop.* 81, 225–232. doi: 10.1016/S0001-706X(01)00213-3

Pawlowski, J., Christen, R., Lecroq, B., Bachar, D., Shahbazkia, H. R., Amaral-Zettler, L., et al. (2011). Eukaryotic richness in the abyss: insights from pyrotag sequencing. *PLoS ONE* 6:e18169. doi: 10.1371/journal.pone.0018169

Poinar, G. O. Jr, Wassink, H. J., Leegwater-van der Linden, M. E., and van der Geest, L. P. (1979). *Serratia marcescens* as a pathogen of tsetse flies. *Acta Trop.* 36, 223–227.

Porter, T. M., Shokralla, S., Baird, D., Golding, G. B., and Hajibabaei, M. (2016). Ribosomal, DNA, and plastid markers used to sample fungal and plant communities from wetland soils reveals complementary biotas. *PLoS ONE* 11:e0142759. doi: 10.1371/journal.pone.0142759

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Rani, A., Sharma, A., Rajagopal, R., Adak, T., and Bhatnagar, R. K. (2009). Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. *BMC Microbiol.* 9:96. doi: 10.1186/1471-2180-9-96

Rio, R. V., Hu, Y., and Aksoy, S. (2004). Strategies of the home-team: symbioses exploited for vector-borne disease control. *Trends Microbiol.* 12, 325–336. doi: 10.1016/j.tim.2004.05.001

Rio, R. V. M., Symula, R. E., Wang, J., Lohs, C., Wu, Y. N., Snyder, A. K., et al. (2012). Insight into the transmission biology and species-specific functional capabilities of tsetse (Diptera: Glossinidae) obligate symbiont Wigglesworthia. *MBio* 3, e00240–e00211. doi: 10.1128/mBio.00240-11

Rose, C., Belmonte, R., Armstrong, S. D., Molyneux, G., Haines, L. R., Lehane, M. J., et al. (2014). An investigation into the protein composition of the teneral *Glossina morsitans* morsitans peritrophic matrix. *PLoS Negl. Trop. Dis.* 8:e2691. doi: 10.1371/journal.pntd.0002691

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O., and Huttenhower, C. (2012). Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat. Methods* 9, 811–814. doi: 10.1038/nmeth.2066

Shaw, A. P., Torr, S. J., Waiswa, C., Cecchi, G., Wint, G. R., Mattioli, R. C., et al. (2013). Estimating the costs of tsetse control options: an example for Uganda. *Prev. Vet. Med.* 110, 290–303. doi: 10.1016/j.prevetmed.2012.12.014

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7:539. doi: 10.1038/msb.2011.75

Simo, G., Njiokou, F., Mbida Mbida, J. A., Njitchouang, G. R., Herder, S., Asonganyi, T., et al. (2008). Tsetse fly host preference from sleeping sickness foci in Cameroon. Epidemiological implications. *Infect. Genet. Evol.* 8, 34–39. doi: 10.1016/j.meegid.2007.09.005

Solano, P., Salou, E., Rayaisse, J.-B., Ravel, S., Gimonneau, G., Traore, I., et al. (2015). "Do tsetse flies only feed on blood?" *Infect. Genet. Evol.* 36, 184–189. doi: 10.1016/j.meegid.2015.09.016

Tchioffo, M. T., Boissière, A., Abate, L., Nsango, S. E., Bayibéki, A. N., Awono-Ambéné, P. H., et al. (2016). Dynamics of bacterial community composition in the malaria mosquito's Epithelia. *Front. Microbiol.* 6:1500. doi: 10.3389/fmicb.2015.01500

Tchouomene-Labou, J., Nana-Djeunga, H., Simo, G., Njitchouang, G. R., Cuny, G., Asonganyi, T., et al. (2014). Spatial and temporal variations relevant to tsetse control in the Bipindi focus of southern Cameroon. *Parasit. Vectors* 6:193. doi: 10.1186/1756-3305-6-193

Terenius, O., Lindh, J. M., Eriksson-Gonzales, K., Bussière, L., Laugen, A. T., Bergquist, H., et al. (2012). Midgut bacterial dynamics in *Aedes aegypti*. *FEMS Microbiol. Ecol.* 80, 556–565. doi: 10.1111/j.1574-6941.2012.01317.x

Vickerman, K., Tetley, L., Hendry, A., and Turner, C. M. (1988). Biology of African trypanosomes in the tsetse fly. *Biol. Cell* 64, 109–119. doi: 10.1016/0248-4900(88)90070-6

Wang, J., Wu, Y., Yang, G., and Aksoy, S. (2009). Interactions between mutualist Wigglesworthia and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12133–12138. doi: 10.1073/pnas.0901226106

Weiss, B. L., Savage, A. F., Griffith, B. C., Wu, Y., and Aksoy, S. (2014). The peritrophic matrix mediates differential infection outcomes in the tsetse fly gut following challenge with commensal, pathogenic, and parasitic microbes. *J. Immunol.* 193, 773–782. doi: 10.4049/jimmunol.1400163

Weiss, B. L., Wang, J., and Aksoy, S. (2011). Tsetse immune system maturation requires the presence of obligate symbionts in larvae. *PLoS Biol.* 9:e1000619. doi: 10.1371/journal.pbio.1000619

Weiss, B. L., Wang, J., Maltz, M. A., Wu, Y., and Aksoy, S. (2013). Trypanosome infection establishment in the tsetse fly gut is influenced by microbiome-regulated host immune barriers. *PLoS Pathog.* 9:e1003318. doi: 10.1371/journal.ppat.1003318

Welburn, S. C., and Maudlin, I. (2012). Priorities for the elimination of sleeping sickness. *Adv. Parasitol.* 79, 299–337. doi: 10.1016/B978-0-12-398457-9.00004-4

Welburn, S. C., Maudlin, I., and Molyneux, D. H. (1994). Midgut lectin activity and sugar specificity in teneral and fed tsetse. *Med. Vet. Entomol.* 8, 81–87. doi: 10.1111/j.1365-2915.1994.tb00391.x

Zélé, F., Nicot, A., Berthomieu, A., Weill, M., Duron, O., and Rivero, A. (2014). Wolbachia increases susceptibility to Plasmodium infection in a natural system. *Proc. Biol. Sci.* 281:20132837. doi: 10.1098/rspb.2013.2837

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 30, 614–620. doi: 10.1093/bioinformatics/btt593

Zouache, K., Raharimalala, F. N., Raquin, V., Tran-Van, V., Raveloson, L. H., Ravelonandro, P., et al. (2011). Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiol. Ecol.* 75, 377–389. doi: 10.1111/j.1574-6941.2010.01012.x

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MINI REVIEW
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Host-*Toxoplasma gondii* Coadaptation Leads to Fine Tuning of the Immune Response

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Toxoplasma gondii has successfully developed strategies to evade host's immune response and reach immune privileged sites, which remains in a controlled environment inside quiescent tissue cysts. In this review, we will approach several known mechanisms used by the parasite to modulate mainly the murine immune system at its favor. In what follows, we review recent findings revealing interference of host's cell autonomous immunity and cell signaling, gene expression, apoptosis, and production of microbicide molecules such as nitric oxide and oxygen reactive species during parasite infection. Modulation of host's metalloproteinases of extracellular matrix is also discussed. These immune evasion strategies are determinant to parasite dissemination throughout the host taking advantage of cells from the immune system to reach brain and retina, crossing crucial hosts' barriers.

Keywords: ***Toxoplasma gondii*, T cells, immunomodulation, cell signalling, immunity**

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INTRODUCTION

Toxoplasma gondii is a parasite acquired through food or water contamination, followed by gut invasion and systemic dissemination. The protozoan *T. gondii* is able to escape the immune system and cross the blood–brain and blood–retina barrier reaching immune privileged sites leading to long-term infection (1). Intracellular pathogen, *T. gondii* subverts innate immunitary system interfering with host signaling pathways according to virulence based on the parasite genotype and the cell type infected (2, 3). Moreover, distinct responses can be triggered depending on inflammatory cells recruited, parasite burden, and the parasites' molecular arrangement (4). *T. gondii* is an example of host–parasite coadaptation, and several studies have unveiled molecular interactions that allow the parasite not to exterminate the host, evading from immune responses at different levels.

INTERFERING WITH CELL-AUTONOMOUS IMMUNITY

Host cell gene transcription is drastically affected by *T. gondii* including those genes involved in energy metabolism, immune responses, and signaling (5–7). Initially, pattern recognition receptors such as toll-like receptors (TLRs) are able to bind parasite molecules. In mice, TLR11 and TLR12 bind to TgPRF triggering a strong IL-12 response that most effective leads to interferon gamma-inducing response genes (IRGs) (8). In humans, those genes are not functional, and TLR2, 4, 8, and 9 are

effective in inducing IL-12 (9). After active invasion of host cell, *T. gondii* surrounds itself with a combination of host membrane and it is able to exclude and to recruit host proteins to the resulting parasitophorous vacuole (PV) in which it develops (3).

Rhoptry organelle initiates vacuole formation by secretion of an array of proteins that are released directly into the host cell, collectively known as RONs, forming the moving junction (MJ) (10). RONs 2/4/5/8 anchor the MJ at host cell membrane during invasion and also function as a selective sieve to host cell proteins that will be incorporated to PV (11, 12). This process assures the formation of a PV devoided of host proteins required for recruitment of endosomes and lysosomes (13).

The non-fusogenic nature of the PV is critical since it inhibits one of the cell-autonomous immunity mechanisms, the autophagy (14). Autophagosomal compartments are generated in eukaryotic cells as part of a bulk degradation system, through the formation of an initial phagophorous derived of membrane cisterna where autophagy-related proteins (ATG) are orderly accumulated leading to the fusion with lysosomal pathway (15). In non-canonical autophagy, ATG proteins can build up from preformed membranes such as the PVM, and not all ATGs are required to participate in the process (16). Extensive experimental data indicate that the autophagy machinery can promote killing of a broad variety of pathogens (17, 18) including *T. gondii*, especially in mouse models, and the IFNγ produced early in infection is crucial for that (19–23). Upon the influence of IFNγ, infected host cells respond regulating nearly 2,000 genes that are called interferon-inducible genes (24). Among those, effector molecules such as the immunity-related p47 GTPases (IRGs) and guanylate-binding proteins (GBPs) rapidly accumulate on and around the PVM, leading to the disruption of the PVM and subsequent death of the parasite in mouse cells (25). In human cells, ubiquitination and recruitment of autophagy adaptors did not require GBPs (26).

The recruitment of IRGs and GBPs to PVM depends on autophagy-related (Atg) gene products (24, 27, 28). In mice, it has been demonstrated that Atg5 and Atg8 (LC3 in humans) are required for the proper targeting of the effectors onto the PVM of *T. gondii* (29–31). In addition, Atg12, Atg16L1, Atg3, and Atg7 are recruited to the PVM to promote parasite killing (26, 32). Infection of Atg5- and Atg3-deficient cells show decreased accumulation of immunity-related GTPase family member b10 (Irgb10) and guanylate-binding protein 2 (Gbp2) at *T. gondii* PVM (33, 34). Genotypes II and III are susceptible to the IRGs resistance system. On the other hand, infection with virulent strains (e.g., type I) has demonstrated that polymorphic *T. gondii* kinase proteins from rhoptries like ROP5, ROP17, and ROP18 phosphorylate IRG proteins in murine cells inactivating them in order to preserve PVM integrity (35–40), suggesting that in type I strain parasites can evade this cell autonomous immunity mechanism (**Figure 1A**).

Until recently, IFN inducible GTPases were thought to be non-functional in *T. gondii* response in human cells. Qin et al. showed that Gbp1 induced by IFNγ in mesenchymal stromal cells was responsible to decrease the number of parasites after 4 h of infection and showed that Gbp1 was found in association with at least 10% of PV (37). On the other hand, Johnston et al. (38) using A549 human epithelial cells showed that in the absence of Gbp1 parasite numbers increase rapidly. However, no Gbp1 was seen in association with PV at any moment. Clearly that are gaps in our knowledge of IRG system in the human autonomous immunity (38). Muniz-Feliciano et al. (22) showed that *T. gondii* microneme proteins (MICs) containing epidermal growth factor (EGF) domains (MIC3 and MIC6) appeared to promote EGF receptor activation in endothelial cells, retinal pigment epithelial cells, and microglia in humans. These findings support the concept that *T. gondii* activates EGFR-Akt signaling in the host cell to prevent targeting of the PVM by LC3 (Atg8 orthologs in humans) and pathogen killing (22).

Moreover, *T. gondii* might also be killed by autophagy in mice macrophages independently of IFNγ, in a mechanism involving CD40, member of TNF receptor superfamily, and activation of ULK1, calcium/calmodulin-dependent kinase kinase b (CaMKKβ), AMP-activated kinase, and Jun-kinase (JKN are involved) (25).

CELL SIGNALING INTERFERENCE

Toxoplasma gondii modulates several signal transduction pathways once inside host cells. At the same time that an effective immune response is generated, intracellular survival strategies are adopted by the parasite. The equilibrium host-*T. gondii* is in the best interest of both allowing the establishment of long-lasting latent infection, increasing the chances of transmission to new hosts. Several *T. gondii* effector molecules have been identified that directly interact with signal transducer and activator of transcription (STAT) pathways, which influence the transcription of both pro and anti-inflammatory molecules such as IFN-γ and major histocompatibility complex class II (MHCII) (STAT1); IL-10 (STAT3); and IL-4 (STAT6) (41–44).

Rhoptry 16 kinase (ROP16) is secreted into the host cytosol during invasion and phosphorylates STAT6 in a rapid and sustained way (45). Phosphorylation of STAT6 by ROP16 mediates induction of arginase-1, resulting in arginine degradation, depriving the parasite from one important metabolite (42). Infection of mice with ROP16 knockout parasites shows that STAT3 is also phosphorylated by this kinase, suppressing TLRs and inhibiting pro-inflammatory responses at some level (42, 46, 47). ROP16 encoded by the type I/III strains, but not type II strains, maintains STAT3/6 activation for 24 h and suppresses IL-12 production from macrophages (45, 47) (**Figure 1B**). A recent report by Jensen et al. showed that ROP16, type II strain, induced the sustained phosphorylation and nuclear translocation of STAT5 in host infected cells, contributing to generation of protective immunity in murine gut mucosal system (48).

Toxoplasma gondii has also been shown to interfere with STAT1 signaling, resulting in blockage of interferon regulatory factor 1 (Irf1), p65 GBPs, inducible nitric oxide synthase (iNOS/Nos2), indoleamine 2,3-dioxygenase 1, and MHC (49–55) (**Figure 1**). Schneider et al. (56) showed that STAT1 is activated during infection of bone marrow-derived murine dendritic cells (BMDCs) through tyrosine 70 (Tyr70) and serine 727 (Ser727) phosphorylation with effective nuclear translocation in a ROP16 independent way. All clonal strains

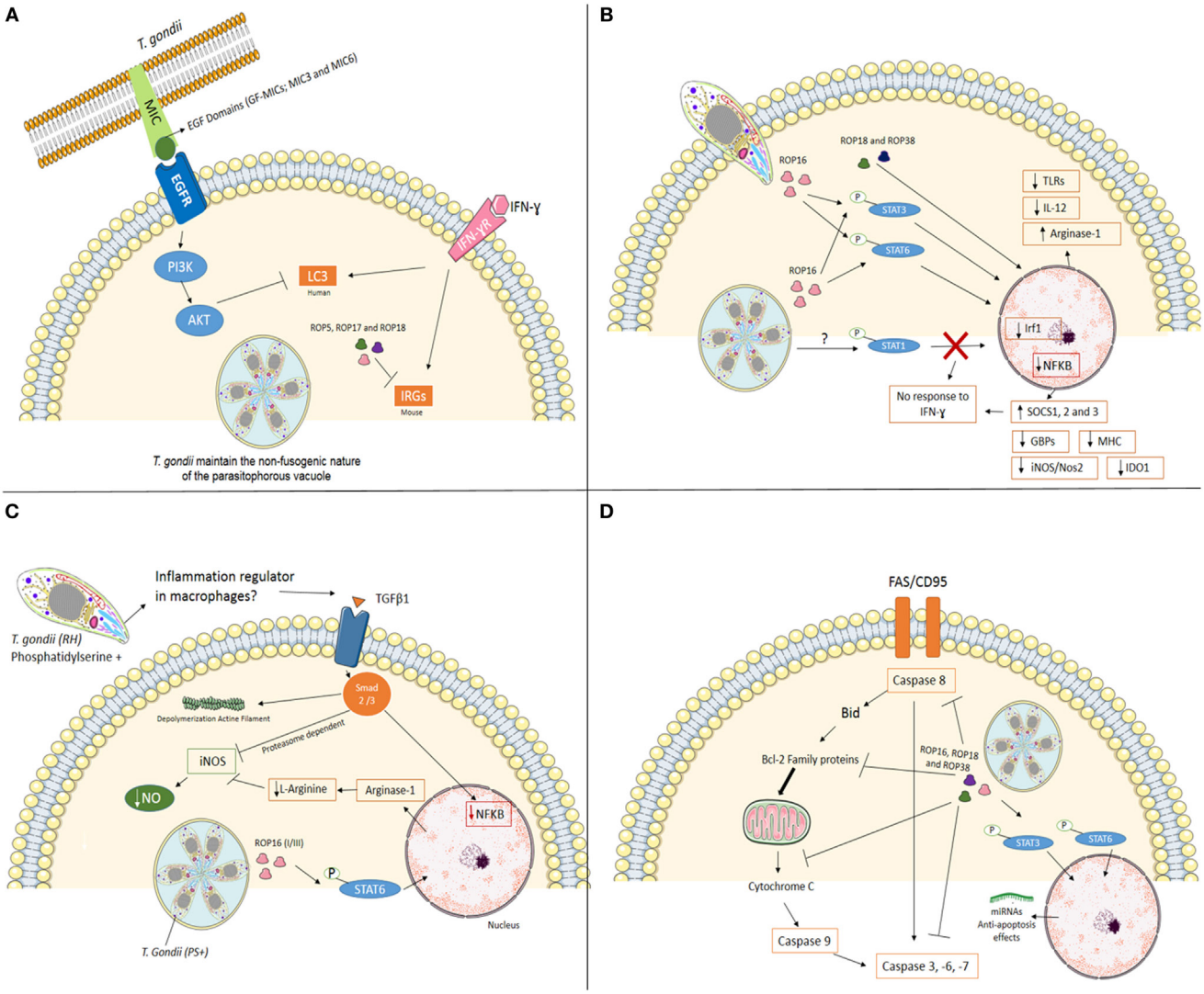

FIGURE 1 | Mechanisms used by *Toxoplasma gondii*, type I strain, to modulate mainly immune system at its favor. **(A)** Interfering with cell-autonomous immunity. In infection with virulent strain (e.g., type I), the polymorphic effector proteins like ROP5, ROP17, and ROP18 cooperate to phosphorylate and inactivate mouse IRG proteins to preserve PVM integrity. In human cells (endothelial, retinal pigment epithelial, and microglia), *T. gondii* activates EGFR-Akt signaling to prevent targeting of the parasite by LC3 structures and pathogen killing dependent on autophagy proteins and lysosomal protease activity. **(B)** Cell signaling interference. The *Toxoplasma* rhoptry 16 kinase (ROP16), ROP18, and 38 can mediate the induction of arginase-1, suppress toll-like receptors (TLRs), IL-12, and nuclear factor-kappa B (NF-κB) through phosphorylation of signal transducer and activator of transcription (STAT) 3 and 6. *T. gondii* also interferes with STAT1 signaling, resulting in blockage of interferon regulatory factor 1 (Irf1), p65 guanylate-binding proteins (GBPs), inducible nitric oxide synthase (iNOS/Nos2), indoleamine 2,3-dioxygenase 1 (IDO1) and major histocompatibility complex (MHC). Furthermore, the infection upregulation SOCS1, 2 and 3. Together, *T. gondii* inhibiting pro-inflammatory response in different ways. **(C)** Silencing microbicide molecules. *T. gondii* [phosphatidylserine positive (PS+)] infection of murine blood monocyte-derived and peritoneal macrophages activated *in vitro* with IFN-γ and lipopolysaccharide (LPS) lead to a substantial decrease in NO production. Decreased mechanisms include phosphorylation of STAT6 by ROP16 resulting in arginine degradation and induction of TGFβ1 through Smad 2 and 3 leading to destruction of iNOS, actin filament (F-actin) depolymerization, and lack of NF-κB in the nucleus. **(D)** Maintaining the host cell alive. *T. gondii* has several strategies for inhibiting the initiation of the apoptotic cascade triggered by mitochondrial pathway or death receptor pathway in infected cells. The effector proteins like ROP16, ROP18, and ROP38 phosphorylate STAT3 and STAT6 and promotes mechanisms that include blocking of mitochondrial cytochrome c release, alterations of the balance between pro- and anti-apoptotic Bcl-2 proteins, degradation of caspase 8, blocking Fas/CD95-mediated apoptosis, and inactivation of effector caspases (-3, -6, -7) in infected cells.

tested (Type I—RH, Type II—PTG, and Type III—M774.1) showed similar results, with a less effective and sustained phosphorylation induced by M774.1 (56). Besides its nuclear translocation, tyrosine-phosphorylated STAT-1 (pYSTAT1) was unable to bind to the Irf1 gene promoter and chromatin immunoprecipitation assays showed the presence of aberrant STAT1 complexes, as earlier described by Lang et al. (57) (**Figure 1B**).

One of the mechanisms of IFN-γ blockage is the dephosphorylation of STAT1 by SOCS1 (suppressor of cytokine signaling phosphatase) induced by positive feedback. *T. gondii* infection has been shown to induce both downregulation of SOCS1 in human fibroblasts (58) and upregulation in murine macrophages (48, 55). Infection of mice with target deletion of SOCS3 in neutrophils and macrophages results in death, as this molecule is upregulated during infection. Furthermore, the administration of anti-IL6 and IL-12 restored mice resistance to the infection (59).

Toxoplasma gondii induces the expression of SOCS2 in DCs through lipoxin A4 (LXA4), an arachidonic acid (Ah) with anti-inflammatory action that stimulates Ah and LXA receptors of the host cell, resulting in decreased expression of chemokine receptor type 5 (CCR5) and IL-12 secretion (60). However, enhancing transcriptions factors can also be an evasion strategy. Dense granule proteins 6 (GRA6) interferes with nuclear factor of activated T cells 4 (NFAT4), activating it *via* calcium-modulating ligand, which might lead to increased migration of inflammatory macrophages (61).

Release of dense granule protein GRA15 by type II strains, but not the type I/III strains, into the host cell cytoplasm mediates nuclear factor-κappaB (NF-κB) activation and initiates IL-12 synthesis (48, 62). On the other hand, type I strains inhibit NF-kB pathway through ROP18 and suppresses pro-inflammatory cytokine expression, resulting in the enhanced survival of the parasites in the hosts (63) (**Figure 1B**).

SILENCING MICROBICIDE MOLECULES

Inflammatory macrophages are able to contain dissemination of infection through microbicide molecules, such as nitric oxide (NO). However, *T. gondii* infection of murine blood monocytes and peritoneal macrophages activated *in vitro* with IFN-γ and lipopolysaccharide leads to a substantial decrease in NO production (51, 52, 54). Interestingly, pretreating *T. gondii* with annexin V, which binds to phosphatidylserine (PS) reverts NO inhibition (64). The authors demonstrated that infection induces TGFβ1 through Smad 2 and 3 leading to destruction of iNOS, actin filament (F-actin) depolymerization, and lack of NF-κB in the nucleus (64). Recently, the same group showed that degradation of iNOS is proteasome dependent (65). iNOS reduced expression is also observed in microglia infected with *T. gondii*, also involving TGFβ pathways, protecting neurons from death (54). PS positive (PS+) but not PS negative (PS−) subpopulations of *T. gondii* were capable of NO inhibition after infection of murine macrophages *in vitro*, and infection *in vivo* with PS+ subpopulations leads to high parasite burden and low inflammatory symptoms at peritoneal cavity, while low or absent infection observed when PS− parasites were used with active inflammatory response observed (66). Thus, PS expression at *T. gondii* cell surface seems to be an interesting regulator of exacerbated inflammation at the entry site (**Figure 1C**).

MAINTAINING THE HOST CELL ALIVE

Toxoplasma gondii-infected cells are resistant to a series of apoptosis inducers (67), allowing intracellular survival and persistence within the host cells (68). In a recent study, He et al. (69) suggested that *T. gondii* (Type I—RH) targets transregulation factors in mouse spleen cells modulating host gene expression. The genes involved in apoptosis or anti-apoptosis were both targeted by differentially expressed miRNAs, which contributes to the fate of host apoptosis process (69). The same group revealed the *T. gondii* infection can alter the transcripts at mitochondria level that are involved in several biosynthetic and metabolic processes and also in apoptosis (70) (**Figure 1D**).

The initiation of the apoptotic cascades is disturbed by *T. gondii* at several key points. Blocking of mitochondrial cytochrome c release is one of the mechanisms affected (71–73). The balance of pro- and anti-apoptotic Bcl-2 proteins (71, 72, 74–76) and direct inhibition of cytochrome c-mediated activation of the caspase cascade (73) were also reported. Inhibition of caspase 8, blocking of Fas/CD95-mediated apoptosis (77–79), inactivation of caspase 3 and PARP (80), as well as abrogation of Granzyme B activity in infected cells (81) are also important in maintaining the host cell alive, in favor of parasite's survival.

Inhibition of apoptosis is regulated also at the transcriptional level. Infection of mouse splenocytes induces activation of host's NF-κB and the transcription of antiapoptotic genes (80). After cell invasion, cells increase levels of active serine threonine kinase/protein kinase B (Akt/PKB), exploiting PI3K in a Gi-dependent way to delay host cell apoptosis (82). Furthermore, *T. gondii* phosphorylates the pro-apoptotic Bad protein to prevent apoptosis (83). These findings suggest that during the early stages of infection *T. gondii* is able to evade induction of apoptosis remaining inside the cell allowing the spreading of infection. However, there are reports indicating that ROP18 from virulent *T. gondii* strains induces apoptosis of neurons *via* RE stress (84). On the other hand, the gp130 expressed by neuronal cells protects them through IL-6, TGFβ, and IL-27 (85).

Signal transducer and activator of transcription molecules are also exploited by *T. gondii* to prevent the apoptosis. Serine proteases, like SERPIN B3 and B4, are significantly expressed in macrophages infected by *T. gondii via* STAT6 activation. Extended parasite intracellular survival in THP-1 is gain through those enzymes that ultimately inhibit apoptosis (86). In a recent work, Cai et al. demonstrated that STAT3 mediates pro-survival by upregulating the miR-17–92 that in turn targets Bim, inhibiting apoptosis in infected macrophages (87).

USING CELLS AS TROJAN HORSES

Inflammatory cells attracted to the primary site of infection are targets of parasites that highjack the cell in order to circulate through the body inside the host cell, in a mechanism similar to a *Trojan horse*, delivering the parasite to deep tissues and immune privileged sites, such as central nervous system and eyes (88).

Our group showed that host extracellular matrix metalloproteases (MMPs) might be involved in infected macrophage dissemination (89, 90). Murine macrophages infected *in vitro* with *T. gondii* exhibit increased membrane type-1 matrix metalloproteinase (MT1-MMP) and disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) while decreased levels of CD44 are observed at cell surface. On the other hand,

augmented active MMP-9 is present at cell supernatant (89) resembling metastasis mechanisms used by invasive tumors (89). Upregulation of MMP-9 and -2 *via* an Erk1/2/NF-κB pathway was also observed in murine mast cells infected with *T. gondii* (91) and human macrophages infected with *T. gondii* showed increased levels of MT1-MMP, with decrease in pro-MMP-2 and pro-MMP-9, maintain the migratory capacity, although decrease some the costimulatory molecules (89).

Regulation of hosts' MMPs processing involves extra- and intracellular mechanisms upon *T. gondii* infection. Urokinase-type PA/urokinase-type PA receptor (uPA/uPAR) pathway is known to be involved with MMPs processing at extracellular space and is regulated by endogenous inhibitor of plasminogen activator inhibitor (PAI-1) and protease nexin-1 (PN-1). We demonstrated that *T. gondii*-infected macrophages secrete a multiprotein complex containing MMP-9/TIMP1/uPAR, and incubation of infected cells with PAI-1 decreases the presence of this complex at cell supernatant (90) (**Figure 2**).

Toxoplasma gondii proteinases were identified (92) and might be involved in intracellular processing of MMPs zymogens, suggesting that hosts and *T. gondii* MMPs would work in favor of parasites' dissemination to secondary organs and to immune privileged sites. Thus, dissemination through lymphatics and leukocytes could be the main form of dissemination, and cumulative information in this subject have been gathered (88, 93).

After oral infection, *T. gondii* is found in the blood inside $CD11b^+$ monocytes and inside mouse $CD11c^+$ DCs at lamina propria Peyer's patches and mesenteric lymph nodes. Infected $CD11b^+$ monocytes are observed at the extravascular space in the mouse brain after 7 days of infection (94). In human infected astroglia cells, the increase of MMP-2 and MMP-9 could promote leukocyte migration during toxoplasmic encephalitis (95). MMP-2 and -9 are higher in the sera and umbilical cord of pregnant women with *T. gondii* infection (96), suggesting that MMPs might be involved in the crossing of *T. gondii* through the placental barrier. Oral infection with *T. gondii* provokes small intestine inflammation as a result of Th1 responses, that depending of mice strain and/or parasite genotype is rapidly contained (3). Muñoz et al. (97) demonstrated that MMP-2 is involved in the development of *T. gondii*-induced immunopathology. The same paper shows that this gelatinase is regulated by IL-23 *via* IL-22 but independent of IL-17 (97).

T cells from $CD4^+$ and $CD8^+$ lineages are essential to control bradyzoites containing cysts at the brain, T cells expressing MMP-10 are present at the brain after 21 days of infection, while T cells expressing MMP-8 are observed at 28th day of infection. At this time, astrocytes express TIMP-1, probably in an attempt to control damage (98). The presence of TIMP-1 at the brain during chronicle stages of toxoplasmosis could control degradation/activation of cytokines, decreasing exacerbated inflammatory response at the brain.

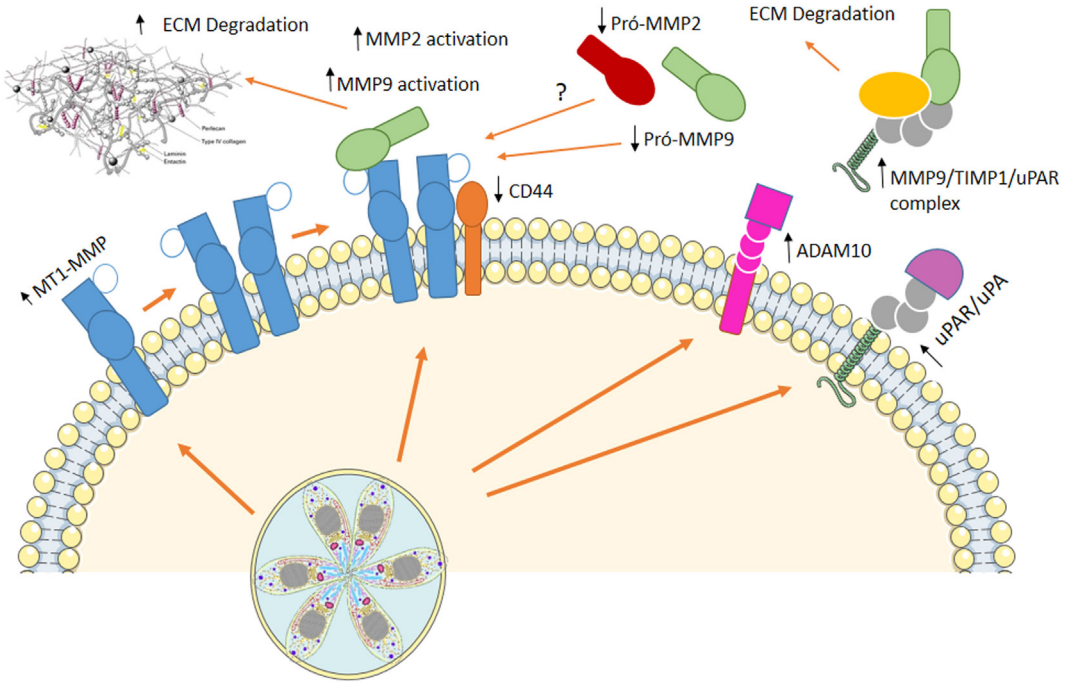

FIGURE 2 | Using cells as Trojan horses. Host extracellular matrix metalloproteases (MMPs) are involved in infected macrophage dissemination. *In vitro* infection of murine macrophages induced an increase in membrane type-1 matrix metalloproteinase (MT1-MMP) and disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), while decreased levels of CD44 are observed at cell surface. On the other hand, augmented active MMP-9, MMP-2, and a multiprotein complex containing MMP-9/TIMP1/urokinase-type PA receptor (uPAR) are present at cell supernatant. This mechanism resembling metastasis allows *Toxoplasma gondii* to disseminate throughout the host, reaching immune-privileged sites, where it remains in low proliferative state, with little damage to the host.

CONCLUSION

Several effector molecules and mechanisms were presented here, which allow *T. gondii* to leave inside host, with little destructive effects to immunocompetent individuals. Both parasite and host have developed several strategies to decrease collateral damaging immediately after infection such as interfering with cell-autonomous immunity and cell signaling and also blocking apoptosis allowing infected host cell to remain alive. Also, controlling dissemination of parasites through metastatic-simile mechanisms, using host cell MMPs and migration, allows parasite to spread to immune-privileged sites, where it remains in low proliferative state, with little damage to the host. In order to successfully reach this semiequilibrium state between parasite–host, the initial events occurring at the parasite entrance site are crucial. Damaging control of ileitis by regulating levels of IFNγ, IL-23, and IL-17 and maintaining the fine tuning of MMPs and other enzymes and pro-zymogen enhancers, inducers, and/or converters are fundamental.

AUTHOR CONTRIBUTIONS

All author contributed equally for the manuscript.

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REFERENCES

1. Harker KS, Ueno N, Lodoen MB. *Toxoplasma gondii* dissemination: a parasite's journey through the infected host. *Parasite Immunol* (2015) 37(3):141–9. doi:10.1111/pim.12163
2. Denkers EY. From cells to signaling cascades: manipulation of innate immunity by *Toxoplasma gondii*. *FEMS Immunol Med Microbiol* (2003) 39(3):193–203. doi:10.1016/S0928-8244(03)00279-7
3. Hunter CA, Sibley LD. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nat Rev Microbiol* (2012) 10:766–78. doi:10.1038/nrmicro2858
4. Pollard AM, Knoll LJ, Mordue DG. The role of specific *Toxoplasma gondii* molecules in manipulation of innate immunity. *Trends Parasitol* (2009) 25(11):491–4. doi:10.1016/j.pt.2009.07.009
5. Blader I, Manger ID, Boothroyd JC. Microarray analysis reveals previously unknown changes in *Toxoplasma gondii* infected human cells. *J Biol Chem* (2001) 276:24223–31. doi:10.1074/jbc.M100951200
6. Stutz A, Kessler H, Kaschel ME, Meissner M, Dalpke AH. Cell invasion and strain dependent induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii*. *Immunobiology* (2012) 217:28–36. doi:10.1016/j.imbio.2011.08.008
7. Zhou CX, Elsheikha HM, Zhou DH, Liu Q, Zhu XQ, Suo X. Dual identification and analysis of differentially expressed transcripts of porcine PK-15 cells and *Toxoplasma gondii* during in vitro infection. *Front Microbiol* (2016) 13(7):721. doi:10.3389/fmicb.2016.00721
8. Gazzinelli RT, Mendonça-Neto R, Lilue J, Howard J, Sher A. Innate resistance against *Toxoplasma gondii*: an evolutionary tale of mice, cats and men. *Cell Host Microbes* (2014) 15(2):132–8. doi:10.1016/j.chom.2014.01.004
9. Morger J, Bajnok J, Boyce K, Craig PS, Rogan MT, Lun ZR, et al. Naturally occurring Toll-like receptor 11 (TLR11) and Toll-like receptor 12 (TLR12) polymorphisms are not associated with *Toxoplasma gondii* infection in wild wood mice. *Infect Genet Evol* (2014) 26:180–4. doi:10.1016/j.meegid.2014.05.032
10. Shen B, Sibley LD. The moving junction, a key portal to host cell invasion by apicomplexan parasites. *Curr Opin Microbiol* (2012) 15(4):449–55. doi:10.1016/j.mib.2012.02.007
11. Mordue DG, Desai N, Dustin M, Sibley LD. Invasion by *Toxoplasma gondii* establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. *J Exp Med* (1999) 190(12):1783–92. doi:10.1084/jem.190.12.1783
12. Charron AJ, Sibley LD. Molecular partitioning during host cell penetration by *Toxoplasma gondii*. *Traffic* (2004) 5(11):855–67. doi:10.1111/j.1600-0854.2004.00228.x
13. Mordue DG, Håkansson S, Niesman I, Sibley LD. *Toxoplasma gondii* resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. *Exp Parasitol* (1999) 92(2):87–99. doi:10.1006/expr.1999.4412
14. Besteiro S. Which roles for autophagy in *Toxoplasma gondii* and related apicomplexan parasites? *Mol Biochem Parasitol* (2012) 184(1):1–8. doi:10.1016/j.molbiopara.2012.04.001
15. Strzyz P. Autophagy: membrane contacts lend a hand. *Nat Rev Mol Cell Biol* (2017) 18(7):404–5. doi:10.1038/nrm.2017.55
16. Latré de Laté P, Pineda M, Harnett M, Harnett W, Besteiro S, Langsley G. Apicomplexan autophagy and modulation of autophagy in parasite-infected host cells. *Biomed J* (2017) 40(1):23–30. doi:10.1016/j.bj.2017.01.001
17. Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunol Rev* (2011) 240(1):92–104. doi:10.1111/j.1600-065X.2010.00995.x
18. Abdelaziz DH, Khalil H, Cormet-Boyaka E, Amer AO. The cooperation between the autophagy machinery and the inflammasome to implement an appropriate innate immune response: do they regulate each other? *Immunol Rev* (2015) 265(1):194–204. doi:10.1111/imr.12288
19. Andrade RM, Wessendarp M, Gubbels MJ, Striepen B, Subauste CS. CD40 induces macrophage anti-*Toxoplasma gondii* activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. *J Clin Invest* (2006) 116(9):2366–77. doi:10.1172/JCI28796
20. Portillo JA, Okenka G, Reed E, Subauste A, Van Grol J, Gentil K, et al. The CD40-autophagy pathway is needed for host protection despite IFN-Γ-dependent immunity and CD40 induces autophagy via control of P21 levels. *PLoS One* (2010) 5(12):e14472. doi:10.1371/journal.pone.0014472
21. Lee YJ, Song HO, Lee YH, Ryu JS, Ahn MH. Proliferation of *Toxoplasma gondii* suppresses host cell autophagy. *Korean J Parasitol* (2013) 51(3):279–87. doi:10.3347/kjp.2013.51.3.279
22. Muniz-Feliciano L, Van Grol J, Portillo JA, Liew L, Liu B, Carlin CR, et al. *Toxoplasma gondii*-induced activation of EGFR prevents autophagy protein-mediated killing of the parasite. *PLoS Pathog* (2013) 9(12):e1003809. doi:10.1371/journal.ppat.1003809
23. Souto XM, Barbosa HS, Menna-Barreto RF. The morphological analysis of autophagy in primary skeletal muscle cells infected with *Toxoplasma gondii*. *Parasitol Res* (2016) 115(7):2853–61. doi:10.1007/s00436-016-5040-3
24. Kim BH, Shenoy AR, Kumar P, Bradfield CJ, MacMicking JD. IFN-inducible GTPases in host cell defense. *Cell Host Microbe* (2012) 12(4):432–44. doi:10.1016/j.chom.2012.09.007
25. Liu E, Lopez Corcino Y, Portillo JA, Miao Y, Subauste CS. Identification of signaling pathways by which CD40 stimulates autophagy and antimicrobial activity against *Toxoplasma gondii* in macrophages. *Infect Immun* (2016) 84(9):2616–26. doi:10.1128/IAI.00101-16
26. Ohshima J, Lee Y, Sasai M, Saitoh T, Su Ma JJ, Kamiyama N, et al. Role of mouse and human autophagy proteins in IFN-gamma induced cell-autonomous responses against *Toxoplasma gondii*. *J Immunol* (2014) 192:3328–35. doi:10.4049/jimmunol.1302822

27. Khaminets A, Hunn JP, Könen-Waisman S, Zhao YO, Preukschat D, Coers J, et al. Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell Microbiol* (2010) 12(7):939–61. doi:10.1111/j.1462-5822.2010.01443.x
28. Hunn JP, Feng CG, Sher A, Howard JC. The immunity-related GTPases in mammals: a fast-evolving cell-autonomous resistance system against intracellular pathogens. *Mamm Genome* (2011) 22(1–2):43–54. doi:10.1007/s00335-010-9293-3
29. Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. *Cell Host Microbe* (2008) 4:458–69. doi:10.1016/j.chom.2008.10.003
30. Selleck EM, Fentress SJ, Beatty WL, Degrandi D, Pfeffer K, Virgin HW, et al. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against *Toxoplasma gondii*. *PLoS Pathog* (2013) 9:e1003320. doi:10.1371/journal.ppat.1003320
31. Park S, Choi J, Biering SB, Dominici E, Williams LE, Hwang S. Targeting by autophagy proteins (TAG): targeting of IFNG-inducible GTPases to membranes by the LC3 conjugation system of autophagy. *Autophagy* (2016) 12(7):1153–67. doi:10.1080/15548627.2016.1178447
32. Choi J, Park S, Biering SB, Selleck E, Liu CY, Zhang X, et al. The parasitophorous vacuole membrane of *Toxoplasma gondii* is targeted for disruption by ubiquitin-like conjugation systems of autophagy. *Immunity* (2014) 40(6):924–35. doi:10.1016/j.immuni.2014.05.006
33. Haldar AK, Piro AS, Pilla DM, Yamamoto M, Coers J. The E2-like conjugation enzyme Atg3 promotes binding of IRG and Gbp proteins to chlamydia- and Toxoplasma-containing vacuoles and host resistance. *PLoS One* (2014) 9(1):e86684. doi:10.1371/journal.pone.0086684
34. Steinfeldt T, Könen-Waisman S, Tong L, Pawlowski N, Lamkemeyer T, Sibley LD, et al. Correction: phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. *PLoS Biol* (2015) 13(7):e1002199. doi:10.1371/journal.pbio.1002199
35. Reese ML, Shah N, Boothroyd JC. The *Toxoplasma pseudokinase* ROP5 is an allosteric inhibitor of the immunity-related GTPases. *J Biol Chem* (2014) 289(40):27849–58. doi:10.1074/jbc.M114.567057
36. Alaganan A, Fentress SJ, Tang K, Wang Q, Sibley LD. Toxoplasma GRA7 effector increases turnover of immunity-related GTPases and contributes to acute virulence in the mouse. *Proc Natl Acad Sci U S A* (2014) 111(3):1126–31. doi:10.1073/pnas.1313501111
37. Qin A, Lai DH, Liu Q, Huang W, Wu YP, Chen X, et al. Guanylate-binding protein 1 (GBP1) contributes to the immunity of human mesenchymal stromal cells against *Toxoplasma gondii*. *Proc Natl Acad Sci U S A* (2017) 114(6):1365–70. doi:10.1073/pnas.1619665114
38. Johnston AC, Piro A, Clough B, Siew M, Virreira Winter S, Coers J, et al. Human GBP1 does not localize to pathogen vacuoles but restricts *Toxoplasma gondii*. *Cell Microbiol* (2016) 18(8):1056–64. doi:10.1111/cmi.12579
39. Etheridge RD, Alaganan A, Tang K, Lou HJ, Turk BE, Sibley LD. The *Toxoplasma pseudokinase* ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host Microbe* (2014) 15:537–50. doi:10.1016/j.chom.2014.04.00
40. Hermanns T, Müller UB, Könen-Waisman S, Howard JC, Steinfeldt T. The *Toxoplasma gondii* rhoptry protein ROP18 is an Irga6-specific kinase and regulated by the dense granule protein GRA7. *Cell Microbiol* (2016) 18(2):244–59. doi:10.1111/cmi.12499
41. Lüder CG, Walter W, Beuerle B, Maeurer MJ, Gross U. *Toxoplasma gondii* down-regulates MHC class II gene expression and antigen presentation by murine macrophages via interference with nuclear translocation of STAT1alpha. *Eur J Immunol* (2001) 31(5):1475–84. doi:10.1002/1521-4141(200105)31:5<1475::AID-IMMU1475>3.0.CO;2-C
42. Butcher BA, Kim L, Panopoulos AD, Watowich SS, Murray PJ, Denkers EY. IL-10-independent STAT3 activation by Toxoplasma gondii mediates suppression of IL-12 and TNF-alpha in host macrophages. *J Immunol* (2005) 174(6):3148–52. doi:10.4049/jimmunol.174.6.3148
43. Ahn HJ, Kim JY, Ryu KJ, Nam HW. STAT6 activation by *Toxoplasma gondii* infection induces the expression of Th2 C-C chemokine ligands and B clade serine protease inhibitors in macrophage. *Parasitol Res* (2009) 105(5):1445–53. doi:10.1007/s00436-009-1577-8
44. Barbosa BF, Lopes-Maria JB, Gomes AO, Angeloni MB, Castro AS, Franco PS, et al. IL10, TGF beta1, and IFN gamma modulate intracellular signaling pathways and cytokine production to control *Toxoplasma gondii* infection in BeWo trophoblast cells. *Biol Reprod* (2015) 92(3):82. doi:10.1095/biolreprod.114.124115
45. Ong YC, Reese ML, Boothroyd JC. *Toxoplasma rhoptry* protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. *J Biol Chem* (2010) 285(37):28731–40. doi:10.1074/jbc.M110.112359
46. Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, Boothroyd JC. Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* (2007) 445(7125):324–7. doi:10.1038/nature05395
47. Yamamoto M, Standley DM, Takashima S, Saiga H, Okuyama M, Kayama H, et al. A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3. *J Exp Med* (2009) 206(12):2747–60. doi:10.1084/jem.20091703
48. Jensen KD, Hu K, Whitmarsh RJ, Hassan MA, Julien L, Lu D, et al. *Toxoplasma gondii* rhoptry 16 kinase promotes host resistance to oral infection and intestinal inflammation only in the context of the dense granule protein GRA15. *Infect Immun* (2013) 81(6):2156–67. doi:10.1128/IAI.01185-12
49. Luder CG, Lang T, Beuerle B, Gross U. Down-regulation of MHC class II molecules and inability to up-regulate class I molecules in murine macrophages after infection with *Toxoplasma gondii*. *Clin Exp Immunol* (1998) 112:308–16. doi:10.1046/j.1365-2249.1998.00594.x
50. Lüder CGK, Gross U, Lopes MF. Intracellular protozoan parasites and apoptosis: diverse strategies to modulate parasite-host interactions. *Trends Parasitol* (2001) 17:480–6. doi:10.1016/S1471-4922(01)02016-5
51. Seabra SH, de Souza W, DaMatta RA. *Toxoplasma gondii* partially inhibits nitric oxide production of activated murine macrophages. *Exp Parasitol* (2002) 100(1):62–70. doi:10.1006/expr.2001.4675
52. Lüder CG, Algner M, Lang C, Bleicher N, Gross U. Reduced expression of the inducible nitric oxide synthase after infection with *Toxoplasma gondii* facilitates parasite replication in activated murine macrophages. *Int J Parasitol* (2003) 33(8):833–44. doi:10.1016/S0020-7519(03)00092-4
53. Lüder CG, Lang C, Giraldo-Velasquez M, Algner M, Gerdes J, Gross U. *Toxoplasma gondii* inhibits MHC class II expression in neural antigen-presenting cells by down-regulating the class II transactivator CIITA. *J Neuroimmunol* (2003) 134(1–2):12–24. doi:10.1016/S0165-5728(02)00320-X
54. Rozenfeld C, Martinez R, Seabra S, Sant'anna C, Gonçalves JG, Bozza M, et al. *Toxoplasma gondii* prevents neuron degeneration by interferon-gamma-activated microglia in a mechanism involving inhibition of inducible nitric oxide synthase and transforming growth factor-beta1 production by infected microglia. *Am J Pathol* (2005) 167(4):1021–31. doi:10.1016/S0002-9440(10)61191-1
55. Zimmermann S, Murray PJ, Heeg K, Dalpke AH. Induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii* contributes to immune evasion in macrophages by blocking IFN-gamma signaling. *J Immunol* (2006) 176(3):1840–7. doi:10.4049/jimmunol.176.3.1840
56. Schneider AG, Abi Abdallah DS, Butcher BA, Denkers EY. *Toxoplasma gondii* triggers phosphorylation and nuclear translocation of dendritic cell STAT1 while simultaneously blocking IFNγ-induced STAT1 transcriptional activity. *PLoS One* (2013) 8(3):e60215. doi:10.1371/journal.pone.0060215
57. Lang C, Hildebrandt A, Brand F, Opitz L, Dihazi H, Lüder CG. Impaired chromatin remodelling at STAT1-regulated promoters leads to global unresponsiveness of *Toxoplasma gondii*-infected macrophages to IFN-γ. *PLoS Pathog* (2012) 8(1):e1002483. doi:10.1371/journal.ppat.1002483
58. Kim SK, Fouts AE, Boothroyd JC. *Toxoplasma gondii* dysregulates IFN-gamma-inducible gene expression in human fibroblasts: insights from a genome-wide transcriptional profiling. *J Immunol* (2007) 178(8):5154–65. doi:10.4049/jimmunol.178.8.5154
59. Whitmarsh RJ, Gray CM, Gregg B, Christian DA, May MJ, Murray PJ, et al. A critical role for SOCS3 in innate resistance to *Toxoplasma gondii*. *Cell Host Microbe* (2011) 10(3):224–36. doi:10.1016/j.chom.2011.07.009
60. Machado FS, Johndrow JE, Esper L, Dias A, Bafica A, Serhan CN, et al. Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat Med* (2006) 12:330–4. doi:10.1038/nm1355
61. Ma JS, Sasai M, Ohshima J, Lee Y, Bando H, Takeda K, et al. Selective and strain-specific NFAT4 activation by the *Toxoplasma gondii* polymorphic dense granule protein GRA6. *J Exp Med* (2014) 211(10):2013–32. doi:10.1084/jem.20131272
62. Rosowski EE, Lu D, Julien L, Rodda L, Gaiser RA, Jensen KD, et al. Strain-specific activation of the NF-B pathway by GRA15, a novel *Toxoplasma*

gondii dense granule protein. *J Exp Med* (2011) 208:195–212. doi:10.1084/jem.20100717
63. Du J, An R, Chen L, Shen Y, Chen Y, Cheng L, et al. *Toxoplasma gondii* virulence factor ROP18 inhibits the host NF-B pathway by promoting p65 degradation. *J Biol Chem* (2014) 289(18):12578–92. doi:10.1074/jbc.M113.544718
64. Seabra SH, de Souza W, Damatta RA. *Toxoplasma gondii* exposes phosphatidylserine inducing a TGF-beta1 autocrine effect orchestrating macrophage evasion. *Biochem Biophys Res Commun* (2004) 324(2):744–52. doi:10.1016/j.bbrc.2004.09.114
65. Padrão Jda C, Cabral GR, da Silva Mde F, Seabra SH, DaMatta RA. *Toxoplasma gondii* infection of activated J774-A1 macrophages causes inducible nitric oxide synthase degradation by the proteasome pathway. *Parasitol Int* (2014) 63(5):659–63. doi:10.1016/j.parint.2014.05.003
66. Santos TA, Portes Jde A, Damasceno-Sá JC, Caldas LA, Souza WD, Damatta RA, et al. Phosphatidylserine exposure by *Toxoplasma gondii* is fundamental to balance the immune response granting survival of the parasite and of the host. *PLoS One* (2011) 6(11):e27867. doi:10.1371/journal.pone.0027867
67. Nash PB, Purner MB, Leon RP, Clarke P, Duke RC, Curiel TJ. *Toxoplasma gondii*-infected cells are resistant to multiple inducers of apoptosis. *J Immunol* (1998) 160(4):1824–30.
68. Sinai AP, Payne TM, Carmen JC, Hardi L, Watson SJ, Molestina RE. Mechanisms underlying the manipulation of host apoptotic pathways by *Toxoplasma gondii*. *Int J Parasitol* (2004) 34(3):381–91. doi:10.1016/j.ijpara.2003.11.009
69. He JJ, Ma J, Li FC, Song HQ, Xu MJ, Zhu XQ. Transcriptional changes of mouse splenocyte organelle components following acute infection with *Toxoplasma gondii*. *Exp Parasitol* (2016) 167:7–16. doi:10.1016/j.exppara.2016.04.019
70. He JJ, Ma J, Wang JL, Xu MJ, Zhu XQ. Analysis of miRNA expression profiling in mouse spleen affected by acute *Toxoplasma gondii* infection. *Infect Genet Evol* (2016) 37:137–42. doi:10.1016/j.meegid.2015.11.005
71. Goebel S, Gross U, Lüder CG. Inhibition of host cell apoptosis by *Toxoplasma gondii* is accompanied by reduced activation of the caspase cascade and alterations of poly(ADP-ribose) polymerase expression. *J Cell Sci* (2001) 114(Pt 19):3495–505.
72. Carmen JC, Sinai AP. The differential effect of *Toxoplasma gondii* infection on the stability of BCL2-family members involves multiple activities. *Front Microbiol* (2011) 2:1. doi:10.3389/fmicb.2011.00001
73. Hippe D, Gais A, Gross U, Lüder CG. Modulation of caspase activation by *Toxoplasma gondii*. *Methods Mol Biol* (2009) 470:275–88. doi:10.1007/978-1-59745-204-5_19
74. Molestina RE, Payne TM, Coppens I, Sinai AP. Activation of NF-kappaB by *Toxoplasma gondii* correlates with increased expression of antiapoptotic genes and localization of phosphorylated IkappaB to the parasitophorous vacuole membrane. *J Cell Sci* (2003) 116(Pt 21):4359–71. doi:10.1242/jcs.00683
75. Hwang IY, Quan JH, Ahn MH, Ahmed HA, Cha GH, Shin DW, et al. *Toxoplasma gondii* infection inhibits the mitochondrial apoptosis through induction of Bcl-2 and HSP70. *Parasitol Res* (2010) 107(6):1313–21. doi:10.1007/s00436-010-1999-3
76. Choi SH, Park SJ, Cha GH, Quan JH, Chang NS, Ahn MH, et al. *Toxoplasma gondii* protects against H(2)O(2)-induced apoptosis in ARPE-19 cells through the transcriptional regulation of apoptotic elements and downregulation of the p38 MAPK pathway. *Acta Ophthalmol* (2011) 89(4):e350–6. doi:10.1111/j.1755-3768.2011.02113.x
77. Keller P, Schaumburg F, Fischer SF, Häcker G, Gross U, Lüder CG. Direct inhibition of cytochrome c-induced caspase activation in vitro by *Toxoplasma gondii* reveals novel mechanisms of interference with host cell apoptosis. *FEMS Microbiol Lett* (2006) 258(2):312–9. doi:10.1111/j.1574-6968.2006.00241.x
78. Vutova P, Wirth M, Hippe D, Gross U, Schulze-Osthoff K, Schmitz I, et al. *Toxoplasma gondii* inhibits Fas/CD95-triggered cell death by inducing aberrant processing and degradation of caspase 8. *Cell Microbiol* (2007) 6:1556e70. doi:10.1111/j.1462-5822.2007.00893.x
79. Hippe D, Lytovchenko O, Schmitz I, Lüder CG. Fas/CD95-mediated apoptosis of type II cells is blocked by *Toxoplasma gondii* primarily via interference with the mitochondrial amplification loop. *Infect Immun* (2008) 76(7):2905–12. doi:10.1128/IAI.01546-07
80. Kim JY, Ahn MH, Jun HS, Jung JW, Ryu JS, Min DY. *Toxoplasma gondii* inhibits apoptosis in infected cells by caspase inactivation and NF-kappaB activation. *Yonsei Med J* (2006) 47(6):862–9. doi:10.3349/ymj.2006.47.6.862
81. Yamada T, Tomita T, Weiss LM, Orlofsky A. *Toxoplasma gondii* inhibits granzyme B-mediated apoptosis by the inhibition of granzyme B function in host cells. *Int J Parasitol* (2011) 41(6):595–607. doi:10.1016/j.ijpara.2010.11.012
82. Kim L, Denkers EY. *Toxoplasma gondii* triggers Gi-dependent PI 3-kinase signaling required for inhibition of host cell apoptosis. *J Cell Sci* (2006) 119(Pt 10):2119–26. doi:10.1242/jcs.02934
83. Quan JH, Cha GH, Zhou W, Chu JQ, Nishikawa Y, Lee YH. Involvement of PI 3 kinase/Akt-dependent Bad phosphorylation in *Toxoplasma gondii*-mediated inhibition of host cell apoptosis. *Exp Parasitol* (2013) 133(4):462–71. doi:10.1016/j.exppara.2013.01.005
84. Wan L, Gong L, Wang W, An R, Zheng M, Jiang Z, et al. *T. gondii* rhoptry protein ROP18 induces apoptosis of neural cells via endoplasmic reticulum stress pathway. *Parasit Vectors* (2015) 21(8):554. doi:10.1186/s13071-015-1103-z
85. Händel U, Brunn A, Drögemüller K, Müller W, Deckert M, Schlüter D. Neuronal gp130 expression is crucial to prevent neuronal loss, hyperinflammation, and lethal course of murine *Toxoplasma encephalitis*. *Am J Pathol* (2012) 181(1):163–73. doi:10.1016/j.ajpath.2012.03.029
86. Song KJ, Ahn HJ, Nam HW. Anti-apoptotic effects of SERPIN B3 and B4 via STAT6 activation in macrophages after infection with *Toxoplasma gondii*. *Korean J Parasitol* (2012) 50(1):1–6. doi:10.3347/kjp.2012.50.1.1
87. Cai Y, Chen H, Mo X, Tang Y, Xu X, Zhang A, et al. *Toxoplasma gondii* inhibits apoptosis via a novel STAT3-miR-17-92-Bim pathway in macrophages. *Cell Signal* (2014) 26(6):1204–12. doi:10.1016/j.cellsig.2014.02.013
88. Da Gama LM, Ribeiro-Gomes FL, Guimarães U Jr, Arnholdt AC. Reduction in adhesiveness to extracellular matrix components, modulation of adhesion molecules and in vivo migration of murine macrophages infected with *Toxoplasma gondii*. *Microbes Infect* (2004) 6(14):1287–96. doi:10.1016/j.micinf.2004.07.008
89. Seipel D, Oliveira BC, Resende TL, Schuindt SH, Pimentel PM, Kanashiro MM, et al. *Toxoplasma gondii* infection positively modulates the macrophages migratory molecular complex by increasing matrix metalloproteinases, CD44 and alpha v beta 3 integrin. *Vet Parasitol* (2010) 169(3–4):312–9. doi:10.1016/j.vetpar.2009.12.042
90. Schuindt SH, Oliveira BC, Pimentel PM, Resende TL, Retamal CA, DaMatta RA, et al. Secretion of multi-protein migratory complex induced by *Toxoplasma gondii* infection in macrophages involves the uPA/uPAR activation system. *Vet Parasitol* (2012) 186(3–4):207–15. doi:10.1016/j.vetpar.2011.11.035
91. Wang MF, Lu CY, Lai SC. Up-regulation of matrix metalloproteinases-2 and -9 via an Erk1/2/NF-κB pathway in murine mast cells infected with *Toxoplasma gondii*. *J Comp Pathol* (2013) 149(2–3):146–55. doi:10.1016/j.jcpa.2013.03.002
92. Song KJ, Nam HW. Protease activity of 80 kDa protein secreted from the apicomplexan parasite *Toxoplasma gondii*. *Korean J Parasitol* (2003) 41(3):165–9. doi:10.3347/kjp.2003.41.3.165
93. JUnno A, Suzuki K, Xuan X, Nishikawa Y, Kitoh K, Takashima Y. Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow. *Parasitol Int* (2008) 57(4):515–8. doi:10.1016/j.parint.2008.06.004
94. Lambert H, Barragan A. Modelling parasite dissemination: host cell subversion and immune evasion by *Toxoplasma gondii*. *Cell Microbiol* (2010) 12(3):292–300. doi:10.1111/j.1462-5822.2009.01417.x
95. Courret N, Darche S, Sonigo P, Milon G, Buzoni-Gâtel D, Tardieux I. CD11c- and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. *Blood* (2006) 107(1):309–16. doi:10.1182/blood-2005-02-0666
96. Wang MF, Lai SC. Fibronectin degradation by MMP-2/MMP-9 in the serum of pregnant women and umbilical cord with *Toxoplasma gondii* infection. *J Obstet Gynaecol* (2013) 33(4):370–4. doi:10.3109/01443615.2013.769501
97. Muñoz M, Heimesaat MM, Danker K, Struck D, Lohmann U, Plickert R, et al. Interleukin (IL)-23 mediates Toxoplasma gondii-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. *J Exp Med* (2009) 206(13):3047–59. doi:10.1084/jem.20090900
98. Clark RT, Nance JP, Noor S, Wilson EH. T-cell production of matrix metalloproteinases and inhibition of parasite clearance by TIMP-1 during

chronic *Toxoplasma* infection in the brain. *ASN Neuro* (2011) 3(1):e00049. doi:10.1042/AN20100027

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Insights into the Cytoadherence Phenomenon of *Plasmodium vivax*: The Putative Role of Phosphatidylserine

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Plasmodium vivax is the most geographically widespread and the dominant human malaria parasite in most countries outside of sub-Saharan Africa and, although it was classically recognized to cause benign infection, severe cases and deaths caused by *P. vivax* have remarkably been reported. In contrast to *Plasmodium falciparum*, which well-known ability to bind to endothelium and placental tissue and form rosettes is related to severity of the disease, it has been a dogma that *P. vivax* is unable to undergo cytoadherent phenomena. However, some studies have demonstrated that red blood cells (RBCs) infected by *P. vivax* can cytoadhere to host cells, while the molecules participating in this host–parasite interaction are still a matter of speculation. In the present overview, we address the evidences currently supporting the adhesive profile of *P. vivax* and, additionally, discuss the putative role of phosphatidylserine—a cell membrane phospholipid with cytoadhesive properties that has been detected on the surface of *Plasmodium*-parasitized RBCs.

Keywords: *Plasmodium vivax*, cytoadhesion, rosetting, eryptosis, phosphatidylserine

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INTRODUCTION

Plasmodium vivax is the most geographically widespread and the second most prevalent parasite causing malaria in the world, with about 35% of global population living at risk of infection (1) and an estimated 8.5 million symptomatic cases in 2015 (2). *P. vivax* contributes significantly to malaria cases outside of sub-Saharan Africa, where it accounts for 41% of the cases, of which 65% occur in South-East Asia, 19% in Eastern Mediterranean, and the remaining in Western Pacific (9%) and American (7%) regions (2).

Despite its considerable impact in global public health, *P. vivax* was for long time a neglected parasite. The reasons for this scenario include: the low global prevalence [4% (2)], when compared with the most prevalent and lethal malaria parasite, *Plasmodium falciparum*; the failure to adapt to *in vitro* culture conditions; as well as the classically recognized benign profile of infection (3). However, in the last decade, severe cases and deaths due to *P. vivax* infection have remarkably been reported in all endemic regions, driving the attention of the academic community to the real importance of *P. vivax* (4). Moreover, the occurrence of severe forms of malaria in *P. vivax* infections, such as cerebral malaria and placental malaria, which were previously reported to be exclusively associated with *P. falciparum*, suggests that *P. vivax* can, to some extent, present pathogenic profiles similar to *P. falciparum* (5–8).

It is known that the main pathological phenomenon related to high virulence of *P. falciparum* is the sequestration of parasitized red blood cells (pRBC) to vascular endothelium and placenta, which allows late-stage forms of parasite to evade splenic phagocytosis, while provoking host damage by obstructing blood flow and inducing local pro-inflammatory response (9). Additional factors contributing to the pathogenesis of *falciparum* malaria comprise rosetting of pRBC with non-parasitized red blood cells (nRBC) as well as clumping of pRBC mediated by platelets (10). All these cytoadhesive events of *P. falciparum*-pRBC are recognized to be mediated by a large and diverse family of parasite antigens, named *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), that is expressed on the surface of pRBC and shows affinity to several host receptors, including intercellular adhesion molecule 1 (ICAM-1), platelet-endothelial cell adhesion molecule 1, chondroitin sulfate A (CSA), hyaluronic acid (HA), thrombospondin (TSP), and CD36 (10). Since homologous of *var* genes, which encode PfEMP1, have not been identified in other *Plasmodium* species and the cytoadherence of pRBC was not consistently documented in non-*falciparum* malaria, it was believed that the cytoadherence phenomenon of pRBC was restricted to *P. falciparum* infection (11–14). However, some evidences currently support that red blood cells (RBCs) infected by *P. vivax* (Pv-pRBC) also undergo cytoadherence events, as addressed below in the present paper.

EVIDENCES OF *P. vivax* CYTOADHESION

Since Pv-pRBC lack adhesive knob structure and, especially, because all parasite stages can be observed in the peripheral blood of patients, it has been a dogma that *P. vivax* lacks the ability to cytoadhere and, therefore, to sequester. However, in 2010 it was demonstrated, for the first time, that Pv-pRBC are able to cytoadhere *in vitro* to host cells (15). In this study, Pv-pRBC obtained from Brazilian non-severe patient samples were tested by static and flow cytoadhesion assays using human lung endothelial cells (HLEC), monkey brain endothelial cells, and human placental cryosections. Although the number of Pv-pRBC adhered under static conditions was lower than that observed for pRBC infected by *P. falciparum*, the strength of interaction with endothelium was similar. Moreover, it was shown using transfected Chinese Hamster Ovarian (CHO) cells that the binding of Pv-pRBC to ICAM-1-expressing cells was twice as high as to non-transfected cells or CD36-expressing cells and that the adherence to placental cryosections and HLEC was inhibited by soluble CSA, suggesting involvement of both ICAM-1 and CSA in adhesive processes of *P. vivax*. In fact, the adhesive capacity of Pv-pRBC to HLEC and the involvement of ICAM-1 were later recorded in studies using *P. vivax* isolates from Colombia (16). Moreover, a treatment with chondroitinase reversed the adherence of *P. vivax* isolates from the Asia-Pacific region to immobilized CSA, while it was additionally observed that hyaluronidase disturbed Pv-pRBC adhesion mediated by HA (17). Nevertheless, the degree of commitment of each host adhesive receptor studied until now is still a matter of speculation. For instance, in the study with Thai patients described above, all *P. vivax* isolates were adherent to immobilized CSA and HA, but none adhered to ICAM-1 (17), and when *P. vivax* isolates from Brazilian Amazon region was evaluated, a low frequency of pRBC adhesion to ICAM-1 and CSA was observed (18).

Corroborating the adhesive profile of *P. vivax*, it was recently reported that the schizont stage was absent in the peripheral circulation in more than half of Brazilian patients diagnosed with *P. vivax* malaria by blood smears and, even when *P. vivax* schizonts were detected, they were mostly present at low frequency (19). Moreover, *in vitro* maturation of *P. vivax* isolates provided a greater ability of Pv-pRBC to cytoadhere to HLEC than the same isolates before maturation, revealing a higher adhesive capacity of mature forms. These data indicate that *P. vivax* might be sequestered in the deep vasculature and that maturation of late stages of *P. vivax* occur outside peripheral circulation. Actually, more than 50 years ago sequestration of Pv-pRBC was proposed by Field et al. (20), who showed a disappearance of schizonts from the peripheral blood of a *P. vivax* patient. In addition, recent discoveries showing accumulation of *P. vivax* schizonts and gametocytes in the bone marrow (21); detection of a large number of intact Pv-pRBC in the spleen (22); and presence of Pv-pRBC within pulmonary microvasculature from a patient with negative blood smear at the time of death (23) support the hypothesis that *P. vivax* has the ability to sequester.

Although *P. vivax* does not present any protein homologous to PfEMP1, a group of variable proteins (VIR proteins) is expressed by this parasite species (24). In contrast to PfEMP1, VIR proteins are not clonally expressed and can additionally be found within pRBC, indicating initially that these *P. vivax* antigens have different functions from PfEMP1 ones (25). However, based on their variant nature and presence on pRBC surface, the role of VIR antigens in Pv-pRBC adhesion has been evaluated. Thus, computational analysis using a *P. falciparum*-based algorithm revealed putative adhesive protein motifs in VIR proteins (26), which could explain the capacity of *vir* gene (VIR-14) to mediate adhesion of pRBC to ICAM-1 when transfected into a non-adhesive *P. falciparum* line (27). Also, consistent with the participation of VIR proteins in the sequester phenomenon of Pv-pRBC, it was previously demonstrated that antibodies against variants of VIR proteins (VIR-A4 and VIR-E5) partially inhibit adhesion of Pv-pRBC to HLEC (15).

Therefore, there is now a growing body of evidence supporting that *P. vivax* parasites possess adhesive phenotypes. Indeed, besides adhesion to endothelium and placental tissue, it is known that *P. vivax* has the ability to form rosettes, which are defined by the binding of a pRBC with two or more nRBC. Rosetting formation in *P. vivax* infection was described more than 20 years ago (28) and has been shown to be more frequent than in *P. falciparum* infection (29, 30), but few studies have been conducted to investigate this *P. vivax* phenomenon; largely due to the absence of a *P. vivax* continuous culture method. Notwithstanding, it was already demonstrated that rosettes in *P. vivax* infection are formed by interaction of pRBC containing trophozoites, schizonts, or gametocytes with mature RBCs (normocytes), a process that involves glycophorin C receptor present on nRBC surface (30). Furthermore, *P. vivax* rosettes were shown to be stable even under high physiological shear stress and rosette formation was closely associated with induction of an increased rigidity of Pv-pRBC,

possibly contributing to sequestration of *P. vivax* in the microvasculature (31). However, differently from *P. falciparum*, both host and parasite antigens intricate on *P. vivax* rosetting as well as the relation of this adhesive phenomenon to the pathogenesis of *vivax* malaria remain unknown.

THE ROLE OF PHOSPHATIDYLSERINE (PS) IN CYTOADHESIVE PHENOMENA

While some research efforts have been dedicated to identifying *P. vivax* antigens participating in cytoadhesion of pRBC, little attention has been given to host RBC factors that have adhesive potentiality, such as PS. PS is a cell membrane phospholipid usually restricted to the inner leaflet of the lipid bilayer (32), but during apoptotic cell death processes PS is exposed on cell surface, promoting recognition and clearance of dying cells by phagocytes (33). Externalization of PS also occurs in activated platelet and transiently in activated lymphocytes and mast cells, where it is associated with procoagulant activity, homing to sites of inflammation and cell degranulation, respectively (34–36). Furthermore, it has been shown that the presence of PS on external leaflet of cell membrane is a hallmark of suicidal erythrocyte death, named eryptosis (37).

Eryptosis occurs in senescent RBC and can precociously be triggered by a variety of endogenous and xenobiotics stimuli (38, 39). Similarly to apoptosis of nucleated cells, eryptotic processes are characterized by many morphological and biochemical changes, i.e., Ca^{2+} influx, cysteine protease activity, PS exposure, cell shrinkage, and plasma membrane microvesiculation, with externalized PS rendering RBC susceptible to clearance by splenic phagocytes (40). Accordingly, overinduction of PS-exposing eryptotic RBC is believed to contribute to the development of anemia related to several clinical disorders, as reported in both experimental and human malaria (41, 42). But, additionally, PS on RBC surface is also considered one of the factors responsible for thrombo-occlusive events in pathologies such as sickle cell disease, chronic renal failure, retinal vein occlusion, and diabetes; in part, by mediating RBC adherence to endothelium as well as cell aggregation (43–46).

Indeed, adherence of PS-exposing RBC to endothelium is observed *in vitro* under dynamic flow conditions mimicking venular wall shear stress (47, 48) and takes place through interaction of PS with the scavenger receptors CD36 or CXC chemokine ligand 16 (CXCL16) expressed on endothelial cell membrane, as well as with TSP, which is found in the basement membrane and extracellular matrix of endothelium and that can be exposed by vascular injury (45, 48, 49). Moreover, soluble plasma TSP can interact with CD36 and, in this manner, could operate as a bridge to adherence of PS-exposing RBC (50, 51). Such interactions involving PS, CD36, TSP, and CXCL16 have also been involved in RBC-platelet aggregation, agreeing with the presence of CD36 and CXCL16 in platelet membrane (52–54). Thus, if parasite antigens able to provide pRBC adhesiveness are absent, *P. vivax* could explore host adhesive molecules to mediate cytoadhesive events of pRBC.

Interestingly, it has previously been demonstrated that intraerythrocytic plasmodia development progressively induces PS exteriorization on pRBC, with larger exposure at the late stages of parasite maturation (55, 56), which possibly result from eryptosis stimulation. Schizogonic process is described to activate non-selective cation channels in host pRBC membrane, allowing the entry of Ca^{2+} necessary for parasite intracellular growth, which, in turn, leads to the activation of phospholipid scramblases responsible for PS exposure (57). Although PS externalization has not been evaluated in *P. vivax* infection, it was already detected by flow cytometry in RBC infected by *P. falciparum*, *P. berghei*, and *P. yoelii* (41, 58, 59) and in *P. falciparum*, the binding of late-stage pRBC exposing PS to CD36-expressing cells as well as immobilized CD36 and TSP was inhibited by annexin V, PS-containing liposomes or glycerophosphorylserine—a soluble form of PS (60), indicating that PS could, at least in part, support cytoadhesive phenomena of pRBC in malaria. Consistent with this possibility, a relationship between cytoadhesive activity and PS exposure was also reported when knobby and knobless *P. falciparum* strains, which differentially induce PS externalization on late-stage pRBC, were studied (60) and, more recently, it was also shown that PS-expressing RBC can operate as nuclei for RBC aggregation induced by *P. falciparum*-conditioned medium (61).

Importantly, studies conducted on *P. berghei* ANKA experimental infection with CD36-deficient rodents have demonstrated that CD36 is an essential receptor for sequestration of schizont-stage pRBC, which occurred mostly in the capillaries of lungs and adipose tissue, but not in the brain, where endothelial expression of CD36 is low or absent (62). Indeed, besides being incriminated in acute tissue injury induced by *P. berghei* ANKA-pRBC accumulation in lung (63), CD36 is known as an important receptor mediating pRBC sequestration, non-related to brain and placental tissue in *P. falciparum* malaria (64), and its expression on surface of platelets and RBC has been implicated in clumping and rosetting processes of *P. falciparum*-pRBC, respectively (65, 66). However, it is noteworthy that, in contrast to *P. falciparum,* which expresses the adhesin PfEMP1, but similarly to *Plasmodium chabaudi*, whose late-stage forms undergo CD36-dependent cytoadhesion *in vitro* (67), no putative parasite ligand for CD36 has been identified in genome of *P. berghei,* or even other species of *Plasmodium* displaying cytoadherence phenotypes, such as *P. vivax* (14, 68), reinforcing the premise that alternative pathways, not based on the expression of parasite adhesins, could mediate CD36-dependent cytoadhesion of late-stage pRBC.

Additional evidences for this proposition are also documented in *P. vivax* malaria. First, a reduction in Pv-pRBC adhesion to HLEC was achieved in the presence of anti-CD36 antibodies, although the small number of samples limited the statistical analysis concerning extension of CD36 participation in *P. vivax* cytoadhesion (15). Second, while studying cellular trafficking and the adhesive propriety of *P. vivax* VIR proteins in *P. falciparum* transgenic lines, it was shown that only one variant of VIR proteins (VIR-14) was exposed at the surface of pRBC, mediating cytoadherence to CHO cells through ICAM-1, but not CD36 (27). Thus, it is tempting to speculate that if antigens encoded by *vir* genes participate in Pv-pRBC adhesive events, it seems that it does not take place through a CD36-dependent mechanism, in which PS could play a role (**Figure 1**). In view of this possibility,

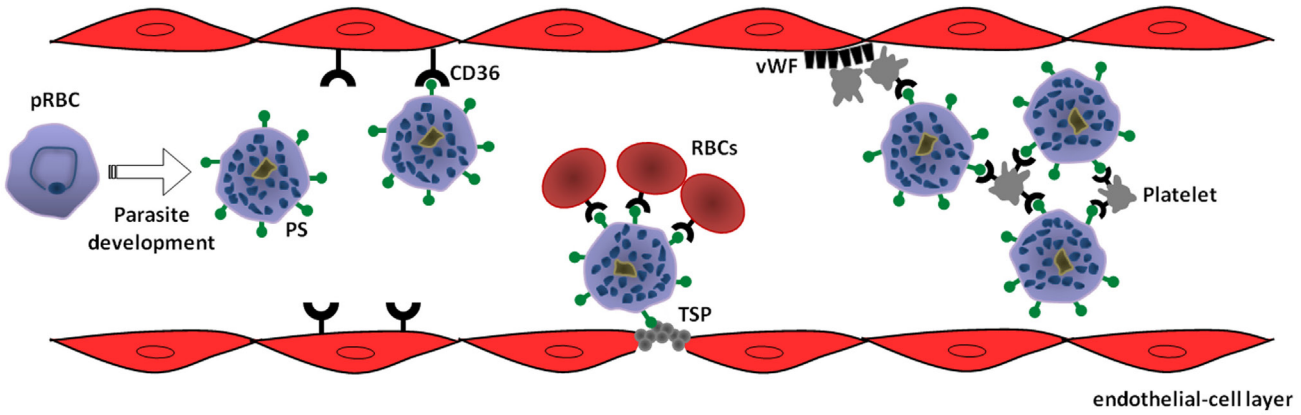

FIGURE 1 | Model of phosphatidylserine (PS) role in cytoadhesive phenomena of *Plasmodium vivax*-parasitized red blood cells (pRBC). Intraerythrocytic parasite development leads to exposure of PS on pRBC surface as a result of suicidal erythrocyte death (eryptosis) induction. In turn, PS mediates sequestration of pRBC to microvasculature through interaction with receptors expressed on endothelial cells, such as CD36, or thrombospondin (TSP) exposed in injured endothelium. In a CD36-dependent manner, PS-exposing pRBC can promote rosetting of non-parasitized red blood cells (RBCs) as well as autoagglutination (clumping) by binding to platelets. Additionally, von Willebrand factor (vWF) can indirectly contribute to the sequestration of PS-exposing pRBC by mediating platelet adhesion at sites of vascular damage (69).

studies evaluating the occurrence of PS externalization in pRBC from *P. vivax* isolates as well as the effect of blocking PS-CD36/TSP interaction on adhesive phenomena of Pv-pRBC may help to confirm the involvement of PS in *vivax* malaria.

AUTHOR CONTRIBUTIONS

PT and SL wrote the paper. Both authors read and approved the final version of the manuscript.

REFERENCES

1. World Health Organization. *Control and Elimination of Plasmodium vivax Malaria: A Technical Brief*. Geneva: World Health Organization (2015).
2. World Health Organization. *World Malaria Report 2016*. Geneva: World Health Organization (2016).
3. Baird JK. Neglect of *Plasmodium vivax* malaria. *Trends Parasitol* (2007) 23: 533–9. doi:10.1016/j.pt.2007.08.011
4. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W. Severe *vivax* malaria: a systematic review and meta-analysis of clinical studies since 1900. *Malar J* (2014) 13:481. doi:10.1186/1475-2875-13-481
5. Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, et al. Multidrug-resistant *Plasmodium vivax* associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med* (2008) 5:e128. doi:10.1371/journal.pmed.0050128
6. Naing C, Whittaker MA, Nyunt Wai V, Mak JW. Is *Plasmodium vivax* malaria a severe malaria? A systematic review and meta-analysis. *PLoS Negl Trop Dis* (2014) 8:e3071. doi:10.1371/journal.pntd.0003071
7. Chaikitgosiyakul S, Rijken MJ, Muehlenbachs A, Lee SJ, Chaisri U, Viriyavejakul P, et al. A morphometric and histological study of placental malaria shows significant changes to villous architecture in both *Plasmodium falciparum* and *Plasmodium vivax* infection. *Malar J* (2014) 13:4. doi:10.1186/1475-2875-13-4
8. Datta M, Biswas J, Dasgupta S, Banerjee K, Choudhury S, Sengupta SK, et al. Comparative study on antenatal and perinatal outcome of vivax and falciparum malaria in a Tertiary Care Hospital of Kolkata, India. *J Clin Diagn Res* (2017) 11:QC01–04. doi:10.7860/JCDR/2017/23051.9195
9. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. *Nature* (2002) 415:673–9. doi:10.1038/415673a
10. Rasti N, Wahlgren M, Chen Q. Molecular aspects of malaria pathogenesis. *FEMS Immunol Med Microbiol* (2004) 41:9–26. doi:10.1016/j.femsim.2004.01.010
11. Korir CC, Galinski MR. Proteomic studies of *Plasmodium knowlesi* SICA variant antigens demonstrate their relationship with *P. falciparum* EMP1. *Infect Genet Evol* (2006) 6:75–9. doi:10.1016/j.meegid.2005.01.003
12. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, et al. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect Dis* (2009) 9:555–66. doi:10.1016/S1473-3099(09)70177-X
13. Maguire JD, Baird JK. The 'non-falciparum' malarias: the roles of epidemiology, parasite biology, clinical syndromes, complications and diagnostic rigour in guiding therapeutic strategies. *Ann Trop Med Parasitol* (2010) 104:283–301. doi:10.1179/136485910X12743554760027
14. Ebbinghaus P, Krücken J. Characterization and tissue-specific expression patterns of the *Plasmodium chabaudi cir* multigene family. *Malar J* (2011) 10:272. doi:10.1186/1475-2875-10-272
15. Carvalho BO, Lopes SC, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, et al. On the cytoadhesion of *Plasmodium vivax*-infected erythrocytes. *J Infect Dis* (2010) 202:638–47. doi:10.1086/654815
16. De las Salas B, Segura C, Pabón A, Lopes SC, Costa FT, Blair S. Adherence to human lung microvascular endothelial cells (HMVEC-L) of *Plasmodium vivax* isolates from Colombia. *Malar J* (2013) 12:347. doi:10.1186/1475-2875-12-347
17. Chotivanich K, Udomsangpetch R, Suwanarusk R, Pukrittayakamee S, Wilairatana P, Beeson JG, et al. *Plasmodium vivax* adherence to placental glycosaminoglycans. *PLoS One* (2012) 7:e34509. doi:10.1371/journal.pone.0034509
18. Marín-Menéndez A, Bardají A, Martínez-Espinosa FE, Bôtto-Menezes C, Lacerda MV, Ortiz J, et al. Rosetting in *Plasmodium vivax*: a cytoadhesion phenotype associated with anaemia. *PLoS Negl Trop Dis* (2013) 7:e2155. doi:10.1371/journal.pntd.0002155
19. Lopes SC, Albrecht L, Carvalho BO, Siqueira AM, Thomson-Luque R, Nogueira PA, et al. Paucity of *Plasmodium vivax* mature schizonts in peripheral blood is associated with their increased cytoadhesive potential. *J Infect Dis* (2014) 209:1403–7. doi:10.1093/infdis/jiu018
20. Field JW, Sandosham AA, Fong YL. *The Microscopial Diagnosis of Human Malaria*. Kuala Lumpur: Institute for Medical Research (1963).
21. Baro B, Deroost K, Raiol T, Brito M, Almeida AC, de Menezes-Neto A, et al. *Plasmodium vivax* gametocytes in the bone marrow of an acute malaria patient and changes in the erythroid miRNA profile. *PLoS Negl Trop Dis* (2017) 11:e0005365. doi:10.1371/journal.pntd.0005365

22. Siqueira AM, Magalhães BML, Melo GC, Ferrer M, Castillo P, Martin-Jaular L, et al. Spleen rupture in a case of untreated *Plasmodium vivax* infection. *PLoS Negl Trop Dis* (2012) 6:e1934. doi:10.1371/journal.pntd.0001934
23. Lacerda MV, Fragoso SC, Alecrim MG, Alexandre MA, Magalhães BM, Siqueira AM, et al. Postmortem characterization of patients with clinical diagnosis of *Plasmodium vivax* malaria: to what extent does this parasite kill? *Clin Infect Dis* (2012) 55:e67–74. doi:10.1093/cid/cis615
24. del Portillo HA, Fernandez-Becerra C, Bowman S, Oliver K, Preuss M, Sanchez CP, et al. A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. *Nature* (2001) 410:839–42. doi:10.1038/35071118
25. Fernandez-Becerra C, Pein O, de Oliveira TR, Yamamoto MM, Cassola AC, Rocha C, et al. Variant proteins of *Plasmodium vivax* are not clonally expressed in natural infections. *Mol Microbiol* (2005) 58:648–58. doi:10.1111/j.1365-2958.2005.04850.x
26. Lopez FJ, Bernabeu M, Fernandez-Becerra C, del Portillo HA. A new computational approach redefines the subtelomeric vir superfamily of *Plasmodium vivax*. *BMC Genomics* (2013) 14:8. doi:10.1186/1471-2164-14-8
27. Bernabeu M, Lopez FJ, Ferrer M, Martin-Jaular L, Razaname A, Corradin G, et al. Functional analysis of *Plasmodium vivax* VIR proteins reveals different subcellular localizations and cytoadherence to the ICAM-1 endothelial receptor. *Cell Microbiol* (2012) 14:386–400. doi:10.1111/j.1462-5822.2011.01726.x
28. Udomsangpetch R, Thanikkul K, Pukrittayakamee S, White NJ. Rosette formation by *Plasmodium vivax*. *Trans R Soc Trop Med Hyg* (1995) 89:635–7. doi:10.1016/0035-9203(95)90422-0
29. Chotivanich KT, Pukrittayakamee S, Simpson JA, White NJ, Udomsangpetch R. Characteristics of *Plasmodium vivax*-infected erythrocyte rosettes. *Am J Trop Med Hyg* (1998) 59:73–6. doi:10.4269/ajtmh.1998.59.73
30. Lee WC, Malleret B, Lau YL, Mauduit M, Fong MY, Cho JS, et al. Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes. *Blood* (2014) 123:e100–9. doi:10.1182/blood-2013-12-541698
31. Zhang R, Lee WC, Lau YL, Albrecht L, Lopes SC, Costa FT, et al. Rheopathologic consequence of *Plasmodium vivax* rosette formation. *PLoS Negl Trop Dis* (2016) 10:e0004912. doi:10.1371/journal.pntd.0004912
32. Op Den Kamp JAF. Lipid asymmetry in membranes. *Ann Rev Biochem* (1979) 48:47–71. doi:10.1146/annurev.bi.48.070179.000403
33. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol* (2006) 16:189–97. doi:10.1016/j.tcb.2006.02.003
34. Lhermusier T, Chap H, Payrastre B. Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome. *J Thromb Haemost* (2011) 9:1883–91. doi:10.1111/j.1538-7836.2011.04478.x
35. Elliott JI, Surprenant A, Marelli-Berg FM, Cooper JC, Cassady-Cain RL, Wooding C, et al. Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat Cell Biol* (2005) 7:808–16. doi:10.1038/ncb1279
36. Rysavy NM, Shimoda LM, Dixon AM, Speck M, Stokes AJ, Turner H, et al. Beyond apoptosis: the mechanism and function of phosphatidylserine asymmetry in the membrane of activating mast cells. *Bioarchitecture* (2014) 4:127–37. doi:10.1080/19490992.2014.995516
37. Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, et al. Mechanisms of suicidal erythrocyte death. *Cell Physiol Biochem* (2005) 15:195–202. doi:10.1159/000086406
38. Bratosin D, Estaquier J, Ameisen JC, Montreuil J. Molecular and cellular mechanisms of erythrocyte programmed cell death: impact on blood transfusion. *Vox Sang* (2002) 83:307–10. doi:10.1111/j.1423-0410.2002.tb05324.x
39. Lang E, Lang F. Triggers, inhibitors, mechanisms, and significance of eryptosis: the suicidal erythrocyte death. *Biomed Res Int* (2015) 2015:513518. doi:10.1155/2015/513518
40. Totino PR, Daniel-Ribeiro CT, Ferreira-da-Cruz MF. Evidencing the role of erythrocytic apoptosis in malarial anemia. *Front Cell Infect Microbiol* (2016) 6:176. doi:10.3389/fcimb.2016.00176
41. Totino PR, Magalhães AD, Silva LA, Banic DM, Daniel-Ribeiro CT, Ferreira-da-Cruz MF. Apoptosis of non-parasitized red blood cells in malaria: a putative mechanism involved in the pathogenesis of anaemia. *Malar J* (2010) 9:350. doi:10.1186/1475-2875-9-350
42. Fendel R, Brandts C, Rudat A, Kreidenweiss A, Steur C, Appelmann I, et al. Hemolysis is associated with low reticulocyte production index and predicts blood transfusion in severe malarial anemia. *PLoS One* (2010) 5:e10038. doi:10.1371/journal.pone.0010038
43. Wali RK, Jaffe S, Kumar D, Kalra VK. Alterations in organization of phospholipids in erythrocytes as factor in adherence to endothelial cells in diabetes mellitus. *Diabetes* (1988) 37:104–11. doi:10.2337/diabetes.37.1.104
44. Bonomini M, Sirolli V, Gizzi F, Di Stante S, Grilli A, Felaco M. Enhanced adherence of human uremic erythrocytes to vascular endothelium: role of phosphatidylserine exposure. *Kidney Int* (2002) 62:1358–63. doi:10.1111/j.1523-1755.2002.kid560.x
45. Setty BN, Kulkarni S, Stuart MJ. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. *Blood* (2002) 99:1564–71. doi:10.1182/blood.V99.5.1564
46. Wautier MP, Héron E, Picot J, Colin Y, Hermine O, Wautier JL. Red blood cell phosphatidylserine exposure is responsible for increased erythrocyte adhesion to endothelium in central retinal vein occlusion. *J Thromb Haemost* (2011) 9:1049–55. doi:10.1111/j.1538-7836.2011.04251.x
47. Closse C, Dachary-Prigent J, Boisseau MR. Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* (1999) 107:300–2. doi:10.1046/j.1365-2141.1999.01718.x
48. Manodori AB, Barabino GA, Lubin BH, Kuypers FA. Adherence of phosphatidylserine-exposing erythrocytes to endothelial matrix thrombospondin. *Blood* (2000) 95:1293–300.
49. Borst O, Abed M, Alesutan I, Towhid ST, Qadri SM, Föller M, et al. Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. *Am J Physiol Cell Physiol* (2012) 302:C644–51. doi:10.1152/ajpcell.00340.2011
50. Silverstein RL, Baird M, Lo SK, Yesner LM. Sense and antisense cDNA transfection of CD36 (glycoprotein IV) in melanoma cells. Role of CD36 as a thrombospondin receptor. *J Biol Chem* (1992) 267:16607–12.
51. Betal SG, Setty BNY. Phosphatidylserine-positive erythrocytes bind to immobilized and soluble thrombospondin-1 via its heparin-binding domain. *Transl Res* (2008) 152:165–77. doi:10.1016/j.trsl.2008.07.007
52. Ohta K, Fukuuchi Y, Tomita M, Tanahashi N, Matsuoka S, Takeda H. Monoclonal antibody against platelet thrombospondin decreases erythrocyte aggregation rate. *Biorheology* (1991) 28:551–6.
53. Wun T, Paglieroni T, Field CL, Welborn J, Cheung A, Walker NJ, et al. Platelet-erythrocyte adhesion in sickle cell disease. *J Investig Med* (1999) 47:121–7.
54. Walker B, Towhid ST, Schmid E, Hoffmann SM, Abed M, Münzer P, et al. Dynamic adhesion of eryptotic erythrocytes to immobilized platelets via platelet phosphatidylserine receptors. *Am J Physiol Cell Physiol* (2014) 306:C291–7. doi:10.1152/ajpcell.00318.2013
55. Joshi P, Dutta GP, Gupta CM. An intracellular simian malarial parasite (*Plasmodium knowlesi*) induces stage-dependent alterations in membrane phospholipid organization of its host erythrocyte. *Biochem J* (1987) 246:103–8. doi:10.1042/bj2460103
56. Schwartz RS, Olson JA, Raventos-Suarez C, Yee M, Heath RH, Lubin B, et al. Altered plasma membrane phospholipid organization in *Plasmodium falciparum*-infected human erythrocytes. *Blood* (1987) 69:401–7.
57. Lang F, Lang PA, Lang KS, Brand V, Tanneur V, Duranton C, et al. Channel-induced apoptosis of infected host cells – the case of malaria. *Pflugers Arch* (2004) 448:319–24. doi:10.1007/s00424-004-1254-9
58. Butthep P, Wanram S, Pattanapanyasat K, Vattanaviboon P, Fucharoen S, Wilairat P. Cytoadherence between endothelial cells and *P. falciparum* infected and noninfected normal and thalassemic red blood cells. *Cytometry B Clin Cytom* (2006) 70:432–42. doi:10.1002/cyto.b.20141
59. Alesutan I, Bobbala D, Qadri SM, Estremera A, Föller M, Lang F. Beneficial effect of aurothiomalate on murine malaria. *Malar J* (2010) 9:118. doi:10.1186/1475-2875-9-118
60. Eda S, Sherman IW. Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell Physiol Biochem* (2002) 12:373–84. doi:10.1159/000067908
61. Balaji SN, Trivedi V. Extracellular methemoglobin primes red blood cell aggregation in malaria: an *in vitro* mechanistic study. *FEBS Lett* (2013) 587:350–7. doi:10.1016/j.febslet.2012.12.015
62. Franke-Fayard B, Janse CJ, Cunha-Rodrigues M, Ramesar J, Büscher P, Que I, et al. Murine malaria parasite sequestration: CD36 is the major receptor, but cerebral pathology is unlinked to sequestration. *Proc Natl Acad Sci U S A* (2005) 102:11468–73. doi:10.1073/pnas.0503386102
63. Lovegrove FE, Gharib SA, Peña-Castillo L, Patel SN, Ruzinski JT, Hughes TR, et al. Parasite burden and CD36-mediated sequestration are determinants

of acute lung injury in an experimental malaria model. *PLoS Pathog* (2008) 4:e1000068. doi:10.1371/journal.ppat.1000068
64. Serghides L, Smith TG, Patel SN, Kain KC. CD36 and malaria: friends or foes? *Trends Parasitol* (2003) 19:461–9. doi:10.1016/j.pt.2003.08.006
65. van Schravendijk MR, Handunnetti SM, Barnwell JW, Howard RJ. Normal human erythrocytes express CD36, an adhesion molecule of monocytes, platelets, and endothelial cells. *Blood* (1992) 80:2105–14.
66. Arman M, Adams Y, Lindergard G, Rowe JA. A method for positive and negative selection of *Plasmodium falciparum* platelet-mediated clumping parasites and investigation of the role of CD36. *PLoS One* (2013) 8:e55453. doi:10.1371/journal.pone.0055453
67. Mota MM, Jarra W, Hirst E, Patnaik PK, Holder AA. *Plasmodium chabaudi*-infected erythrocytes adhere to CD36 and bind to microvascular endothelial cells in an organ-specific way. *Infect Immun* (2000) 68:4135–44. doi:10.1128/IAI.68.7.4135-4144.2000
68. Frech C, Chen N. Variant surface antigens of malaria parasites: functional and evolutionary insights from comparative gene family classification and analysis. *BMC Genomics* (2013) 14:427. doi:10.1186/1471-2164-14-427
69. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost* (2003) 1:1335–42. doi:10.1046/j.1538-7836.2003.00260.x

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The P2X7 Receptor Mediates *Toxoplasma gondii* Control in Macrophages through Canonical NLRP3 Inflammasome Activation and Reactive Oxygen Species Production

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Toxoplasma gondii (T. gondii) is the protozoan parasite that causes toxoplasmosis, a potentially fatal disease to immunocompromised patients, and which affects approximately 30% of the world's population. Previously, we showed that purinergic signaling *via* the P2X7 receptor contributes to *T. gondii* elimination in macrophages, through reactive oxygen species (ROS) production and lysosome fusion with the parasitophorous vacuole. Moreover, we demonstrated that P2X7 receptor activation promotes the production of anti-parasitic pro-inflammatory cytokines during early *T. gondii* infection *in vivo*. However, the cascade of signaling events that leads to parasite elimination *via* P2X7 receptor activation remained to be elucidated. Here, we investigated the cellular pathways involved in *T. gondii* elimination triggered by P2X7 receptor signaling, during early infection in macrophages. We focused on the potential role of the inflammasome, a protein complex that can be co-activated by the P2X7 receptor, and which is involved in the host immune defense against *T. gondii* infection. Using peritoneal and bone marrow-derived macrophages from knockout mice deficient for inflammasome components (NLRP3$^{-/-}$, Caspase-1/11$^{-/-}$, Caspase-11$^{-/-}$), we show that the control of *T. gondii* infection *via* P2X7 receptor activation by extracellular ATP (eATP) depends on the canonical inflammasome effector caspase-1, but not on caspase-11 (a non-canonical inflammasome effector). Parasite elimination *via* P2X7 receptor and inflammasome activation was also dependent on ROS generation and pannexin-1 channel. Treatment with eATP increased IL-1β secretion from infected macrophages, and this effect was dependent on the canonical NLRP3 inflammasome. Finally, treatment with recombinant IL-1β promoted parasite elimination *via* mitochondrial ROS generation (as assessed using Mito-TEMPO). Together, our results support a model where P2X7 receptor activation by eATP inhibits *T. gondii* growth in macrophages by triggering NADPH-oxidase-dependent ROS production, and also by activating a canonical NLRP3 inflammasome, which increases IL-1β production (*via* caspase-1 activity), leading to mitochondrial ROS generation.

Keywords: P2X7 receptor, *Toxoplasma gondii*, NLRP3 inflammasome, caspase-1, caspase-11, IL-1β, reactive oxygen species

INTRODUCTION

Toxoplasma gondii is a parasitic protozoan from the phylum Apicomplexa and the etiologic agent of toxoplasmosis, an important disease that affects 30% of the world's population (1, 2). Toxoplasmosis can be transmitted by organ transplantation, blood transfusion or congenital infection, due to the parasite's ability to cross biological barriers (3, 4). In most cases, *T. gondii* causes asymptomatic disease, but toxoplasmosis can be fatal in immunocompromised patients, such as those with HIV/AIDS, cancer, and organ transplants (2). Also, severe ocular disease and multiple organ failure in immunocompetent individuals have been observed in endemic countries, due to the circulation of atypical parasite strains (5, 6). The current therapy for toxoplasmosis is based on drug combinations such as sulfadiazine/pyrimethamine, which reduce parasite replication, controlling host damage and symptoms; however, combination therapy causes important side effects and does not eliminate the resistant *T. gondii* cysts (7, 8).

T. gondii is an obligate intracellular parasite, able to infect virtually all nucleated cell types in a wide variety of hosts (2). It has been proposed that *T. gondii* utilizes innate immune cells, like macrophages and dendritic cells, to migrate to preferential infection sites, such as the immune "shielded" environment of the central nervous system, establishing lifelong chronic infection (9, 10). The parasite survives inside host cells by overcoming many host antimicrobial mechanisms, including reactive oxygen species (ROS) and nitric oxide (NO) production, lysosome fusion (to the parasitophorous vacuole), host cell death induction, and the secretion of pro-inflammatory cytokines and chemokines (11). Concomitantly, *T. gondii* triggers the activation of host cell anti-inflammatory transcription factors, inducing the over expression of receptors involved in host cell migration to the brain, which characterizes the chronic phase of toxoplasmosis (12). The ability to subvert immune system mechanisms and the fast establishment of the latent (chronic) phase of the disease directly jeopardize treatment for toxoplasmosis.

Inflammasomes are multimeric protein complexes that produce active caspase-1 by cleavage of procaspase-1 (13), and whose activation triggers maturation of IL-1β/IL-18 and pyroptosis, a type of inflammatory cell death (14). The inflammasome NLRP3 are formed by nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins, an apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) protein adaptor and an inactive zymogen, procaspase-1 (Casp-1) (14). In mice, activation of the NLRP3 inflammasome requires two signals. First, recognition of a pathogen-associated molecular pattern by cell surface molecules known as pattern recognition receptors (PRRs) leads to the transcription of pro-inflammatory cytokines (15). Then, activation of the cytosolic PRRs results in the oligomerization of inflammasome components and procaspase-1 cleavage into active caspase-1 (13). The active caspase-1 subsequently cleaves the pro-inflammatory IL-1-family cytokines into their bioactive forms IL-1β/IL-18 and induces pyroptosis (14). Inflammasomes involved in caspase-1 activation are known as canonical inflammasomes, while those related to caspase-11 (in mice) or caspase-4/5 (in humans) are known as non-canonical inflammasomes (16, 17). Activation of the canonical NLRP3 inflammasome is trigged by cytosolic stress conditions such as K^+ efflux, leakage of lysosome components, mitochondrial damage, and ROS production (14, 18). Interestingly, the P2X7 nucleotide receptor activation involved in the elimination of different intracellular parasites, such as *Leishmania amazonensis* (19), *Porphyromonas gingivalis* (20), *Mycobacterium tuberculosis* (21–24), and *T. gondii* (25–27), induces most of these stress conditions and, thus, is capable of activating the NLRP3 inflammasome (28–30). Polymorphisms in the human P2X7 receptor are directly associated with host susceptibility to congenital or acquired toxoplasmosis, in immunocompetent patients (25, 31). Also, P2X7 absence or disruption of P2X7 receptor function increases toxoplasmosis severity in murine infection with virulent (RH, type I) or non-virulent (Me-49, type II) strains of *T. gondii* (27, 32).

Our group demonstrated that P2X7 receptor activation by extracellular ATP (eATP) in *T. gondii*-infected macrophages contributes to parasite elimination by ROS production and lysosome fusion with the parasitophorous vacuole (26). We also showed that the P2X7 receptor is an important activator of the anti-parasitic pro-inflammatory response that occurs in early *T. gondii* infection *in vivo* (32). However, the cascade of intracellular events trigged by purinergic signaling—which results in parasite control in early infection—remains to be elucidated.

In this work, we used macrophages from knockout mice lacking inflammasome components to examine the hypothesis that the inflammasome is involved in *T. gondii* infection control *via* eATP and the P2X7 receptor. We also studied the roles of ROS production and IL-1β secretion induced by P2X7 receptor in infected cell. Our combined data provide an overall model of the cellular pathways involved in *T. gondii* infection control mediated by the P2X7 receptor, in murine macrophages.

MATERIALS AND METHODS

Reagents

Adenosine-5'-triphosphate (ATP), *N*-acetyl cysteine (NAC), penicillin, streptomycin, HEPES, Mito-TEMPO, apocynin, carbenoxolone, paraformaldehyde, and bovine serum albumin were purchased from Sigma Aldrich (USA). Fetal bovine serum (FBS) was from Gibco/Life Technologies (USA). Z-YVAD and recombinant IL-1β were from R&D Systems (USA).

Mice

The following mouse strains were used in this work: Swiss CF1, C57BL/6 (wild-type strain), P2X7$^{-/-}$ (originally from the Jackson Laboratory), Caspase-11$^{-/-}$, Caspase1/11$^{-/-}$ (Genentech, USA), and NLRP3$^{-/-}$ mice generated on the C57BL/6 background (8–12 weeks old) were obtained from the Isogenic Breeding Unit at Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil. Mice were maintained at the Animal House for Transgenic Mice of the Federal University of Rio de Janeiro (UFRJ), at 22°C in a 12-h light/dark cycle. Mice aged between 8 and 12 weeks were used in all experiments. The procedures for the care and use of animals were according to the guidelines of the Brazilian College of Animal Experimentation (COBEA). All

efforts were made to minimize animal suffering and to reduce the number of animals used in this study. This study was approved and followed all the guidelines established by the Ethics Committee on the Use of Animals (CEUA) of the Biophysic Institute Carlos Chagas Filho (IBCCF, UFRJ, no. 082/15).

Parasites

T. gondii tachyzoites from the RH strain were maintained in Swiss CF-1 mice as previously described in Ref. (33). Briefly, 5-week-old mice were infected intraperitoneally with 10^6 tachyzoites. After 72 h of infection, parasites were harvested from the peritoneal cavity by PBS washing and then collected by centrifugation at 1,000 × *g* for 10 min. Harvested parasites were counted in a hemocytometer for experimental infections or for the next passage in mice.

Bone Marrow-Derived Macrophages (BMDMs) and Peritoneal Macrophages

Bone marrow-derived macrophages were generated using L929 cell conditioned media (LCCM) as a source of macrophage colony-stimulating factor, as previously described (34, 35). RPMI containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate was used throughout the procedure. In brief, fresh bone marrow from C57BL/6, NLRP3$^{-/-}$, P2X7$^{-/-}$, Caspase-11$^{-/-}$, and Caspase-1/11$^{-/-}$ mice were obtained and 5×10^6 cells were plated in the petri dishes in RPMI with 20% FBS and 30% LCCM. On day 3 after plating, fresh medium was added to the cultures. On day 7 after plating, cells were harvested, washed with PBS, counted and plated in RPMI with 10% FBS and 5% LCCM, for cytokine detection assays (in 6-well plates, at 2×10^6 cells/well) or light microscopy analysis (in 24-well plates with 13 mm coverslips, at 2×10^5 cells/well). Cells were then incubated at 37°C for at least 18 h before the start of experiments. All experimental infections and treatments were performed in RPMI with 10% FBS (but without LCCM). Macrophage differentiation to >95% purity was confirmed by flow cytometry as described previously (35), before the start of each experiment.

Peritoneal macrophages were collected as previously described (33). Briefly, peritoneal exudates from C57BL/6 mice were obtained by washing the peritoneal cavity with 10 mL of sterile PBS. Peritoneal cells were counted by light microscopy, resuspended in DMEM medium, and 2×10^5 cells were plated in 24-well plates with 13-mm coverslips for light microscopy analyzes. Cells were left to adhere for 1 h, at 37°C (and 5% CO_2), and then non-adherent cells were removed by PBS washes before "supplemented DMEM" (DMEM with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES) was added to cultures.

Infection Assays

Cells were infected in supplemented DMEM medium, at 37°C (and 5% CO_2). BMDMs or peritoneal macrophages were infected at a ratio of 3:1 tachyzoites to host cells for 2 h at 37°C (and 5% CO_2), and non-internalized parasites were removed by PBS washes. Where required, cells were then incubated for 30 min in medium containing 3 mM eATP and/or 1 ng/mL IL-1β. In some experiments, cells were pretreated for 40 min with 10 mM NAC, 2 µM Z-YVAD, 1 µM Apocynin, 50 µM carbenoxolone, or 10 nM Mito-TEMPO, before ATP or IL-1β treatment. Then, cells were maintained at 37°C (and 5% CO_2). After 18 h post-infection, aliquots of culture supernatants were collected and kept at −20°C, for IL-1β quantification (see "ELISA") and cultures were processed for light microscopy, as described below.

Light Microscopy

Infected cells were fixed in 4% paraformaldehyde and stained with Panoptic ("Panotico Rapido kit," LaborClin, Brazil) following the manufacturer's instructions. A minimum of 300 cells/sample were evaluated by light microscopy, in a Zeiss microscope (manufacturer). As previously described (33), the percentage of infection and infection index, which represents the overall infection load, were determined using the formula:

$$\%\ \text{of infection} = \frac{\text{iC} \times 100}{\text{TotalC}}$$

$$\text{Infection Index} = \frac{\{\%\ \text{of infection} \times \text{IntP}\}}{\text{TotalC}} \div 100$$

where iC is number of infected cells; totalC is the total number of cells; and IntP is the number of intracellular parasites.

Cytokine Assay—ELISA

IL-1β levels were quantified in supernatant samples from infected BMDM cultures (see Infection Assays), using the Mouse IL-1 beta/IL-1F2 DuoSet ELISA #DY401 kit, according to the manufacturer's instructions (R&D Systems).

ROS Assay

To estimate ROS production, cells were plated (at a density of 2×10^5 cells/well) in opaque-black 96-well plates with a transparent and flat bottom. After 2 h of infection with *T. gondii,* cells were kept untreated or were subjected to one or both of the following treatments, in the presence of 2 µM of H_2DCFDA: 10 mM NAC for 30 min, followed by 3 mM ATP for up to 60 min. Fluorescence was measured at 40, 50, and 60 min of exposure to ATP, using a Spectra Max^3 spectrophotometer (Molecular Devices), at 37°C, at excitation and emission wavelengths of 490 and 520 nm, respectively.

Statistical Analyses

A two-tailed *t*-test was used for comparisons of two groups, while multiple comparisons were performed by one-way ANOVA followed by Tukey's post-test. All statistical analyses were performed using Graph Pad Prism 5 (La Jolla, CA, USA).

RESULTS

P2X7 Receptor Activation Promotes *T. gondii* Control in Macrophages, *via* Inflammasome Activation, Caspase-1 and ROS Production

In a previous study, our group demonstrated that P2X7 receptor activation *via* eATP reduces *T. gondii* infection burden in

peritoneal macrophages and J774.G8 cells (26). However, the mechanism of P2X7-induced *T. gondii* infection control in these cells remained unclear. On the other hand, eATP is capable of activating the inflammasome in infected cells (30) and promotes pathogen elimination in different models of infection (36). Therefore, we investigated whether *T. gondii* elimination in infected cells *via* eATP treatment was dependent on inflammasome activation.

To address this question, we infected BMDMs from wild-type C57BL/6 mice, as well as from NLRP3$^{-/-}$ and Caspase1/11$^{-/-}$ mice with *T. gondii*, and treated infected cells with eATP (3 mM), which activates the P2X7 receptor (26). Light microscopy-based quantification of the percentage of infected cells and the infection index (which combines the proportion of infected cells and the number of parasites per cell) showed that treatment with eATP decreased the parasite load in wild-type macrophages, while the parasite load remained high in cells from NLRP3$^{-/-}$ and Caspase1/11$^{-/-}$ mice (**Figure 1**). These data suggest that, in macrophages, the control of *T. gondii* infection mediated by eATP depends on the assembly of the NLRP3 inflammasome.

We demonstrated previously that P2X7 receptor activation reduces *T. gondii* load in macrophages, while also generating ROS (26). Given that, in macrophages, P2X7 receptor activation is associated with activation of the NLRP3 inflammasome (15), we sought to determine if ROS and caspase-1—the hallmark of the canonical NLRP3 inflammasome—are required for the reduction of *T. gondii* burden in BMDMs, *via* P2X7 receptor signaling.

To address this issue, we infected BMDMs from C57BL/6 (WT) mice with *T. gondii* tachyzoites followed by pretreatment with NAC or Z-YVAD (which inhibit ROS and caspase-1 activities, respectively) and then treated infected cells with eATP. We confirmed our previous observations that eATP reduced the percentage of infected cells (**Figure 2A**) and the infection index (**Figure 2B**), in wild-type macrophage cultures. This effect was abolished by treatment with either NAC or

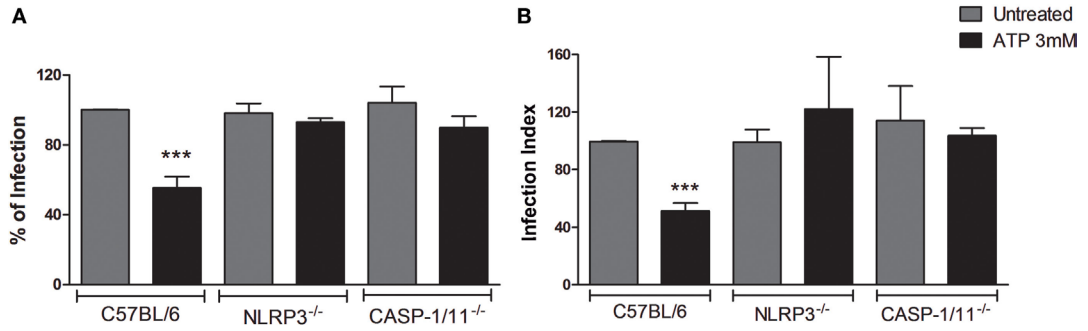

FIGURE 1 | Extracellular ATP (eATP)-induced *T. gondii* control is dependent on NLRP3 and caspase-1/11 molecules. Bone marrow-derived macrophages from C57BL/6 (WT), NLRP3$^{-/-}$ or Caspase-1/11$^{-/-}$ mice were infected with *T. gondii* tachyzoites for 2 h and kept untreated, or were treated with 3 mM eATP for 30 min. Then, cells were incubated at 37°C for a total of 18 h, and stained with Panoptic, for light microscopy quantification of the percentage of infection **(A)** and the infection index **(B)**. Treatment with eATP reduced the percentage of infected cells and the infection index in wild-type (C57BL/6) macrophages, but not in the knockout cells. Normalized data represent mean ± SEM of three independent experiments performed in triplicates. ***$p \leq 0.001$ vs. untreated (by two-tailed *t*-test).

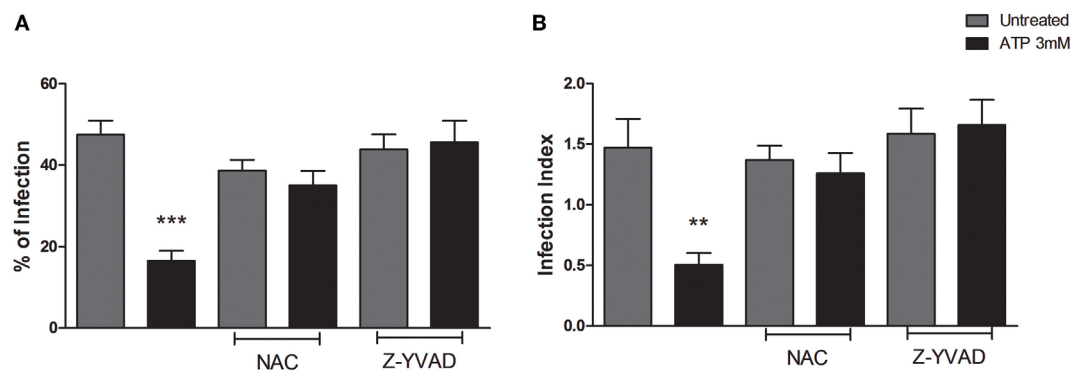

FIGURE 2 | The decrease in *T. gondii* infection upon extracellular ATP (eATP) treatment depends on reactive oxygen species (ROS) generation and caspase-1 activation. Wild-type (C57BL/6) bone marrow-derived macrophages were infected with *T. gondii* tachyzoites for 2 h and then left untreated, or subjected to one of the following treatments: 10 mM *N*-acetyl cysteine (NAC) or 2 μM Z-YVAD for 30 min followed by 3 mM eATP, for 30 min. After treatments, cells were incubated for a further 18 h at 37°C, and stained with Panoptic, for light microscopy quantification of the percentage of infection **(A)** and the infection index **(B)**. Treatment with NAD or Z-YVAD before exposure to eATP prevented the eATP-dependent reduction in *T. gondii* infection. Data represent mean ± SEM of three independent experiments performed in triplicates. **$p \leq 0.01$ and ***$p \leq 0.001$ vs. untreated (by one-way ANOVA followed by Tukey's test).

Z-YVAD (**Figure 2**). Thus, the reduction in *T. gondii* infection load *via* eATP and the P2X7 receptor depends on activation of the canonical NLRP3 inflammasome, and also on ROS production and caspase-1.

A Canonical, but Not a Non-Canonical, Inflammasome Mediates *T. gondii* Elimination *via* P2X7 Receptor Signaling

Given that P2X7 receptor activation reduces *T. gondii* infection load in a caspase1/11-dependent manner (**Figures 1** and **2**), we investigated whether canonical and/or non-canonical inflammasome were involved in parasite elimination. To differentiate between canonical and non-canonical inflammasome contributions, we quantified the parasite load after eATP treatment in BMDMs from C57BL/6 (wild-type), Caspase11$^{-/-}$, and Caspase1/11$^{-/-}$ mice. Treatment with eATP reduced the parasite load in wild-type and Caspase-11$^{-/-}$ macrophages, while no reduction in parasite load was observed after nucleotide treatment in Caspase1/11$^{-/-}$cells (**Figure 3**). Thus caspase-1, but not caspase-11, is required for eATP-mediated *T. gondii* infection control. Combined with the data shown in **Figures 1** and **2**, these results confirm that the activation of the canonical NLRP3 inflammasome is crucial for the control of *T. gondii* infection in macrophages *via* P2X7 receptor activation by eATP. These data also show that caspase-11 is dispensable for pathogen elimination *via* eATP, while caspase-1 is required for this effect, defining a role for the canonical inflammasome only (and not for the

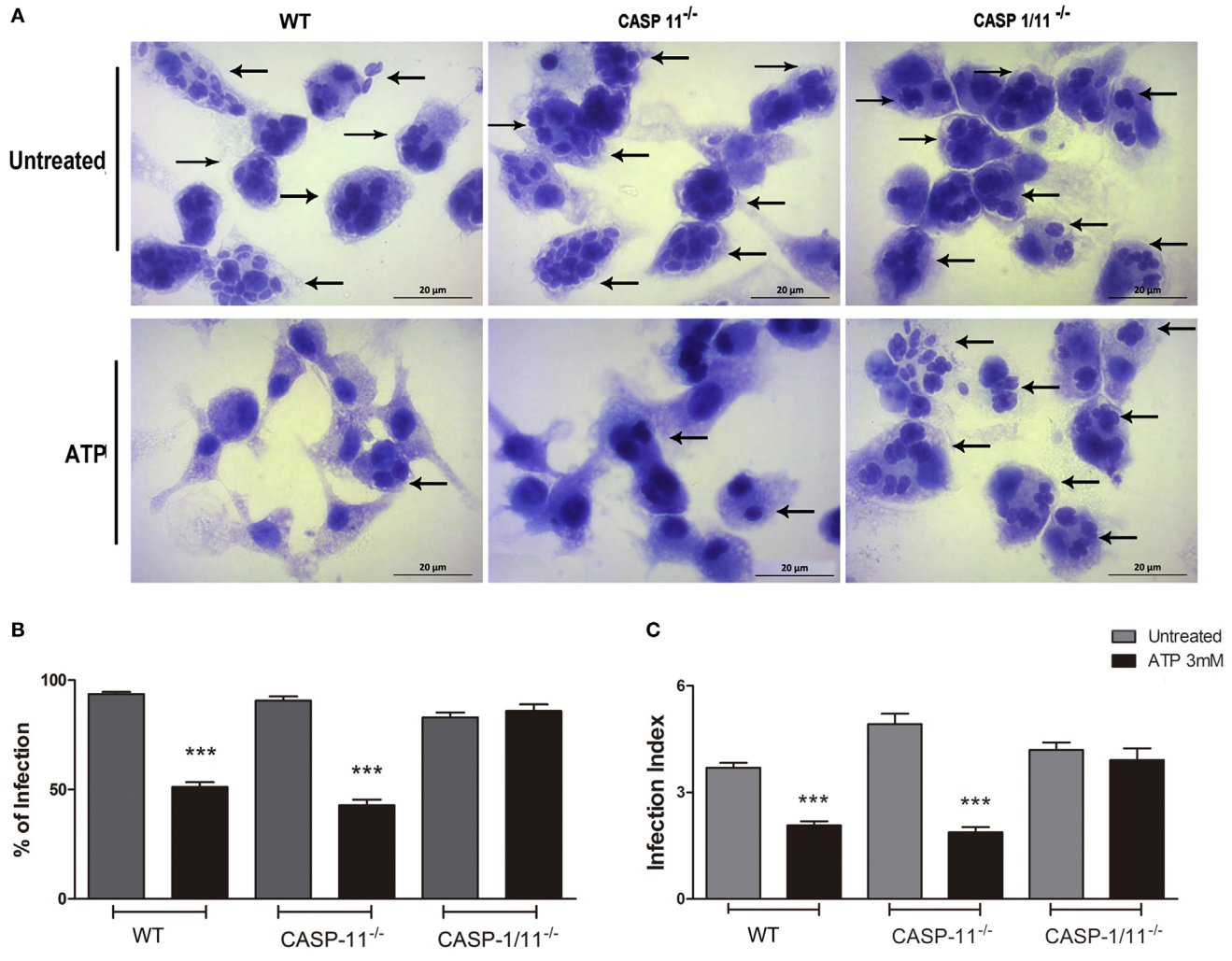

FIGURE 3 | P2X7 receptor activation triggers *T. gondii* elimination *via* a canonical (but not *via* a non-canonical) inflammasome. Bone marrow-derived macrophages (BMDMs) from C57BL/6 (wild-type), and from Caspase-11$^{-/-}$ and Caspase1/11$^{-/-}$knockout mice (lacking non-canonical and canonical inflammasome effectors, respectively) were infected with *T. gondii* tachyzoites for 2 h and then kept untreated, or were treated 3 mM extracellular ATP for 30 min. Then, cells were incubated at 37°C for a total of 18 h, and stained with Panoptic, for light microscopy observation [**(A)**; representative images of three independent experiments] and for the quantification of the % of infection **(B)** and the infection index **(C)**. We observed reduced parasite load in wild-type and Caspase-11$^{-/-}$ BMDMs, but not in Caspase-1/11$^{-/-}$ cells. Black arrows indicate parasitophorous vacuoles containing *T. gondii* tachyzoites. Micrographs are representative of three independent experiments. Data represent mean ± SEM of three independent experiments performed in triplicates. ***$p \leq 0.001$ vs. untreated (by two-tailed *t*-test).

non-canonical inflammasome) in *T. gondii* infection control mediated by purinergic signaling.

eATP Induces NLRP3 Inflammasome-Dependent IL-1β Secretion and ROS Generation in Infected Macrophages

IL-1β is an important pro-inflammatory cytokine in response to different pathogens (37), and its secretion is induced by inflammasome activation (14). Therefore, we investigated whether activation of the canonical inflammasome *via* eATP/P2X7 receptor resulted in the secretion of IL-1β by *T. gondii*-infected cells.

T. gondii infection itself induced discrete (and similar) amounts of IL-1β release by WT (C57BL/6), NLRP3$^{-/-}$and Caspase1/11$^{-/-}$ BMDMs (**Figure 4A**). When infected cells were treated with eATP, IL-1β secretion was potentiated in WT macrophages but did not increase in cells from NLRP3$^{-/-}$ or Caspase1/11$^{-/-}$ mice, showing that activation of the

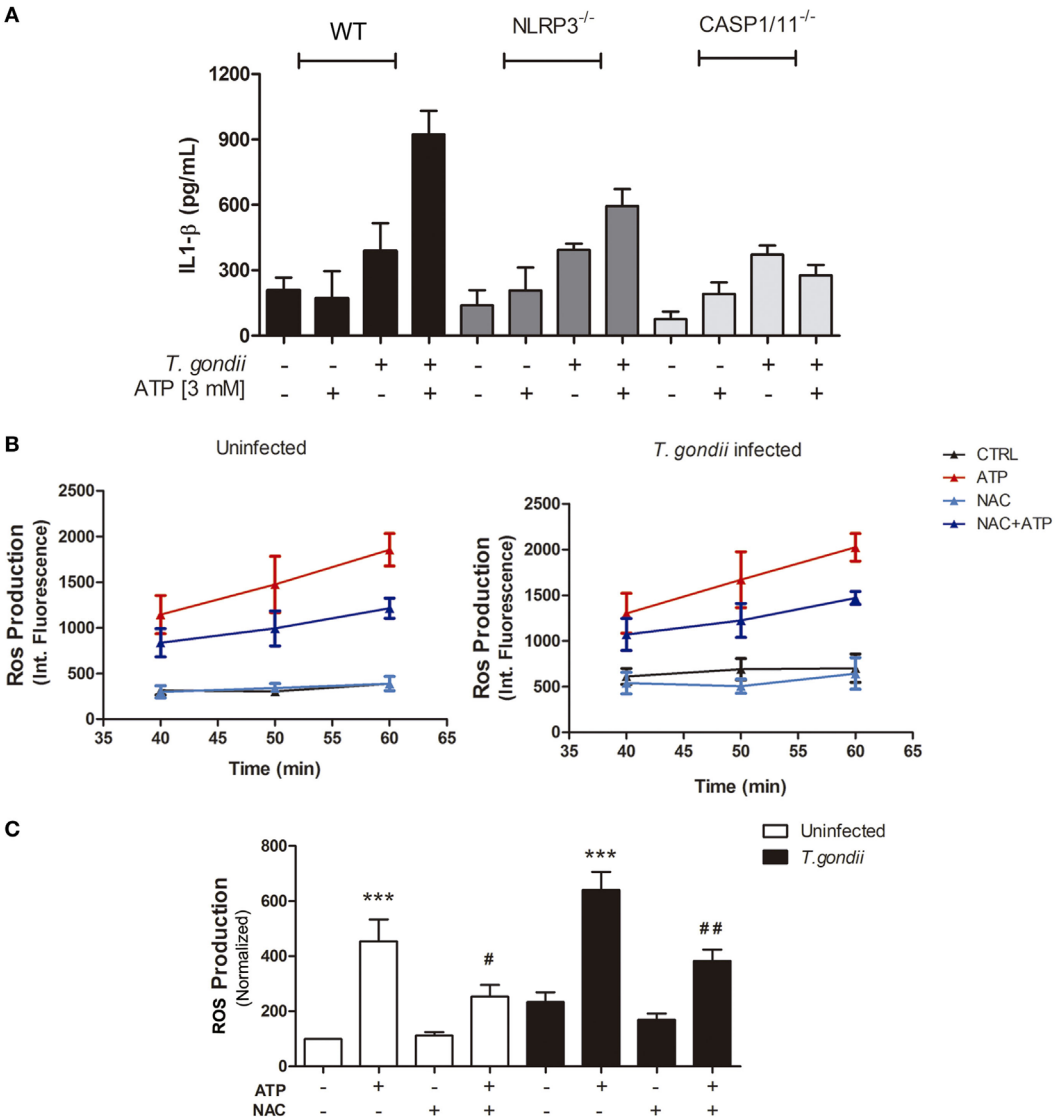

FIGURE 4 | Extracellular ATP (eATP) is important for IL-1β secretion *via* canonical NLRP3 inflammasome induction and reactive oxygen species (ROS) production, in *T. gondii*-infected macrophages. Bone marrow-derived macrophages from C57BL/6 (WT), NLRP3$^{-/-}$ or Caspase-1/11$^{-/-}$ mice, either uninfected or infected with *T. gondii* tachyzoites, were kept untreated or were treated with ATP, and then culture supernatant samples were used to estimate IL-1β secretion **(A)**. Treatment with 3 mM eATP (for 30 min) was performed 2 h post-infection, and all cells were incubated for 24 h prior to IL-1β quantification. Data represent mean ± SEM from triplicates. For ROS detection, before eATP treatment, some cultures were incubated with 10 mM *N*-acetyl cysteine (NAC) and ROS production was detected. **(B,C)** ROS levels in cells treated for 30 min, with NAC and then with 3 mM eATP (for up to 60 min), 2 h post-infection. ROS levels were estimated by H_2DCFDA fluorescence, at different time-points during treatment with eATP **(B)**, with normalized data from the 50-min time-point in **(C)**. Data represent mean ± SEM of three independent experiments performed in triplicates. $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ vs. corresponding samples treated with eATP only; $^{***}p \leq 0.001$ vs. the corresponding untreated control (by one-way ANOVA followed by Tukey's test).

canonical NLRP3 inflammasome by eATP (*via* the P2X7 receptor) triggers IL-1β secretion in *T. gondii*-infected macrophages.

ROS production is one of the most potent microbicidal mechanisms against intracellular pathogens, and *T. gondii* efficiently block ROS production in order to survive and evade host immune mechanisms (38). In a previous study, we showed that treatment with eATP induces ROS production in murine macrophages (26). To test if *T. gondii* infection subverts eATP-induced ROS generation, we quantified ROS levels in *T. gondii*-infected peritoneal macrophages at different time-points (40, 50, and 60 min), after eATP. Exposure to eATP increased ROS levels, from 40 to 60 min of treatment (**Figure 4B**), even in *T. gondii*-infected cells (**Figure 4B**). The ROS level increase triggered by eATP after 40 min of treatment was reduced significantly by exposure to the ROS production inhibitor NAC (**Figure 4C**). Together, these results show that eATP induced the production of key molecules for the control of intracellular parasite infection—ROS and IL-1β—even in the presence of *T. gondii* and its evasion mechanisms.

T. gondii Infection Control *via* eATP and IL-1β Depends on ROS Production and Pannexin-1

Thus far, we showed that eATP treatment inhibits *T. gondii* infection in macrophages by a mechanism dependent on the canonical NLRP3 inflammasome and on ROS production, and that NLRP3 inflammasome activation in cells treated with eATP leads to the secretion of IL-1β. To examine in more detail the relationships between P2X7 receptor activation (by eATP), IL-1β production and ROS generation, during *T. gondii* infection control by macrophages, we treated infected peritoneal macrophages with eATP or recombinant IL-1β, alone or in combination with the antioxidant NAC. As expected, treatment with eATP or recombinant IL-1β reduced *T. gondii* infection in wild-type macrophages (**Figure 5A**). Interestingly, the antioxidant NAC completely inhibited the control of *T. gondii* infection by eATP and IL-1β in peritoneal macrophages (**Figures 5B,C**). These results show that ROS production induced downstream of P2X7 receptor activation or IL-1β secretion is essential for the control of *T. gondii* infection mediated by purinergic signaling.

Importantly, treatment with recombinant IL-1β reduced *T. gondii* infection load even in macrophages lacking the P2X7 receptor (from P2X7$^{-/-}$mice) (**Figures 5D,E**). These data indicate that IL-1β is downstream of P2X7 receptor activation in the signaling network that results in *T. gondii* infection control by macrophages. In addition, these experiments confirmed that the effects of eATP treatment described here depends on the P2X7 receptor, since eATP treatment did not reduce the percentage of infected cells (**Figure 5D**) or the infection index (**Figure 5E**) in P2X7$^{-/-}$ macrophages.

In order to reinforce the role of IL-1β promoting parasite killing, we assessed if the blockade of pannexin-1 channel, which is required for IL-1β secretion in macrophages (39) can inhibit eATP-induced killing of *T. gondii*. As shown in **Figures 5F,G**, carbenoxolone pretreatment abolished the eATP-induced control of the parasite load. These data confirm that eATP-induced IL-1β secretion from macrophages is crucial to *T. gondii* elimination.

T. gondii Elimination *via* eATP and IL-1β Depends on Distinct ROS Generation Sources

The precise role and origin of the ROS pool that acts as an antiparasitic effector during P2X7 receptor activation remain poorly defined. Considering that our data support the notion that P2X7 receptor activation induces ROS generation *via* eATP treatment, we decided to examine which source of ROS pool is required for *T. gondii* infection control triggered by eATP, and whether this phenomenon is mediated by the canonical inflammasome effector IL-1β. To examine this question, we pretreated infected peritoneal macrophages with Mito-TEMPO, an inhibitor of mitochondrial ROS production, and then treated infected cells with eATP or recombinant IL-1β. Treatment with Mito-TEMPO effectively inhibited the control of *T. gondii* infection by IL-1β, while eATP treatment still reduced the parasite load even in the presence of Mito-TEMPO (**Figures 6A,B**). Then, we pretreated infected peritoneal macrophages with apocynin, an inhibitor of NADPH oxidase ROS production, and treated infected cells with eATP. Treatment with Apocynin completely blocked the eATP-induced reduction of parasite load (**Figures 6C,D**). Therefore, while both eATP and IL-1β reduce *T. gondii* infection load *via* ROS generation (**Figure 5**), the mitochondrial ROS pool is important only for the effector activity of IL-1β on *T. gondii* infection control. IL-1β-independent pathways of *T. gondii* elimination triggered by eATP are likely to depend on ROS production *via* NADPH oxidase.

DISCUSSION

Toxoplasmosis is a major parasitic disease transmitted by food, with a widespread distribution and a global human infection rate of ~30% (1). As toxoplasmosis is an inflammatory disease, the killing of *T. gondii* requires both innate and adaptive immune responses (40). Several lines of evidence—from gene polymorphism data to mouse knockout analysis—support the notion that P2X7 receptor activity contributes to control toxoplasmosis infection *in vivo*, by triggering antimicrobial activities in the intracellular environment (such as ROS production and lysosome fusion to the parasitophorous vacuole), and by stimulating pro-inflammatory events, such as the production of IL-12, IL-1β, and IFN-γ (27, 32). However, the downstream pathways that lead to parasite elimination *via* P2X7 receptor activation had not been dissected previously. Here, we described the cellular pathways that contribute to *T. gondii* killing induced by P2X7 receptor activation, showing that the control of *T. gondii* infection triggered by purinergic signaling requires the activity of a canonical NLRP3 inflammasome, and involves the inflammasome-dependent production of ROS and IL-1β (**Figure 7**).

IL-1β secretion, ROS production, and cell death are the most common cellular events linked to P2X7 receptor activation, and also seem to depend on inflammasome activation (20, 29, 35). ATP, a P2X7 receptor ligand, acts as a second signal to activate the NLRP3 inflammasome (41). Both NLRP1 and NLRP3

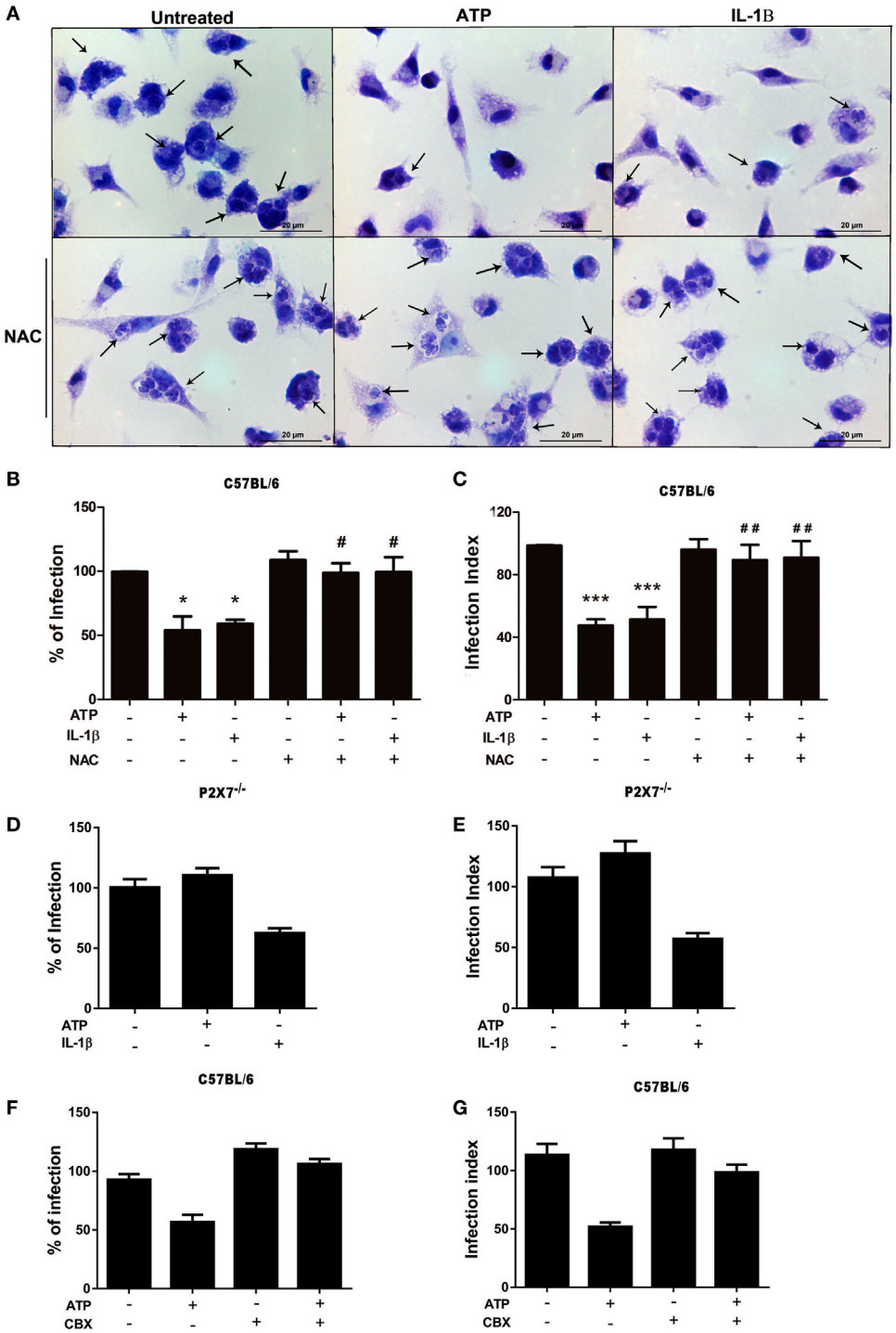

FIGURE 5 | The control of *T. gondii* infection induced by extracellular ATP (eATP) and IL-1β requires the generation of reactive oxygen species and pannexin-1. Peritoneal macrophages from C57BL/6 (WT) and P2X7$^{-/-}$ mice were infected with *T. gondii* tachyzoites for 2 h, and then left untreated or subjected to one or both of the following treatments as indicated: 10 mM *N*-acetyl cysteine (NAC) or 50 µM carbenoxolone for 40 min, followed by 3 mM eATP or 1 ng/mL IL-1β for 30 min. Then, cells were incubated at 37°C for a further 18 h, and stained with Panoptic for light microscopy analysis **(A)** representative images of three independent experiments, which was used to quantify the percentage of infection **(B,D,F)** and the infection index **(C,E,G)** in C57BL/6 **(B,C,F,G)** and P2X7$^{-/-}$ **(D,E)** cells. In **(A)**, black arrows indicate parasitophorous vacuoles containing *T. gondii* tachyzoites in C57BL/6. Normalized data represent mean ± SEM of three independent experiments [in **(B–E)**] and 2 independent experiments [in **(F,G)**] performed in triplicates. **(F,G)** are representative experiments. Treatment with eATP or IL-1β reduced the parasite load in C57BL/6 cells, and this effect was abolished by NAC pretreatment **(B,C)**. Treatment with eATP had no effect in the parasite load in P2X7$^{-/-}$ cells **(D,E)** or C57BL/6 cells pretreated with carbenoxolone **(F,G)**. $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ vs. sample treated with ATP only; $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$ vs. untreated (by one-way ANOVA followed by Tukey's test).

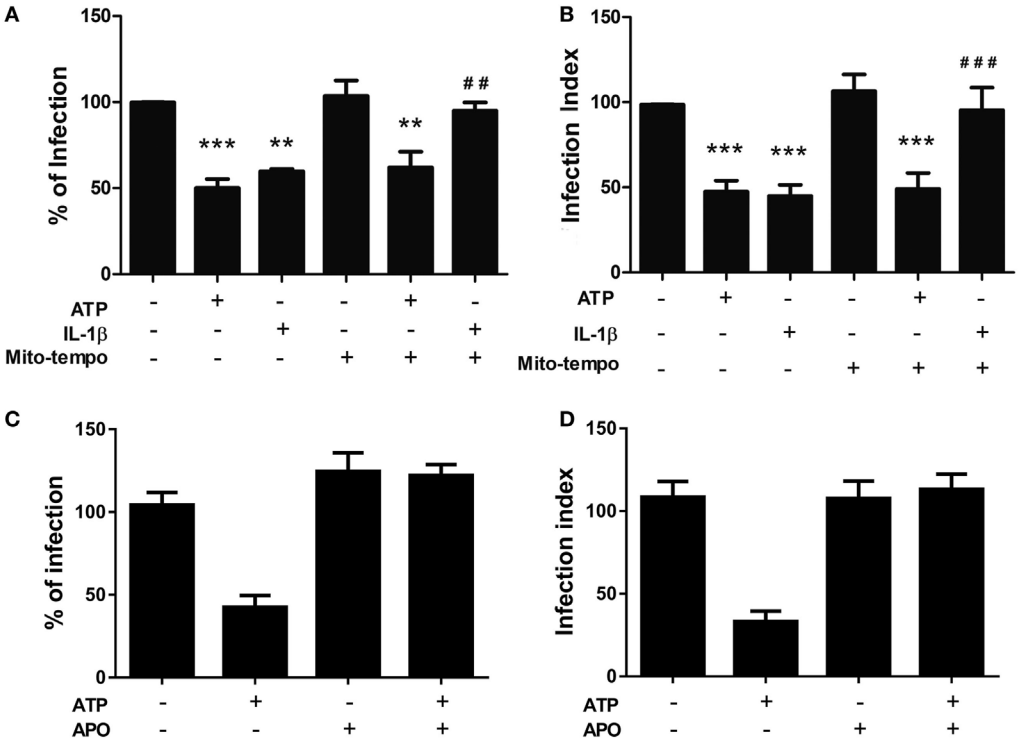

FIGURE 6 | The control of *T. gondii* infection induced by extracellular ATP (eATP) and IL-1β depends on different sources of reactive oxygen species generation. Peritoneal macrophages from C57BL/6 mice were infected with *T. gondii* tachyzoites for 2 h and left untreated or subjected to one or more of the following treatments: 100 nM Mito-TEMPO or 1 μM apocynin for 40 min, followed by 3 mM eATP or 1 ng/mL IL-1β for 30 min. Then, cells were incubated at 37°C for a further 18 h, and the percentage of infected cells **(A,C)**, and number of parasites per host cell **(B,D)** were estimated by light microscopy examination, after staining with Panoptic. Normalized data represent mean ± SEM of three independent experiments performed in triplicates in **(A,B)** and two independent experiments in **(C,D)**. $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$ vs. sample treated with ATP only; $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ vs. untreated (by one-way ANOVA followed by Tukey's test).

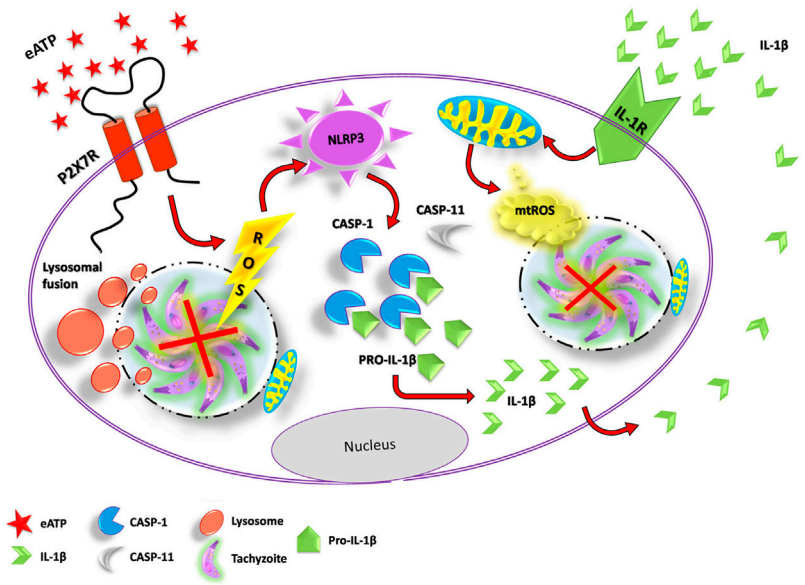

FIGURE 7 | Proposed mechanism of *T. gondi* infection control mediated by extracellular ATP (eATP)/P2X7 receptor and IL-1β, in macrophages. Binding of eATP to the P2X7 receptor may lead to pathogen elimination *via* different mechanisms: the fusion of lysosomes to the parasitophorous vacuole (forming a phagolysosome), reactive oxygen species (ROS) generation and the activation of the canonical NLRP3 inflammasome. NLRP3 inflammasome activation by eATP potentiates IL-1β release through caspase-1 activity and, in turn, IL-1β in the extracellular compartment binds to its receptor, triggering *T. gondii* elimination *via* mitochondrial ROS generation.

inflammasomes are involved in the immune responses against *T. gondii* infection, and mice lacking the inflammasome effectors caspase-1 and caspase-11 have 95% mortality after *T. gondii* infection (42). In agreement with these data, we show here that the control of *T. gondii* infection by P2X7 activation in macrophages depends on the NLRP3 inflammasome, and that the inflammasome effector caspase-1 is essential for *T. gondii* infection control by the P2X7 receptor, because lack of inflammasome components (NLRP3 and caspase-1) or inhibition of caspase-1 activity by Z-YVAD abolished the reduction in parasite load after treatment with ATP. In addition, the release of IL-1β in NLRP3 deficient mice can be explained by activation of NLRP1 inflammasome in macrophages of those mice (42). By examining separately the contributions of caspase-1 and caspase-11 to *T. gondii* infection control (using mouse knockout cells) we also show that the reduction in infection load caused by ATP treatment requires only caspase-1, and not caspase-11, supporting the hypothesis that a canonical NLRP3 is activated by the P2X7 receptor, to reduce *T. gondii* infection in macrophages.

Pyroptosis was shown to occur in response to P2X7 activation in macrophages (43), and this cell death mechanism can contribute to the clearance of intracellular pathogens (44). We did not detect significant cell death after 30 min or 18 h of eATP treatment (data not shown). In addition, our data suggest that, as have been shown for several pathogenic bacteria (45), the infection with *T gondii* may block pyroptosis. Thus, it is possible to conceive that different intracellular pathogens, not only bacteria, may subvert the immune response by operating in the inhibition of pyroptosis.

Even before it interacts with host cells, *T. gondii* secretes molecules that downregulate PRRs and, consequently, reduce the production of pro-inflammatory mediators (46). PRR activation acts as a first signal for inflammasome activation, inducing the transcription of genes coding for pro-inflammatory molecules. When PRR activation is followed by a second signal, it culminates in the cleavage and secretion of IL-1β (47). Our data show that upon eATP treatment and activation of the P2X7 receptor, *T. gondii* is not capable of downregulating the inflammasome-induced IL-1β secretion efficiently, since we observed that eATP treatment promotes IL-1β secretion in macrophages from WT mice. In this context, it is already described that P2X7 receptor activation leads to pannexin-1 pore formation and it is important for IL-1β release from macrophages (39). Our data show that inhibiting pannexin-1 channels, eATP-treated macrophages lack the ability to control *T. gondii* infection, which suggests that eATP-induced IL-1β secretion is crucial for the parasite killing. The results shown here reinforce the notion that IL-1β secretion potentiates the microbicidal effect of P2X7 activation.

Reactive oxygen species production is another key mechanism of intracellular pathogen elimination (48). *T. gondii* posses a specialized arsenal of antioxidant molecules and can successfully neutralize oxidative stress in the host (38). However, our result show that eATP treatment induced ROS production in murine macrophages even during *T. gondii* infection. Importantly, treatment with the inflammasome final product IL-1β reduced *T. gondii* infection load in macrophages, and ROS production was required for *T. gondii* infection control by eATP and IL-1β, because the pan ROS inhibitor NAC prevented the parasite load reduction caused by ATP and IL-1β. Our data show that, upon eATP treatment, ROS production is maintained during *T. gondii* infection, suggesting that P2X7 receptor activation overcomes sophisticated antioxidant mechanisms triggered by the pathogen. In phagocytes, NADPH oxidase activity and mitochondria are the two most common sources of ROS involved in immune response events (48), and the mitochondrial-derived ROS also activates the NLRP3 inflammasome (49). Here, we found that ROS derived from mitochondria participates in the control of *T. gondii* infection downstream of IL-1β signaling only, while IL-1β-independent signaling triggered by eATP is capable of controls *T. gondii* infection even upon inhibition of mitochondrial ROS production (by Mito-TEMPO). Therefore, we consider that NADPH oxidase-derived ROS are involved in the control of *T. gondii* infection in macrophages, upon P2X7 signaling activation.

CONCLUSION

Our data demonstrate that eATP treatment reduces *T. gondii* infection load in macrophages through activation of the NLRP3 inflammasome, and that eATP promotes IL-1β secretion, and leads to ROS production, in infected cells. By using knockout mice for different inflammasome molecules, such as NLRP3 and caspase1 and 11, we also identified that a canonical (but not a non-canonical) inflammasome is involved in the eATP-induced IL-1β secretion and elimination of the intracellular parasites, in macrophages.

We conclude that P2X7 receptor activation inhibits *T. gondii* growth *via* ROS generated, most likely, from NADPH oxidase (rather than mitochondrial ROS), while also activating the canonical NLRP3 inflammasome, which leads to IL-1β secretion and infection control *via* mitochondrial ROS production.

ETHICS STATEMENT

The procedures for the care and use of animals were according to the guidelines of the Brazilian College of Animal Experimentation (COBEA). All efforts were made to minimize animal suffering and to reduce the number of animals used in this study. This study was approved and followed all the guidelines established by the Ethics Committee on the Use of Animals (CEUA) of the Biophysic Institute Carlos Chagas Filho (IBCCF, UFRJ, no. 082/15).

AUTHOR CONTRIBUTIONS

ACAMS and CLCAS designed and performed the experiments, analyzed the results, and wrote the manuscript. TPR and GCR performed experiments and analyzed the results. MB, DZ, and RCV analyzed the results and revised the manuscript. RCS designed the experiments, analyzed the results, and revised the manuscript.

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REFERENCES

1. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet* (2004) 363:1965–76. doi:10.1016/S0140-6736(04)16412-X
2. Wang ZD, Liu HH, Ma ZX, Ma HY, Li ZY, Yang ZB, et al. *Toxoplasma gondii* infection in immunocompromised patients: a systematic review and meta-analysis. *Front Microbiol* (2017) 8:389. doi:10.3389/fmicb.2017.00389
3. Hill D, Dubey JP. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin Microbiol Infect* (2002) 8:634–40. doi:10.1046/j.1469-0691.2002.00485.x
4. Barragan A, Sibley LD. Migration of *Toxoplasma gondii* across biological barriers. *Trends Microbiol* (2003) 11:426–30. doi:10.1016/S0966-842X(03)00205-1
5. Carme B, Bissuel F, Ajzenberg D, Bouyne R, Aznar C, Demar M, et al. Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana. *J Clin Microbiol* (2002) 40:4037–44. doi:10.1128/JCM.40.11.4037-4044.2002
6. Khan A, Jordan C, Muccioli C, Vallochi AL, Rizzo LV, Belfort R Jr, et al. Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. *Emerg Infect Dis* (2006) 12:942–9. doi:10.3201/eid1206.060025
7. Lima GS, Saraiva PG, Saraiva FP. Current therapy of acquired ocular toxoplasmosis: a review. *J Ocul Pharmacol Ther* (2015) 31:511–7. doi:10.1089/jop.2015.0059
8. Dannemann B, McCutchan JA, Israelski D, Antoniskis D, Leport C, Luft B, et al. Treatment of toxoplasmic encephalitis in patients with AIDS. A randomized trial comparing pyrimethamine plus clindamycin to pyrimethamine plus sulfadiazine. The California Collaborative Treatment Group. *Ann Intern Med* (1992) 116:33–43. doi:10.7326/0003-4819-116-1-33
9. Hitziger N, Dellacasa I, Albiger B, Barragan A. Dissemination of *Toxoplasma gondii* to immunoprivileged organs and role of Toll/interleukin-1 receptor signalling for host resistance assessed by in vivo bioluminescence imaging. *Cell Microbiol* (2005) 7:837–48. doi:10.1111/j.1462-5822.2005.00517.x
10. Bierly AL, Shufesky WJ, Sukhumavasi W, Morelli AE, Denkers EY. Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during *Toxoplasma gondii* infection. *J Immunol* (2008) 181:8485–91. doi:10.4049/jimmunol.181.12.8485
11. Denkers EY, Kim L, Butcher BA. In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during *Toxoplasma gondii* infection. *Cell Microbiol* (2003) 5:75–83. doi:10.1046/j.1462-5822.2003.00258.x
12. Silva NM, Manzan RM, Carneiro WP, Milanezi CM, Silva JS, Ferro EA, et al. *Toxoplasma gondii*: the severity of toxoplasmic encephalitis in C57BL/6 mice is associated with increased ALCAM and VCAM-1 expression in the central nervous system and higher blood-brain barrier permeability. *Exp Parasitol* (2010) 126:167–77. doi:10.1016/j.exppara.2010.04.019
13. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci* (2016) 41:1012–21. doi:10.1016/j.tibs.2016.09.002
14. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol* (2016) 16:407–20. doi:10.1038/nri.2016.58
15. Prochnicki T, Mangan MS, Latz E. Recent insights into the molecular mechanisms of the NLRP3 inflammasome activation. *F1000Res* (2016) 5:1–15. doi:10.12688/f1000research.8614.1
16. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. *Nature* (2011) 479:117–21. doi:10.1038/nature10558
17. Roberts JS, Yilmaz Ö. Dangerous liaisons: caspase-11 and reactive oxygen species crosstalk in pathogen elimination. *Int J Mol Sci* (2015) 16:23337–54. doi:10.3390/ijms161023337
18. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. *Cell* (2014) 157:1013–22. doi:10.1016/j.cell.2014.04.007
19. Chaves MM, Marques-da-Silva C, Monteiro AP, Canetti C, Coutinho-Silva R. Leukotriene B4 modulates P2X7 receptor-mediated *Leishmania amazonensis* elimination in murine macrophages. *J Immunol* (2014) 192:4765–73. doi:10.4049/jimmunol.1301058
20. Almeida-da-Silva CL, Morandini AC, Ulrich H, Ojcius DM, Coutinho-Silva R. Purinergic signaling during *Porphyromonas gingivalis* infection. *Biomed J* (2016) 39:251–60. doi:10.1016/j.bj.2016.08.003
21. Amaral EP, Ribeiro SC, Lanes VR, Almeida FM, de Andrade MR, Bomfim CC, et al. Pulmonary infection with hypervirulent mycobacteria reveals a crucial role for the P2X7 receptor in aggressive forms of tuberculosis. *PLoS Pathog* (2014) 10:e1004188. doi:10.1371/journal.ppat.1004188
22. Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, Kumararatne DS. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity* (1997) 7:433–44. doi:10.1016/S1074-7613(00)80364-7
23. Kusner DJ, Adams J. ATP-induced killing of virulent *Mycobacterium tuberculosis* within human macrophages requires phospholipase D. *J Immunol* (2000) 164:379–88. doi:10.4049/jimmunol.164.1.379
24. Kusner DJ, Barton JA. ATP stimulates human macrophages to kill intracellular virulent *Mycobacterium tuberculosis* via calcium-dependent phagosome-lysosome fusion. *J Immunol* (2001) 167:3308–15. doi:10.4049/jimmunol.167.6.3308
25. Lees MP, Fuller SJ, McLeod R, Boulter NR, Miller CM, Zakrzewski AM, et al. P2X7 receptor-mediated killing of an intracellular parasite, *Toxoplasma gondii*, by human and murine macrophages. *J Immunol* (2010) 184:7040–6. doi:10.4049/jimmunol.1000012
26. Correa G, Marques da SC, de Abreu Moreira-Souza AC, Vommaro RC, Coutinho-Silva R. Activation of the P2X(7) receptor triggers the elimination of *Toxoplasma gondii* tachyzoites from infected macrophages. *Microbes Infect* (2010) 12:497–504. doi:10.1016/j.micinf.2010.03.004
27. Miller CM, Zakrzewski AM, Robinson DP, Fuller SJ, Walker RA, Ikin RJ, et al. Lack of a functioning P2X7 receptor leads to increased susceptibility to toxoplasmic ileitis. *PLoS One* (2015) 10:e0129048. doi:10.1371/journal.pone.0129048
28. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* (2006) 440:228–32. doi:10.1038/nature04515
29. Coutinho-Silva R, Monteiro da CC, Persechini PM, Ojcius DM. The role of P2 receptors in controlling infections by intracellular pathogens. *Purinergic Signal* (2007) 3:83–90. doi:10.1007/s11302-006-9039-6
30. Coutinho-Silva R, Kanellopoulos JM, Perfettini JL. Cellular alarms and whispers contribute to the polyphonic melody of danger signals required for immunity. *Microbes Infect* (2012) 14:1239–40. doi:10.1016/j.micinf.2012.10.002
31. Jamieson SE, Peixoto-Rangel AL, Hargrave AC, Roubaix LA, Mui EJ, Boulter NR, et al. Evidence for associations between the purinergic receptor P2X(7) (P2RX7) and toxoplasmosis. *Genes Immun* (2010) 11:374–83. doi:10.1038/gene.2010.31
32. Corrêa G, Almeida Lindenberg C, Moreira-Souza AC, Savio LE, Takiya CM, Marques-da-Silva C, et al. Inflammatory early events associated to the role of P2X7 receptor in acute murine toxoplasmosis. *Immunobiology* (2017) 222:676–83. doi:10.1016/j.imbio.2016.12.007
33. Moreira-Souza AC, Marinho Y, Correa G, Santoro GF, Coutinho CM, Vommaro RC, et al. Pyrimidinergic receptor activation controls *Toxoplasma gondii* infection in macrophages. *PLoS One* (2015) 10:e0133502. doi:10.1371/journal.pone.0133502

34. Marim FM, Silveira TN, Lima DS Jr, Zamboni DS. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PLoS One* (2010) 5:e15263. doi:10.1371/journal.pone.0015263
35. Morandini AC, Savio LE, Coutinho-Silva R. The role of P2X7 receptor in infectious inflammatory diseases and the influence of ectonucleotidases. *Biomed J* (2014) 37:169–77. doi:10.4103/2319-4170.127803
36. Lamkanfi M, Dixit VM. Modulation of inflammasome pathways by bacterial and viral pathogens. *J Immunol* (2011) 187:597–602. doi:10.4049/jimmunol.1100229
37. Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol* (2010) 10:89–102. doi:10.1038/nri2691
38. Ding M, Kwok LY, Schluter D, Clayton C, Soldati D. The antioxidant systems in *Toxoplasma gondii* and the role of cytosolic catalase in defence against oxidative injury. *Mol Microbiol* (2004) 51:47–61. doi:10.1046/j.1365-2958.2003.03823.x
39. Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J* (2006) 25:5071–82. doi:10.1038/sj.emboj.7601378
40. Dupont CD, Christian DA, Hunter CA. Immune response and immunopathology during toxoplasmosis. *Semin Immunopathol* (2012) 34:793–813. doi:10.1007/s00281-012-0339-3
41. Bording-Jorgensen M, Alipour M, Wine E, Danesh G. Inflammasome activation by ATP enhances *Citrobacter rodentium* clearance through ROS generation. *Cell Physiol Biochem* (2017) 41:193–204. doi:10.1159/000455988
42. Gorfu G, Cirelli KM, Melo MB, Mayer-Barber K, Crown D, Koller BH, et al. Dual role for inflammasome sensors NLRP1 and NLRP3 in murine resistance to *Toxoplasma gondii*. *MBio* (2014) 5:1–12. doi:10.1128/mBio.01117-13
43. Le Feuvre RA, Brough D, Iwakura Y, Takeda K, Rothwell NJ. Priming of macrophages with lipopolysaccharide potentiates P2X7-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J Biol Chem* (2002) 277:3210–8. doi:10.1074/jbc.M104388200
44. Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. *Immunol Rev* (2015) 265:130–42. doi:10.1111/imr.12287
45. Cunha LD, Zamboni DS. Subversion of inflammasome activation and pyroptosis by pathogenic bacteria. *Front Cell Infect Microbiol* (2013) 3:76. doi:10.3389/fcimb.2013.00076
46. Morampudi V, Braun MY, D'Souza S. Modulation of early beta-defensin-2 production as a mechanism developed by type I *Toxoplasma gondii* to evade human intestinal immunity. *Infect Immun* (2011) 79:2043–50. doi:10.1128/IAI.01086-10
47. Kesavardhana S, Kanneganti TD. Mechanisms governing inflammasome activation, assembly and pyroptosis induction. *Int Immunol* (2017) 29(5):201–10. doi:10.1093/intimm/dxx018.:10
48. Dupre-Crochet S, Erard M, Nubetae O. ROS production in phagocytes: why, when, and where? *J Leukoc Biol* (2013) 94:657–70. doi:10.1189/jlb.1012544
49. Heid ME, Keyel PA, Kamga C, Shiva S, Watkins SC, Salter RD. Mitochondrial reactive oxygen species induces NLRP3-dependent lysosomal damage and inflammasome activation. *J Immunol* (2013) 191:5230–8. doi:10.4049/jimmunol.1301490

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Potential Sabotage of Host Cell Physiology by Apicomplexan Parasites for Their Survival Benefits

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Plasmodium, *Toxoplasma*, *Cryptosporidium*, *Babesia*, and *Theileria* are the major apicomplexan parasites affecting humans or animals worldwide. These pathogens represent an excellent example of host manipulators who can overturn host signaling pathways for their survival. They infect different types of host cells and take charge of the host machinery to gain nutrients and prevent itself from host attack. The mechanisms by which these pathogens modulate the host signaling pathways are well studied for *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, and *Theileria*, except for limited studies on *Babesia*. *Theileria* is a unique pathogen taking into account the way it modulates host cell transformation, resulting in its clonal expansion. These parasites majorly modulate similar host signaling pathways, however, the disease outcome and effect is different among them. In this review, we discuss the approaches of these apicomplexan to manipulate the host–parasite clearance pathways during infection, invasion, survival, and egress.

Keywords: ***Plasmodium*, *Toxoplasma*, *Theileria*, *Babesia*, *Cryptosporidium*, host signaling pathways**

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INTRODUCTION

The Apicomplexan parasites represent a major class of pathogens with a wide host range. They have emerged as one of the most successful intracellular parasites, which efficiently modulate the host for their survival benefits. In this review, we focus on the potential sabotage mechanisms adopted by the five well-studied pathogens of human and veterinary importance: *Plasmodium falciparum* (malaria), *Babesia bovis* (babesiosis), *Theileria annulata* (theileriosis), *Toxoplasma gondii* (toxoplasmosis), and *Cryptosporidium parvum* (cryptosporidiosis). These parasites are morphologically similar; however, variations exist in the context of host range, mode of infection, invasion, and replication inside the host (**Table 1**).

Beginning with transmission, *P. falciparum, T. annulata*, and *B. bovis* are vector borne; however, *C. parvum* and *T. gondii* do not require a vector and the host is infected by oocyst-ingestion (**Table 1**). *T. annulata* solely infects animals impacting their health and causing huge economic loss, whereas other parasites have broader host preference range. *P. falciparum and T. gondii* infections affect human health and cause mortality worldwide. On the other hand, *B. bovis* and *C. parvum* are comparatively less pathogenic with fewer reported cases of mortality and morbidity.

In this review, we epitomize the major blueprint of the pathways targeted by these parasites to sabotage the host defense mechanism for their survival and consequent disease progression.

TABLE 1 | A generalized comparative account among *Theileria, Plasmodium, Babesia, Toxoplasma*, and *Cryptosporidium* parasites.

	Theileria	***Plasmodium***	***Babesia***	***Toxoplasma***	***Cryptosporidium***
Cells infected	Lymphocytes and RBC	Hepatocytes and RBC	Lymphocytes and RBC	Intestinal cells	Enterocytes
Dense granules	Yes	Yes	Spherical bodies	Yes	Yes
Parasitophorous Vacuole (PV)	No	Yes	No	Yes	Yes
Apicoplast	Yes	Yes	Yes	Yes	No
Host	Animals	Human Animals	Animals Humans	Animals Humans	Animals Humans
Invasion process	Zippering	Gliding	Gliding	Gliding	Gliding
Conoid structure	No	No	No	Yes	Yes
Vector	Tick	Mosquito	Tick	No	No
Major species	*Theileria annulata, T. parva, T. equi, T. orientalis*	*Plasmodium falciparum, P. vivax, P. ovale, Plasmodium berghei, P. knowlesi, P. malariae*	*B. bigemina, Babesia bovis, B. major, B. divergens, B. microti*	*Toxoplasma gondii*	*Cryptosporidium parvum, C. hominis, C. canis, C. felis, C. meleagridis, C. muris*
Pathogenic stage	Schizont	Schizont	Sporozoite	Tachyzoites	Trophozoite
Zoonotic	No	No except *P. knowlesi*	Yes	Yes	Yes

Plasmodium: THE MALARIA PARASITE

Plasmodium falciparum is considered the most lethal among the *Plasmodium* species, as it accounts for serious illness and high mortality (1–5). Two hundred fourteen million new cases of malaria are reported worldwide with a 35% mortality rate reported for children below 5 years of age (6).

Malaria transmission cycle starts with the female *Anopheles* feeding on a mammalian host. Thereafter, *Plasmodium* smartly exploits host cell machinery in numerous ways discussed hereafter to complete its life cycle (7–9). The sporozoites harbored in the salivary gland enter the host blood stream and passes on to the hepatic sinusoid (10–12). The presence of antihistamines and immunomodulators in the salivary gland secretion protects *Plasmodium* from the initial host immune response (10, 11, 13). The endothelial cell lining the liver sinusoid, guarded by kupffer cells (liver macrophages) prevents sporozoite entry into the hepatocytes (12, 14, 15). The circumsporozoite protein (CSP) of the parasite interacts with LRP-1 (low-density lipoprotein receptor-related protein) present on the kupffer cells thereby upregulating cAMP. Thereafter, cAMP mediates EPAC (exchange protein activated by cAMP) inhibition of reactive oxygen species (ROS) production ultimately suppressing the macrophage defense (15–17) (**Figure 1**). Simultaneously, the expression of TNFα, IL-6, and monocyte chemoattractant protein-1 (MCP-1) is downregulated and there is an increased production of anti-inflammatory IL-10 cytokine (15). The sporozoite also downregulates expression of kupffer cells MHC-1 and IL-12 to overturn their antigen presenting ability and ease infiltration of sporozoites into hepatocytes (15, 17). All these events result in the successful invasion.

Furthermore, the role of calcium (Ca^{2+}) in activating various parasite proteins involved in the process of invasion, egress, motility, and cell cycle regulation has been observed (18–21). In *Plasmodium*, endoplasmic reticulum and acidocalcisomes are the major Ca^{2+} reservoirs which are also observed in *Toxoplasma*. The activation of protein kinase G (PKG) by an unknown parasitic signal during invasion or egress releases Ca^{2+} from the parasite endoplasmic reticulum mediated by cyclic guanosine monophosphate (cGMP) (20). Furthermore, phosphoinositide phospholipase C (PI-PLC) is activated by cGMP-dependent PKG which results in hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The translocation of IP3 on to the ER surface causes efflux of Ca^{2+} to the cytoplasm by the formation of IP3-Ca^{2+} channel (20, 22) (**Figure 1**). The increase in the cytoplasmic Ca^{2+} levels activates various calcium-dependent proteases and kinases, like calcium-dependent protein kinases (PfCDPK), double C2 domain protein (PfDOC2) which induce the secretion of microneme and rhoptry proteins for cell adherence and invasion (20, 23, 24). The hepatocyte invasion of the sporozoites occurs *via* Ca^{2+}-mediated activation and secretion of microneme proteins, CSP and thrombospondin-related adhesion protein (TRAP) such as Trap-like protein (TLP) (12, 15, 25) (**Figure 1**). The CSP secreted to the apex in association with actin covers the surface of the sporozoites and its glycosyl phosphatidyl inositol (GPI) anchored C terminus helps in the invasion of sporozoites (12, 26, 27). PfTRAP (TLP) protein interaction with actomyosin motor complex helps in gliding movement of the parasite (15, 25, 28).

The transcellular migration by sporozoites is mediated by the secretion of perforin protein SPECT or perforin like protein1 (PfPLP1), which is also demonstrated to be important in cell traversal, to perforate the hepatocytes (12, 29). Hepatocytic growth factor (HGF) is released by the perforated hepatocyte (30, 31), which activates c-MET receptor tyrosine kinase (c-MET RTK) on them resulting in the activation of tyrosine residues at the cytoplasmic domain of the c-MET receptor (32, 33). This recruits phosphoinositide 3-kinase (PI3-K) which phosphorylates and sequesters proapoptotic proteins of the BCL-2 family (Bad, Bim, PUMA) through AKT (32–35). AKT, which activates anti-apoptotic proteins (BCl-2, BCL-XL, A1), inhibits Bax on the outer mitochondrial membrane and hinders the permeabilization of the mitochondrial membrane and the subsequent release of proapoptotic signaling molecule such as cytochrome-*c* (Cyt-*c*) and eventually blocks apoptosis (**Figure 2**) (12, 27, 32, 33).

Once the parasite has already invaded the hepatocyte, host cell apoptosis block is independent of the PI3-K pathway. It seems that the direct intervention of parasite proteins is necessary for

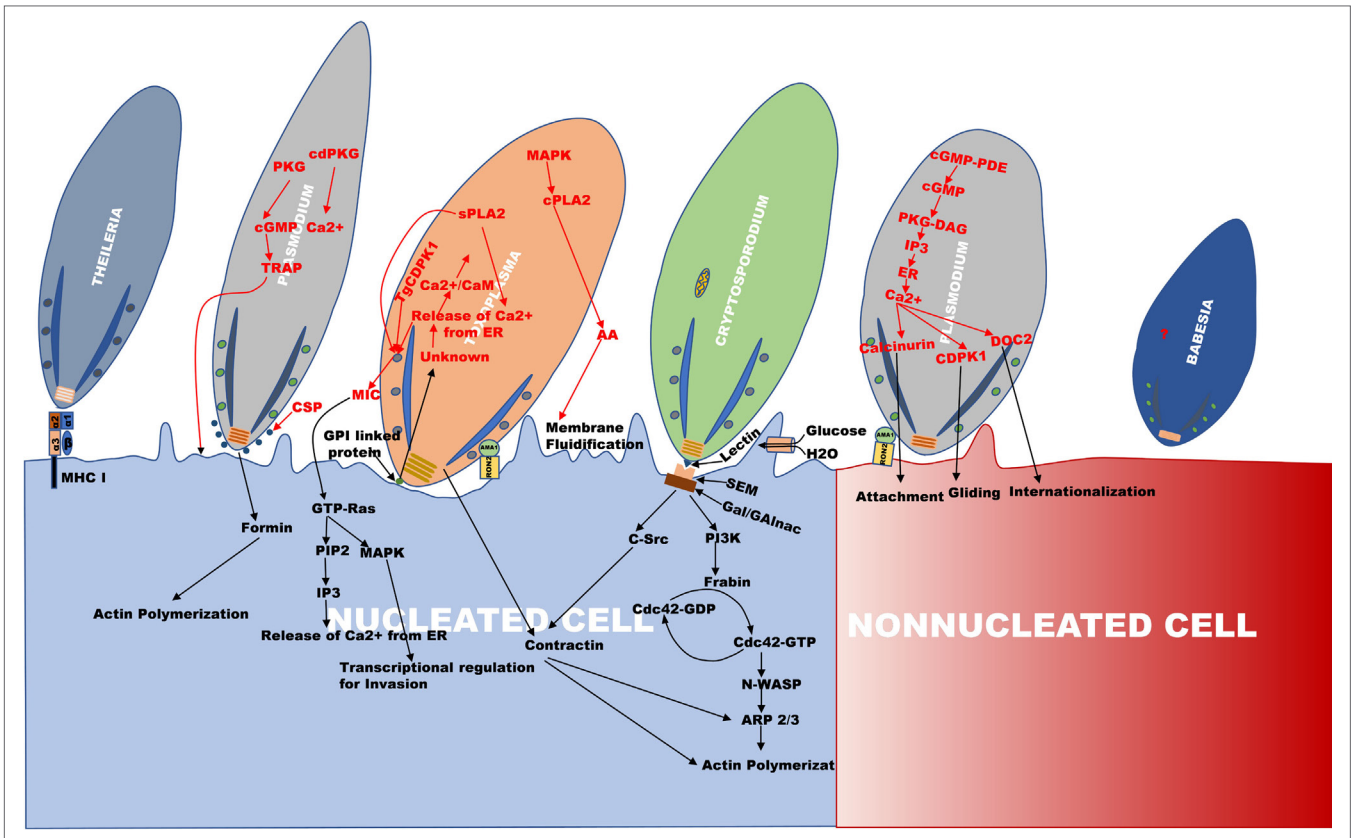

FIGURE 1 | An outline of the invasion mechanism used by *Theileria, Plasmodium*, *Toxoplasma*, *Cryptosporidium*, and *Babesia*. The figure is the representation of the invasion process, which happens during internalization of *Theileria, Plasmodium, Toxoplasma, Cryptosporidium*, *and Babesia.* The apical region of all the parasites faces toward the cell surface for their entry. In *Plasmodium,* invasion occurs in two types of cells, erythrocyte (non-nucleated cell) and hepatocyte (nucleated cell) as compared to other parasites where invasion occurs in the nucleated cell.

modulating the host survival signal (36). One such example is hypoxia mediated by host-dependent HIF-α through AMPK activation which promotes proliferation and parasite survival in the liver (37). The presence of the autophagy marker Atg8 on *P. falciparum* might suggest the parasite's involving degradative functions, but it instead majorly contributes toward biogenic process (38, 39). After the exoerythrocytic merogony, they trigger apoptosis, although this does not seem to occur by activating the caspase-dependent pathway nor *via* the expression of phosphatidylserine (36, 40). However, a serine-repeated antigen (SERA), a cysteine protease identified in *Plasmodium berghei*, is upregulated and is reported to be playing a role in parasite-induced cell death, parasitophorous vacuole (PV) disruption and merosome formation at the time of exoerythrocytic merozoite egress (12). It suggests that the parasite secretory proteins are mediators of host cell apoptosis in the late liver stage. Among seven calcium-dependent protein kinases (CDPK1–7) known in *Plasmodium,* inhibition of PfCDPK5 leads to schizont stage arrest (20).

Erythrocyte invasion of *Plasmodium* occurs in two stages. First, the interaction of merozoite with the erythrocyte causes host cytoskeletal distortion with the help of high Ca^{2+} level, increasing the contact area between the two favoring merozoite entry and the alignment of its apical pole (14, 19, 20). The role of calcineurin (CnA, CnB) has been implicated in merozoite attachment to erythrocytes, which when knocked down results in impaired invasion (20, 41). Furthermore, DOC2 activation induces microneme secretion of erythrocyte binding antigen (EBA175) and AMA1 (microneme apical antigen1), which are involved in the attachment (20, 42) (**Figure 1**). At the second stage of invasion, AMA1, in association with RON2 (rhoptry neck protein), binds to the erythrocyte ligand resulting in the formation of tight junction *via* TRAP (43). It has also been observed that the localization of formin at the apical pole nucleates the parasite F-actin with its FH2 domain and helps in parasite motility (44). The forward propulsion of the actin-myosin filament helps in the invasion of merozoite and encapsulation into the PV in the host cytoplasm (14, 45–47). *Plasmodium* invasion entails increased erythrocyte membrane permeability in order to gain nutrients from the extracellular fluid for its survival (45, 48) and further, utilizes the NF-kB-dependent pathway to inhibit host cell apoptosis (49). Following the invasion, the parasite secretes proteins essential for survival, cell adhesion, and pathogenicity. These are transported from the cytosol to the plasma membrane through vesicular transport. The interplay of protein export elements (PEXEL) and *Plasmodium* translocon of exported proteins (PTEX), cause the

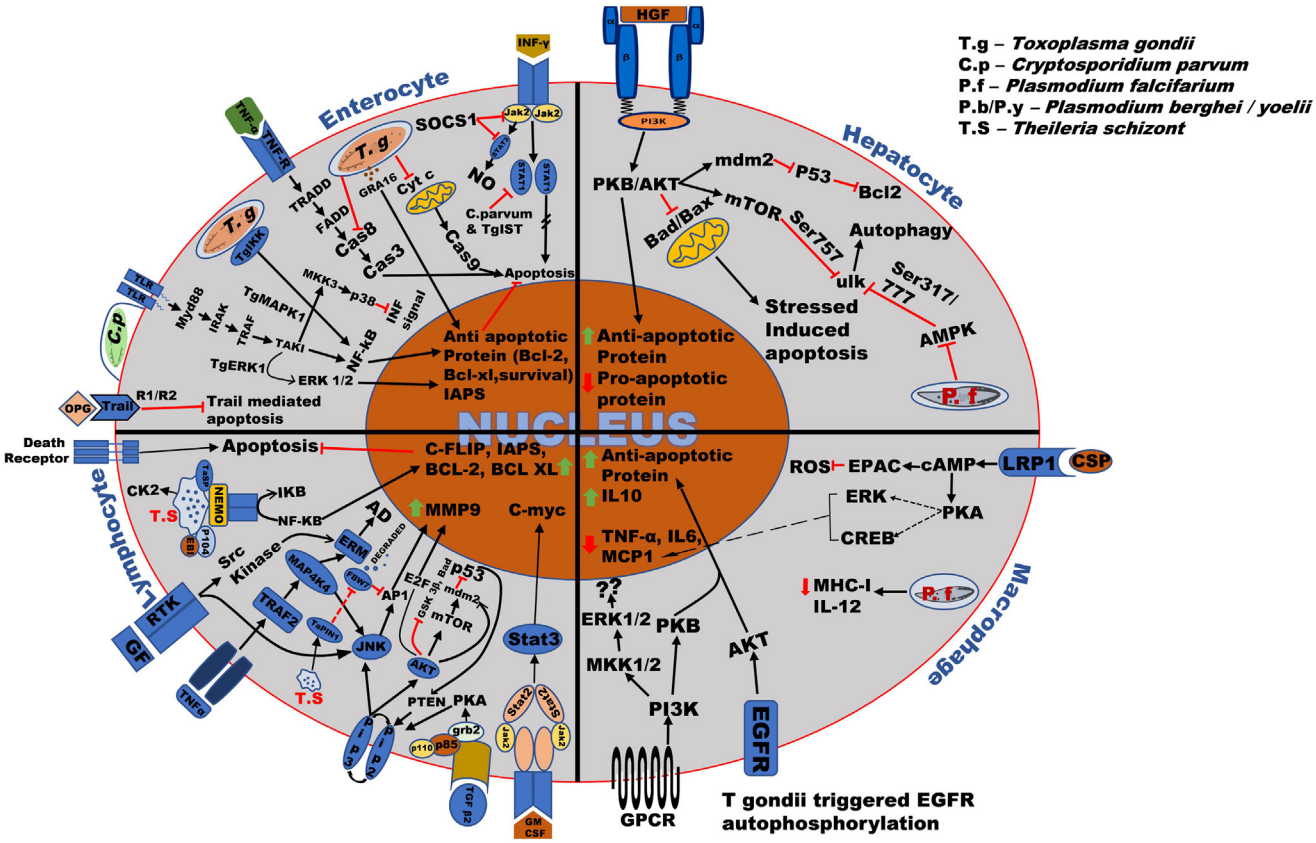

FIGURE 2 | Overall survival mechanism used by the Apicomplexan parasites in different host cells. *Toxoplasma* and *Cryptosporidium* bind to the surface receptor of host cells through the ligands such as EGF, TNF-α, and parasitic surface proteins such as circumsporozoite protein (CSP). After invasion into the host cells such as enterocytes, macrophages, hepatocytes, etc., the parasite modify the host signaling pathway such as TRADD, NF-kB, PKB/AKT resulting in production and upregulation of anti-apoptotic proteins such as Bcl-2, Bcl-xl, and anti-inflammatory cytokines such as IL-10 thereby stopping cytochrome-*c* (Cyt-*c*), TNF-alpha-related-apoptosis-inducing ligand (TRAIL) and BAD, BAX production, and ensuring its survival in the host. *Plasmodium* parasite mainly modifies host PKB/AKT signaling pathway causing upregulation of anti-apoptotic protein and downregulation of pro-apoptotic proteins such as BAD/BAX. *Theileria* schizont proliferates uncontrollably within the host macrophages and lymphocytes. Right after invasion, it upregulates anti-apoptotic proteins such as c-FLIP, IAPs, Bcl-2, Bcl-XL, and proto-oncogenic proteins such as C-myc, antiapoptotic genes such as C-FLIP, Bcl-2, and matrix metallo-protein (MMP9) by majorly targeting host signaling pathways such as NF-κB, JNK/AKT, JAK/STAT, phosphoinositide 3-kinase (PI3-K)/MAPK, and TGF-β2. The regulation of these host signaling pathways causes continuous survival and proliferations of the parasite infected cells which are also common in some cases.

exported proteins to be transported to the plasma membrane of the RBC (50–52).

Cell cytolysis and infecting new cells are an essential strategy associated with disease progression in *Plasmodium*. Parasite egress is a tightly regulated proteolytic activity of parasite proteases PfSUB1 and dipeptidyl peptidase 3 (DPAP3) in response to increased Ca^{2+} level in the cytosol of the parasite. The PfSUB1, an exoneme secreted protease acts on the PV and the plasma membrane of RBC causing cell rupture and egress (53, 54). Millholland et al. 2013 showed the role of host calpains in cytolysis. Krebs cycle intermediates, formed during replication, act through host GPCR, diffused from host plasma membrane to the PV, also help in cytolysis. The ligand activation of GPCR signal through Gαq activates PKC *via* PLC activation. PKC phosphorylates and liberates adducin (a protein that maintains cytoskeletal integrity) resulting in host cell cytoskeletal deformation, which in turn opens the TRPC6 cation channel on the plasma membrane of RBC. The influx of Ca^{2+} from the extracellular fluid activates CaMK (Ca^{2+}/calmodulin dependent kinase) *via* calcium-dependent calmodulin, which phosphorylates host cytoskeletal substrates also resulting in a rapid influx of Ca^{2+}. This Ca^{2+} activates and releases host calpain, which causes lysis and dissolution of host cytoskeleton facilitating parasite release (55, 56). The modulation of host survival signaling by *Plasmodium* allows them to successfully establish a specific environment where they can proliferate and differentiate leading to pathogenesis.

Despite substantial progress in the malaria research, restraining the disease still remains a challenge. *P falciparum* parasites owing to their multiple forms/stages, antigenic polymorphisms and AT-rich genome have further impended the problem. Current chemotherapy is based on using artemisinin and artemisinin-based combination therapies (ACTs), however, reports of drug resistance have already emerged. An effective vaccine should be the ultimate goal for long-term control of the disease. To date only

the RTS, S/AS01 vaccine, targeting the CSP 178 of *P. falciparum* has reached phase three trials, but has not shown much efficacy (57). There are still many gaps in the understanding of the invasion process of *P. falciparum*, such as which molecules signal the release of Ca^{2+}, which leads to adherence and invasion by activating many pathways. These pathways playing role in Ca^{2+} release can be targeted for identifying the novel antigens for developing future vaccines and therapeutics.

Babesia: MALARIA LIKE PARASITE

Babesiosis is a hemolytic disease prevalent in tropical and subtropical parts of the world with a broad host range. *B. microti and B. divergens* infect humans and have emerged as a public health concern predominantly in the United States and Europe, respectively (58). Human cases of babesiosis have surfaced recently in Asian countries, including India and Korea (59). *B. bovis and B. bigemina* both infect cattle but, however, higher morbidity and mortality are associated with *B. bovis* resulting in a huge economic loss (60). There are very limited studies investigating the parasite and its host interactions as compared to the other apicomplexan parasites. Owing to the striking similarity between *Babesia* and *Plasmodium* (61), the disease pathogenesis is considered to be similar to malaria during infection in cattle (60).

Babesia multiplies in the host erythrocyte in a similar fashion to *Plasmodium,* and they are transovarially transmitted in the vector except for *B. microti* (**Table 1**) (62). The life cycle of the parasite begins with tick feeding on host blood and simultaneously releasing sporozoites into the host bloodstream (63). Invasion occurs in a similar fashion as observed for *Plasmodium*, *Toxoplasma*, and *Cryptosporidium via* gliding mechanism using microneme and rhoptry secretions (61, 64). Parasite loosely attaches to the surface of the host RBC by its surface GPI anchored proteins and within the apical secretory organelles. Variant erythrocyte surface antigen 1 (VESA1), a heterodimeric protein of *Babesia* is known to play role in cytoadherence to the host erythrocyte surface (62, 65). After entry of the sporozoites into the red blood cells, they divide by binary fission and produce merozoites. Erythrocyte lysis further allows each merozoite to invade a new RBC and successive merogonies follow (62, 64, 66, 67). Merozoites interact with the RBC surface receptors and play a major role in invasion.

In *Babesia*, the role of Ca^{2+} has been primarily described in invasion and egress mechanism of the parasite, however, the modulation of the host signaling pathways are not thoroughly understood. In *B. bovis,* Ca^{2+}-dependent protein kinase inhibitor showed growth limiting effects (68), though, in *B. divergens*, it impacted egress of the merozoites from erythrocytes (69, 70). Since there is no PV formation in *Babesia*, less Ca^{2+} is released during egress as compared to *P. falciparum* parasites (**Table 2**) (70, 71).

Transovarial transmissions in tick vector and straight entry of sporozoites into erythrocytes are some key features, which make *Babesia* parasites distinct from *Plasmodium* or *Theileria* parasites. Few studies to understand the mechanism of disease pathogenesis during *Babesia* infections have been published. Also, the mechanism of entry and transmission of the parasites are poorly defined. It will be important to investigate the parasite invasion and evasion strategies along with parasite vector interactions for identifying key genes that might play an important role in immune evasion or disease pathogenesis.

Theileria: A LIVESTOCK PATHOGEN

Theileria annulata and T. parva cause tropical theileriosis and east coast fever, respectively, in ruminants predominantly in cattle causing enormous economic loss to the livestock industry (106, 107). Tick vector transmits the parasite upon feeding on animal through the saliva (108–110). After entering the blood stream, it infects WBC of different lineage, *T. parva* infects B cells and T cells whereas *T. annulata* infects B cells and cells of monocyte lineage. The sporozoites, i.e., the infective stage of the parasite passively invade the host cell by zippering mechanism, which is unlike other apicomplexan discussed in which a tight continuous junction is formed between the host cell surface and the parasite sporozoites (108). In addition, the role of MHC class I molecule (**Figure 1**) (111), intrasporozoite Ca^{2+} and protein kinases of host and parasite and the G-protein linked signaling has been shown in invasion (75, 112). After entry into the host cell, parasite rhoptries and its microsphere discharges dissolve the enveloping PV membrane (108), and move to the host cell cytoplasm rather than to PV in comparison to other apicomplexan parasites and provides it with an advantage of escaping lysosomal degradation (**Table 1**). Additional advantage of staying in the host cytoplasm allows the parasite to modulate several signaling pathways, such as TGF-β, JNK, PI3-K, NF-κB, src kinase, and casein kinase 2 (CK2) (80, 100, 113–115).

Theileria transforms their host cell into a cell with a cancerous phenotype by modulating several host cell kinases and activating transcription factors (116). Several studies have been done to identify parasite protein instigating epigenetic changes that may lead to successful transformation. *T. annulata* protein, TaPIN (secretory prolyl isomerase Pin) has been reported to promote transformation by degrading FBW7, a host ubiquitin ligase *via* stabilizing c-JUN (117). p104 and TaSP are surface proteins which have been reported to be phosphorylated in a host cell cycle-dependent manner and might be involved in transformation (118). Two more proteins are TashAT group of protein, which contains AT hook DNA-binding motif and nuclear localization signal and found to be localized in host nucleus (119). *SuAT1*, a parasite gene, contains AT hook DNA-binding polypeptide and predicted signal peptide, PEST motifs and nuclear localization signals, which may interact with the host cell and play a role in transformation (120). Studies to identify epigenetics changes are scarce, only one study has shown the role of oncomiR mir155 in repressing DET1 protein (involve in c-Jun ubiquitination) and stabilizing c-Jun (121). The parasite schizont hijacks the host mitotic assembly resulting in its clonal expansion (122–124).

Theileria transformed cells can be reversed, unlike tumor cells upon treatment with BW720c (122). The transformation occurs by modulating several signaling pathways which ultimately inhibits apoptosis, increases proliferation, and encourages metastatis (116). NF-κB is constitutively expressed in *Theileria* infected cells, which in turn upregulates many anti-apoptotic proteins, such as c-FLIP, IAPs, Bcl-2, and Bcl-XL, and induces Gadd45β that blocks the pro-JNKK2-mediated apoptotic JNK pathway. NF-κB

TABLE 2 | A number of host signaling pathways modulated by *Theileria, Plasmodium, Babesia, Toxoplasma*, *and Cryptosporidium* during its invasion, survival, expansion, and egress in the host cell.

Host factor	Parasite	Mechanism	Benefit to parasite	Reference
CYTOSKELETAL REMODELING				
Actin	*Theileria*	Actin rearrangement through ERM proteins	Helps in cell motility and dissemination	Baumgartner et al. (72)
	Plasmodium	Parasite formin-mediated F-actin nucleation	Key event for Parasite motility/invasion of erythrocytes.	Baum et al. (44) Bargieri et al. (43)
	Toxoplasma	*Via* F-actin and Arp2/3 recruitment.	Parasite motility and entry.	Gonzalez et al. (73)
	Cryptosporidium	Activating Arp2/3 *via* c-src kinase and phosphoinositide 3-Kinase (PI3-K).	Parasite entry Helps invasion	Chen et al. (74)
Ca^{2+} SIGNALING				
Ca^{2+}	*Theileria*	Intrasporozoite calcium	Favors internalization	Shaw (75)
	Plasmodium	Mobilization of intracellular Ca^{2+}	Helps invasion	Gao et al. (42)
	Toxoplasma	Mobilization of extra/intracellular Ca^{2+}	Microneme secretion required for cell motility	Lourido and Moreno (21)
	Cryptosporidium	PKCα depended on leaky tight junctions	Favors invasion	Hashim et al. (76)
	Babesia	Mechanism unknown	Parasite entry and egress	Mossaad et al. (70)
SURVIVAL OR APOPTOSIS				
NF-kβ	*Theileria*	Direct activation through IKK recruitment	Helps survival	Heussler et al. (77)
	Plasmodium	Activated by infected erythrocyte	Helps survival by upregulating anti-apoptotic pathway	Tripathi et al. (49)
	Toxoplasma	Activated either by host or parasite IKK	Helps survival	Molestina and Sinai (78)
	Cryptosporidium	Parasite-induced activation	Helps survival	Chen et al. (79)
PI3-K	*Theileria*	Activated *via* TGF-β2 receptor	Promotes survival *via* inhibiting host apoptosis	Haidar et al. (80)
	Plasmodium	Activated *via* c-MET receptor tyrosine kinase receptor in hepatocytes	Helps survival	Rodrigues et al. (33)
	Toxoplasma	Activated *via* Gα-PCR (Protein Coupled Receptor)	Promotes survival *via* inhibiting host apoptosis	Kim (81)
	Cryptosporidium	Recruitment of PI3-K by sporozoite attachment	Helps in invasion	Chen et al. (74)
JAK/STAT	*Theileria*	Activated *via* Granulocyte-macrophage colony-stimulating factor (GM-CSF) autocrine signaling	Promotes proliferation *via* enhancing host c-myc levels	Dessauge et al. (82)
	Toxoplasma	Prolong phosphorylated state of STAT3/6. STAT1 inactivation by T. *gondii* inhibitor of STAT1 (TgIST)	Promotes survival *via* limiting IL12 and IFNγ. Promotes survival *via* preventing IFNγ signaling.	Laliberté and Carruthers (83) Olias et al. (84)
	Cryptosporidium	Inhibited *via* STAT1 α depletion	Promotes survival *via* inhibition of NO production	Lean et al. (85)
p53	*Theileria*	Sequestration of p53 and degradation	Aids survival	Haller et al. (86)
	Plasmodium	Mdm2-mediated p53 inhibition	Promotes liver stage infection	Kaushansky et al. (87)
	Toxoplasma	GRA16-mediated p53 regulation	Benefits the parasite by altering p53 levels.	Bougdour et al. (88)
MAP KINASE PATHWAY				
JNK	*Theileria*	Activated *via* grb2 association with TGF-β2	Promotes survival and metastasis.	Lizundia (89)
	Toxoplasma	JNK is inhibited	Escaping JNK-mediated apoptosis	Kim (81)
	Cryptosporidium	JNK is inhibited	Escaping JNK-mediated apoptosis	Liu et al. (90)
p38 MAPK	*Toxoplasma*	IFN-γ signaling-mediated production of iNOS is inhibited.	Facilitates survival	Brumlik et al. (91)
	Cryptosporidium	Induces NETosis	Killing of parasite	Muñoz-Caro et al. (92)
ERK1/2	*Toxoplasma*	Activated *via* TgERK7	Ensures survival and reinfection	Li et al. (93)
	Cryptosporidium	Parasite-induced NETosis	Favors killing of parasite	Muñoz-Caro et al. (92)
AUTOPHAGY				
	Theileria	Inhibits	Promotes survival	Duszenko et al. (94)
	Plasmodium	AKT-activated mammalian target of rapamycin inhibits autophagy	Promotes liver stage infection	Kaushansky et al. (87)
	Toxoplasma	*Via* EGFR/AKT pathway	Helps parasite bypass autophagy	Muniz-Feliciano et al. (95)
CELLULAR METABOLIC STRESS				
Reactive oxygen species (ROS)	*Theileria*	Activates NF-kβ and PI3-K signalling pathways	Promoting survival	Metheni et al. (96)
	Plasmodium	ROS accumulation in RBCs	Inhibit parasite growth	Usynin et al. (17) Zheng et al. (15)
	Toxoplasma	Alters ROS levels by downregulating nox4 and inhibiting p38.	Promotes survival	Treeck et al. (97)
	Cryptosporidium	Scavenges ROS by parasite peroxidase	Promotes survival	Hong et al. (98)
	Babesia	Scavenges ROS by parasite peroxidase	Promotes survival	Bosch et al. (99)

(*Continued*)

TABLE 2 | Continued

Host factor	Parasite	Mechanism	Benefit to parasite	Reference
NOS	*Theileria*	Upregulation of iNOS by NF-kβ	Promotes survival *via* NO-mediated inhibition of Fas apoptosis	Durrani et al. (100)
	Plasmodium	Infection-mediated upregulation of iNOS	Parasite clearance	Chiwakata et al. (101)
	Toxoplasma	TgMAPK1-mediated NO reduction	Promotes survival	Brumlik et al. (91)
	Cryptosporidium	Inhibition of IFN-γ-mediated NO upregulation	Promote survival	Lean et al. (85)
	Babesia	IFNγ-mediated upregulation	Parasite growth arrest	Goff et al. (102)
Hypoxia	*Theileria*	Induces transcription of proteins required for the metabolic shift	Enhances survival	Metheni et al. (103)
	Plasmodium	HIF-induced AMPK activation	Promotes development of exoerythrocytic forms (EEF) and increases iron uptake	Ng et al. (37)
	Toxoplasma	Protects HIF1α degradation and enhanced HK2 expression	Promotes parasite growth *via* increasing glycolytic flux	Menendez et al. (104)
CYTOLYSIS AND EGRESS				
GPCR	*Toxoplasma and Plasmodium*	PKC-mediated Ca^{2+} influx, finally activating calpain which proteolyse host cytoskeleton.	Parasite egress	Chandramohanadas et al. (105) Millholland et al. (56)

is activated by recruitment and phosphorylation of IKK signalosome α and β subunits, which further phosphorylates inhibitory κB (iκB) setting NF-κB free to translocate to the nucleus (125). Infected cells release a plethora of cytokines and growth factors that activate TGF-β receptor (I and II) and TNF-α receptor (126). TGF-β2 activates smad2/3 and subsequently smad4, which overexpresses COX-2 resulting in increased levels of prostaglandins and downregulates PKIG, a potent inhibitor of PKA pathway. Simultaneously, TGF-β2 is accounted for parasite motility and invasiveness by activating Rho–ROCK kinase and recruiting an adaptor protein growth factor receptor-bound protein 2 (Grb2) to TGF-RII receptor. The signaling descends by Grb2, activating downstream PI3-K/AKT and JNK pathway (80, 127). Activator protein 1 (AP1), a JNK activated transcription factor drives B cell integration cluster (BIC) transcription upregulating miRNA 155 which inhibits DET1 resulting in accumulation of c-Jun and increased proliferation (89, 121, 128–131). B-1 a bovine analog of MMP9 (ECM degrading proteinase) containing AP1 binding sites also helps in detachment and metastasis of the infected cell to other organs (89, 132, 133).

Theileria modulates the host PI3-K/AKT pathway to be regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), depending on an autocrine loop and, hence, sharing a major role in cell proliferation (134, 135). Phosphorylation of the AKT protein by class I PI3-K facilitates the release of Rb bound E2F transcription factor, activating MDM2 (E3 protein ubiquitin ligase and negative regulator of p53 tumor suppressor gene) directly or through mammalian target of rapamycin (mTOR). AKT-mediated inhibition of several proapoptotic genes (*bad, foxo*) and GSK-3β help the infected cells to combat the stress-induced mitochondrial-mediated apoptosis and to maintain elevated c-Myc levels, respectively (**Table 2**; **Figure 2**) (136). Hypoxia-inducing factor (HIF-1α) is activated by mTOR pathway and by constitutive NF-κB and AP1 production (137). Increased levels of ROS during infection and HIF-1 expression induce the Warburg effect allowing the parasite to establish uncontrolled proliferation (96, 103, 138, 139). PI3-K/AKT pathway, therefore, plays a very important role in survival and proliferation as well as in metastasis of *Theileria*. PTEN, an inhibitor of the PI3-K pathway and activator of p53 are suppressed majorly by NF-κB and CK2 (82, 140, 141). CK2 also dampens TNF/Fas-mediated apoptosis and accelerates iκB degradation augmenting NF-κB activation. Activation of JAK/STAT pathway *via* GM-CSF autocrine loop enhances c-myc expression, whereas phosphorylation by CK2 stabilizes this potent oncogene primarily upregulated in *Theileria* infected cells (**Figure 2**) (82, 115).

The proliferation of infected cells is followed by evasion and metastasis, which involves cytoskeleton alteration. TNF-α binding to its receptor recruits TNF receptor-associated factor 2 (TRAF2), which may either activates NF-κB or a mitogen-activated protein kinase, MAP4K4 (134). MAP4K4 bifurcates into JNK activation and ERM (ezrin/radixin/moesin) cytoskeletal protein phosphorylation, helping in actin dynamics through Rho kinase. ERM may also be activated through src kinases (72). Interfering with autophagic mechanisms also augments survival of the parasite within the cell. Dampening peptide presentation by $CD4^+$ and $CD8^+$ infected cells is a way opted by the parasite to increase its chances of survival enhancing the establishment of infection (94). In contrast to other apicomplexan parasites, *Theileria* usually does not egress and remains attached to the host mitotic assembly. Although under unfavorable circumstances the schizont ruptures releasing merozoites, which invade RBCs, forming piroplasm, which is taken up by the tick and the cycle resumes. *Theileria* parasites have thereby evolved a wide range of strategies to help them survive and proliferate inside the host cells.

Theileria parasites are considered as the smartest among the apicomplexan group for their ability to manipulate the host cells. However, the parasite proteins and molecular mechanisms behind the host cell manipulation are still not clear. Very few proteins are identified till now, which are involved in the host–parasite interaction. The presence of a heterogeneous population of *T. annulata* parasite strains in the field is making it difficult for a currently used attenuated vaccine (schizont stage) to be effective against *T. annulata* parasites. Resistance against burpaquone, a

drug currently being used for the treatment of theileriosis, and acaricide, used for controlling the tick vector, has hampered the control of the disease. In this post genomic era with the availability of advanced genomic and proteomic tools, better studies are needed to dissect the pathways modulated by *Theileria* in detail and select new targets for the development of second-generation drugs and vaccines for disease control.

ENTERIC AND ZOONOTIC APICOMPLEXAN PATHOGEN

Toxoplasma

Toxoplasmosis, caused by *Toxoplasma gondii*, is one of the most well-studied zoonoses (142). *Toxoplasma* appears to be one of the most feared apicomplexan parasites due to a considerable number of congenital transmission incidents and subsequent fetal damage in animals and humans (143, 144). It also causes neurologic deficits (145) and chorioretinitis (146). The life cycle of the parasite circulates between definitive (feline) and intermediate hosts (mammals/birds, etc.). Sexual phase occurs in the small intestine of the feline host from which the oocysts/tissue cysts are excreted along with the feces and ingested by the intermediate host through multiple routes. The oocysts release sporozoites, which invade the intestinal lining. On the other hand, tissue cysts release bradyzoites which differentiate into tachyzoites (147–149). The tachyzoites further replicate in the host and may again differentiate into bradyzoites in the brain, liver, and muscle tissue forming cysts (147, 150, 151). This inter-conversion between tachyzoites and bradyzoites appears essential to the life cycle and infective potential of the parasite (**Table 1**) (152).

Host–parasite interactions are mostly *via* secreted parasite proteins from their rhoptries, micronemes, and dense granules, which help parasite in cell invasion, survival, and egress. Invasion by *T. gondii* involves gliding mobility prompted by an actin-myosin motor based complex (153) and interrelated signaling cascades as well. The parasite attaches loosely with the host cell surface *via* GPI-linked proteins, surface antigens (SAGs), SAG-related sequences (SRSs), and SAG unrelated surface antigens (SUSAs) (154, 155). After the secretion of *Toxoplasma* microneme adhesion proteins (MICs) into the host, there is an increased activity of Calcium/Calmodulin (Ca/CAM)-dependent processes leading to the secretion of Phospholipases (sPLA2 and PLA2) (**Figure 1**). *T. gondii* calcium-dependent protein kinase1 (TgCDPK1) has been reported to be involved in microneme secretion and can thereby regulate cell motility which is essential for invasion (156). sPLA2 secretion causes the release of microneme proteins MIC3/MIC2, which induces Ca^{2+} release from host ER *via* Protein Kinase C-Inositol 1,4,5 triphosphate (PKC–IP3) pathway. cPLA2 activated by parasite MAPK causes membrane fluidification by hydrolyzing host membrane phospholipids (157). Therefore, Ca^{2+}signaling induce a lot of complex cascades facilitating parasite invasion (**Figure 1**; **Table 2**). Apicomplexan have been reported to have several Ca^{2+}ATPases and CA^{2+}/H^{+} exchangers, which help in invasion (21).

Toxoplasma invades *via* gliding movement, which results in actin remodeling by F-actin ring formation at the point of entry with the subsequent recruitment of Arp2/3 complex (**Figure 1**) (73, 158). Parasite rhoptries are secreted followed by microneme secretion, which consists of RON and traditional rhoptries proteins (ROP). RON2 and AMA1 associate together to form a tight junction between the host and the parasite referred to as moving junction (MJ) facilitating the formation of PV (159–161). Sporozoites also invade using paralogs of AMA1 and RON2 conveniently named as sporoAMA1 and sporoRON2 (162). Traditional rhoptry proteins such as Rop 17, Rop 18 (kinase), and Rop 5 (pseudokinase) reside on the PV membrane inhibiting the accumulation of immunity-related GTPases (IRGs). This complex also has a dense granule protein, namely GRA 7 which has a definite impact on IRG turnover (163, 164).

Once inside the host cell, the parasite thrives on host nutrients by expressing various parasite transporters, enzymes, and following complex cascades (165). *T. gondii* inhibits apoptosis and dodges autophagy by manipulating PI3-K pathway, the immediate downstream effector protein kinase B (PKB/Akt), JAK/STATs, mTOR, NF-kβ, ERK1/2, C-myc, and microRNAs to promote its survival (165, 166). The parasite avoids lysosomal degradation by cleverly maintaining the non-fusogenic nature of the PV. Studies suggest that *T. gondii* micronemal proteins (MICs) with epidermal growth factor (EGF) domains activate Epidermal Growth Factor Receptor (EGFR) on endothelial, retinal cells, and microglia keeping the parasite protected in the vacuole (95). It has been reported that the parasite causes mTOR activation in an infected host cell even in the absence of phosphorylation of 4E-BP1 and S6K1 (167). Later, a study confirmed the role of mTORC1 and C2 in host cell invasion and persistence of infection (168).

Toxoplasma gondii disrupts host apoptotic pathways primarily by affecting the release of Cyt-*c* and thereby preventing activation of caspase 3 (166). The parasite modulates the host NF-kβ pathway in line with *Theileria* causing increased expression of anti-apoptotic genes, although, there is also a role of *Toxoplasma* IKK (TgIKK) in maintaining the NF-kβ response which declines after the initial activation by the host IKK (78). A dense granule protein GRA 15 also activates this pathway *via* TNF receptor-associated factor 6 (TRAF6) thereby inducing the release of pro-inflammatory cytokines (169). Rop 16, on the other hand, might be playing a role in inhibiting cytokine synthesis by host macrophages (170). Hence, opposed the effect of two of these factors determines macrophage polarization in the host (171). *Toxoplasma* polymorphic effectors determine macrophage polarization and intestinal inflammation (171) *Toxoplasma* inhibits the proapoptotic genes (BCL-2, Bad, caspase-9) by modulating the host–PI3-K pathway. It also hinders apoptosis by downregulating phosphorylated c-Jun N-terminal kinase levels (81). AKT/PKB pathway is upregulated, which serves the parasite by inhibiting forkhead transcription factor (FKHRI) resulting in decreased levels of proapoptotic factors, such as Bim and FasL (172). Infected cells also display increased expression of anti-apoptotic proteins, such as BCL-2, BFL1, BCL-XI, BCL-W, and MCI-I, and reduced expression of proapoptotic factors Bad and Bax (**Figure 2**).

Toxoplasma ERK-7 (TgERK7) protein has been recently demonstrated to play an important role in the intracellular proliferation of the parasite in the host (93). *T. gondii* also protects itself from host interferon-γ (IFN-γ)-mediated pathway by

obstructing the expression of IFN-γ activated genes. *T. gondii* inhibitor of STAT1 (TgIST) has been shown to bind to activated STAT1 in the host cell membrane and recruits host Mi2/NURD complex which keeps the STAT1 in inactivated stage thereby preventing pro-inflammatory gene expression (84, 173). Elevated Ca^{2+} during T. *gondii* infection activates the protein kinase C cascade which further activates COX-2 resulting in increased prostaglandin E2 (PGE2) levels helping in the resolution of inflammation (174). Furthermore, a dual role of TGF-β during infection by inducing or suppressing the immune system has been reported (175).

During infection, TRAF6 is activated by the parasite dense granule protein GRA7 which leads to unusually increased levels of ROS in the cells (97). Increased levels of ROS, few cytokines and growth factors, causes elevated HIF-1 levels *via* dampening prolyl hydroxylase domain containing protein 2 (PHD-2) levels downstream to Type I TGFβ receptor signaling. Influencing the levels of such a crucial host factor as PHD-2 is pivotal for the maintenance of a secure haunt of the parasite (176). A host kinase-HK2 also activates HIF-1 expression resulting in glycolytic flux and Warburg effect, as identified by siRNA screening (104). A microarray-based study reveals that increased HIF-1 level in infected cells lead to activation of EGR1 and AP1 which play roles in inducing resistance against drugs and proliferation, respectively (177, 178). The parasite also seizes the IFN-γ-induced iNOS production by *T. gondii* expressed MAP kinase (TgMAPK1), which reduces NO production by p38 MAPK (91). But on the other hand, a dense granule protein GRA24 also plays a role in maintaining p38α autophosphorylation, forming a complex, which consequently activates EGR1 and cFOS which induce the release of MCP-1 and IL-12, which can keep the parasite load in check (179).

Toxoplasma also modulates p53 levels for its own benefit by GRA16, another parasite dense granule protein which binds to two host enzymes-HAUSP and PP2A phosphatase in the host nucleus (88). *T. gondii* reportedly alters dopaminergic and GABAergic signaling due to elevated levels of mi-RNA132 which might be the underlying cause for the neuronal abnormalities often found associated with the infection (180). The parasite utilizes GABA to partially satisfy its carbon requirements and also in egress (181). Once it has successfully established infection, egress mainly occurs *via* GPCR-coupled signaling pathway similar to *Plasmodium*. TgCDPK1 and TgCDPK3 activated by Ca^{2+} influx have been reported to play a role in egress. Studies suggest that a parasite pore forming protein TgPLP1 might be responsible for making the PV perforated to make egress easier (182). Recently, cGMP-dependent PKG has been identified to play an important role in controlling egression (156).

As it appears from the above discussion, the cunning parasite can steer a staggering number of host signaling pathways in direction of its own purpose. However, despite the fact that very specific knowledge is available about particular such proteins, it is not clear how they affect host gene expression since such nucleus targeted proteins do not really resemble host transcription factors neither can they bind to the host cell DNA (164). Interestingly, not all of these secreted proteins benefit the parasite. Some actually trigger the host immune system to call up its guards. Now, how might the parasite strike a balance to sustain infection or how might we use such kind of knowledge to limit infection still remains to be worked out.

Cryptosporidium

Cryptosporidium commonly causes gastrointestinal diseases worldwide, which albeit minimally invasive in the immunocompetent host (both human and animals) can be deadly in immunosuppressed patients (183, 184). *C. parvum* with a broad host range and zoonoses is considered a more important pathogen in comparison to *C. hominis,* which only infects human. The disease prevalence ranges from 1 to 37% in countries such as Africa, Asia, Australia, South America, and Central America (185, 186).

Its life cycle comprises of a sexual and an asexual stage, which takes place in a single host (187). Similar to *Toxoplasma*, *Cryptosporidium* infection occurs by ingestion of oocysts through contaminated water followed by excystation and release of sporozoites. These zoites then invades the enterocytes by gliding movement (**Table 1**, **Figure 1**) (188). *Cryptosporidium* form an intracellular but extra cytoplasmic PV wherein they get developed into spherical trophozoites (184, 189). Invasion of host epithelial cells occurs *via* aggregating the host actin and actin binding protein, villin at the site of parasite attachment and further inducing host tyrosine kinase signaling cascades (189, 190). Reports of numerous *C. parvum* proteins have been implicated in attachment, invasion, and intracellular development (191, 192). p30, a galactose-*N*-acetylgalactosamine (Gal/GalNAc) lectin parasite protein has been identified which forms an adhesion complex along with gp40 and gp900 (193). Furthermore, the cryptosporidial binding leads to the formation of sphingolipid-enriched membrane microdomains which attracts Gal/GalNAc epitope containing glycoproteins on the host membrane parasite interface, activating PI3-K (192). The PI3-K cascade successively activates Cdc42, N-WASP, and Arp2/3 (actin-related protein 2/3) resulting in the formation of actin plaque (74, 124, 194). The parasite recruited src tyrosine kinase subsequently phosphorylates cortactin stimulating the polymerization and rearrangement of the actin cortex in the cell periphery through activation of Arp2/3 complex proteins (**Figure 1**) (74, 195). Increase in local cell volume by accumulation of host aquaporin AQP1 and Na+/Glucose co-transporter also aid in efficient membrane protrusions (196). Few studies have also shown the role of host calpain in remodeling host cytoskeleton which is essential during parasite invasion (197).

Ca^{2+}-ATPase located at the *Cryptosporidium* sporozoites apical and perinuclear regions helps it in fulfilling its Ca^{2+} requirement during the invasion (193). *Cryptosporidium* also possesses 7 CDPKs, which has a role in invasive and regulatory processes similar to *Plasmodium* and *Toxoplasma*. *Cryptosporidium* invasion is promoted by a Ca^{2+}-dependent PKC signaling pathway, which disrupts the cell–cell junction. PKC causes downstream activation of PKCα which has been associated with tight junctional leakiness in renal epithelial cells (76, 193, 198). *Cryptosporidium* embodies a novel Ca^{2+}-activated nucleoside diphosphatase (apyrase, CApy), which interfere with extracellular nucleotide and modulates inflammatory pathways delaying the response against parasite clearance (199). The trophozoite

stage of the parasite inhibits apoptosis; however, schizont- and merozoite-affected cells are handled by host apoptosis through Fas/FasL signaling (200, 201).

Cryptosporidium activates NF-κB pathway by inducing IL-8 secretion and acting synergistically with AP1 and IL-6 (79) (**Figure 2**; **Table 2**). It has also been reported to play a role in activating other survival signals, e.g., over expression of antiapoptotic proteins (bcl-2, IAP, survivin) and inhibition of proapoptotic proteins (bax) (77, 202). Myc, an oncogenic protein plays a role in positive regulation of parasite survival, whereas PTEN, an inhibitor of PI3-K, negatively regulates the anti-apoptotic protein (90). Also, microarray analysis revealed that TNF-superfamily receptor osteoprotegerin (OPG) is upregulated in infected host intestinal mucosa by microarray. The overexpression of OPG helps in evading host defense by inhibiting TNF-alpha-related-apoptosis-inducing ligand (TRAIL)-mediated apoptosis and supporting the parasite to complete its life cycle (184, 203). The host tries to control the propagation of *Cryptosporidium* by enhancing Th1 response characterized by the production of IFNγ and IL-12. The parasite, too, in turn, erodes the JAK/STAT-mediated IFNγ signaling by depletion of STAT1-α (85, 204). TNF-α and TGF-β play roles in providing the host protective immunity and healing effect against the infection (85, 205, 206). Again, increased mucin levels in the host by COX-2-mediated PGE2 protects the host (183), ERK1/2 and p38 MAPK pathway also assist the host cells to destroy the parasite by inducing NETosis (Formation of the neutrophil extracellular trap) (92).

Absence of Apicoplast in *C. parvum* parasites and complications in their *in vitro* propagation has posed problems for researchers involved in drug or vaccine development. Despite many efforts by *Cryptosporidium* to modulate the host signaling pathways, the parasite loses the battle against the host. At present, only one drug (nitazoxanide) with limited efficacy is approved for treatment of Cryptosporidiosis. Further studies are needed to better understand the egress mechanism of *Cryptosporidium* (74). Susceptibility to the parasite has shown to be linked with the immune status of the host. Understanding the host–pathogen interaction will be critical in designing new tools for effective control of the disease.

FINAL CONCLUSION

A substantial amount of research has been done to gain insights into pathways by which these parasites modulate and undermine the host defense, yet gaps in knowledge still prevail and many questions remain unanswered. In this review, we have attempted to include all the major work carried out in this field. Advancement in gene editing technologies and whole genome sequencing of these pathogens lead us to better understand the manipulation strategies used by the parasites. Emerging problems of either drug resistance or unavailability of an effective vaccine against some of the parasites make the precise comprehension of the sabotage techniques employed by the parasites a primary requisite in order to curb the morbidity rate.

As discussed above, it is apparent that few of the host defense pathways targeted by these parasites to survive and proliferate in the host cell are common among the mentioned pathogens. Host cell invasion by *Plasmodium*, *Babesia*, *Toxoplasma*, and *Cryptosporidium* occur *via* gliding movement, whereas in *Theileria* an overall different process known as zippering takes place. *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* reside and replicate inside the PV in the host, however, *Theileria* and *Babesia* survives in the host cytoplasm. *Theileria* clearly takes an advantage of staying in the host cytoplasm by modulating numerous pathways, though any such information about *Babesia* has to be still investigated. Several pathways are modulated by majority of these parasites such as host cytoskeleton remodeling, Ca^{2++} modulated signaling pathways, and apoptotic pathways which helps in their survival. In the review, we have discussed the cross talks happening between the parasite and the host and observed that the multifaceted nature of the parasite gives them an upper hand over the host.

Future studies focusing on

(i) Exploring the parasite proteins and their role in host–parasite interface interaction will provide in-depth understanding of the invasion process. These targets can be further utilized to develop vaccine or drugs.
(ii) What are the alteration in the host cell that leads to the nutrient acquisition after invasion and the host factors contributing to parasite replication?
(iii) Studies are also required to develop inhibitors against known molecules/pathways, which help the in intracellular survival of parasite in the host cell.

Therefore, targeting the common pathways playing crucial role in all parasites survival and dissemination may be a good approach to understand disease pathogenesis and controlling the disease.

In order to deal with these cunning pathogens, we need all the necessary information to be able to target important molecules for a vaccine or drug development. However, a considerable amount of research and thorough screening of presently available literature is still required to better understand how these parasites exploit their hosts for their own survival. Apicomplexan parasites infecting human beings such as *Plasmodium* is hugely funded and globally studied; however, so is not the case when it comes to parasites such as *Babesia* and *Theileria,* which are of veterinary importance. Therefore, for more in-depth understanding of these pathogens, tenacious research is expected which would only be possible through the combined efforts of researchers and support from funding agencies on a global range.

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All authors mentioned have made a significant effort and contributed intellectually to the work and approved it for publication.

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REFERENCES

1. Boussaroque A, Fall B, Madamet M, Camara C, Benoit N, Fall M, et al. Emergence of mutations in the K13 Propeller Gene of *Plasmodium falciparum* Isolates from Dakar, Senegal, in 2013-2014. *Antimicrob Agents Chemother* (2015) 60:624–7. doi:10.1128/aac.01346-15
2. Cui L, Rosenthal PJ, Rathod PK, Ndiaye D, Mharakurwa S. Antimalarial drug resistance: literature review and activities and findings of the ICEMR network. *Am J Trop Med Hyg* (2015) 93:57–68. doi:10.4269/ajtmh.15-0007
3. Huang B, Deng C, Yang T, Xue L, Wang Q, Huang S, et al. Polymorphisms of the artemisinin resistant marker (K13) in *Plasmodium falciparum* parasite populations of Grande Comore Island 10 years after artemisinin combination therapy. *Parasit Vectors* (2015) 8:634. doi:10.1186/s13071-015-1253-z
4. Isozumi R, Uemura H, Kimata I, Ichinose Y, Logedi J, Omar AH, et al. Novel mutations in K13 propeller gene of artemisinin-resistant *Plasmodium falciparum*. *Emerg Infect Dis* (2015) 21:490–2. doi:10.3201/eid2103.140898
5. Sharma RK, Thakor HG, Saha KB, Sonal GS, Dhariwal AC, Singh N. Malaria situation in India with special reference to tribal areas. *Indian J Med Res* (2015) 141:537–45. doi:10.4103/0971-5916.159510
6. World Health Organization. (WHO fact sheet: world malaria report; 9 December 2015). *World Health* (2015) 243:10–13.
7. Mota MM, Rodriguez A. Migration through host cells: the first steps of *Plasmodium* sporozoites in the mammalian host. *Cell Microbiol* (2004) 6:1113–8. doi:10.1111/j.1462-5822.2004.00460.x
8. Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. *Cell Mol Life Sci* (2014) 71:1245–63. doi:10.1007/s00018-013-1491-1
9. Silva LS, Silva-Filho JL, Caruso-Neves C, Pinheiro AAS. New Concepts in malaria pathogenesis: the role of the renin-angiotensin system. *Front Cell Infect Microbiol* (2016) 5:103. doi:10.3389/fcimb.2015.00103
10. Baldacci P, Ménard R. The elusive malaria sporozoite in the mammalian host. *Mol Microbiol* (2004) 54:298–306. doi:10.1111/j.1365-2958.2004.04275.x
11. Ishino T, Yano K, Chinzei Y, Yuda M. Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biol* (2004) 2:e4. doi:10.1371/journal.pbio.0020004
12. Garcia JE, Puentes A, Patarroyo ME. Developmental biology of sporozoite-host interactions in *Plasmodium falciparum* malaria: implications for vaccine design. *Clin Microbiol Rev* (2006) 19:686–707. doi:10.1128/cmr.00063-05
13. Dhar R, Kumar N. Role of mosquito salivary glands. *Curr Sci* (2003) 85:1308–13.
14. Dhangadamajhi G, Kar SK, Ranjit M. The survival strategies of malaria parasite in the red blood cell and host cell polymorphisms. *Malar Res Treat* (2010) 2010:1–9. doi:10.4061/2010/973094
15. Zheng H, Tan Z, Xu W. Immune evasion strategies of pre-erythrocytic malaria parasites. *Mediators Inflamm* (2014) 2014:1–6. doi:10.1155/2014/362605
16. Coppi A, Tewari R, Bishop JR, Bennett BL, Lawrence R, Esko JD, et al. Heparan sulfate proteoglycans provide a signal to *Plasmodium* sporozoites to stop migrating and productively invade host cells. *Cell Host Microbe* (2007) 2:316–27. doi:10.1016/j.chom.2007.10.002
17. Usynin I, Klotz C, Frevert U. Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells. *Cell Microbiol* (2007) 9:2610–28. doi:10.1111/j.1462-5822.2007.00982.x
18. Mota MM, Hafalla JCR, Rodriguez A. Migration through host cells activates *Plasmodium* sporozoites for infection. *Nat Med* (2002) 8:1318–22. doi:10.1038/nm785
19. Brochet M, Collins MO, Smith TK, Thompson E, Sebastian S, Volkmann K, et al. Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca2+ signals at key decision points in the life cycle of malaria parasites. *PLoS Biol* (2014) 12:e1001806. doi:10.1371/journal.pbio.1001806
20. Brochet M, Billker O. Calcium signalling in malaria parasites. *Mol Microbiol* (2016) 100:397–408. doi:10.1111/mmi.13324
21. Lourido S, Moreno SNJ. The calcium signaling toolkit of the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium* (2015) 57:186–93. doi:10.1016/j.ceca.2014.12.010
22. Hopp CS, Bowyer PW, Baker DA. The role of cGMP signalling in regulating life cycle progression of *Plasmodium*. *Microbes Infect* (2012) 14:831–7. doi:10.1016/j.micinf.2012.04.011
23. Cheemadan S, Ramadoss R, Bozdech Z. Role of calcium signaling in the transcriptional regulation of the apicoplast genome of *Plasmodium falciparum*. *Biomed Res Int* (2014) 2014:1–12. doi:10.1155/2014/869401
24. Jean S, Zapata-Jenks MA, Farley JM, Tracy E, Mayer DCG. *Plasmodium falciparum* double C2 domain protein, PfDOC2, binds to calcium when associated with membranes. *Exp Parasitol* (2014) 144:91–5. doi:10.1016/j.exppara.2014.06.015
25. Moreira CK, Templeton TJ, Lavazec C, Hayward RE, Hobbs CV, Kroeze H, et al. The *Plasmodium* TRAP/MIC2 family member, TRAP-like protein (TLP), is involved in tissue traversal by sporozoites. *Cell Microbiol* (2008) 10:1505–16. doi:10.1111/j.1462-5822.2008.01143.x
26. Plassmeyer ML, Reiter K, Shimp RL, Kotova S, Smith PD, Hurt DE, et al. Structure of the *Plasmodium falciparum* circumsporozoite protein, a leading malaria vaccine candidate. *J Biol Chem* (2009) 284:26951–63. doi:10.1074/jbc.m109.013706
27. Lüder CGK, Stanway RR, Chaussepied M, Langsley G, Heussler VT. Intracellular survival of apicomplexan parasites and host cell modification. *Int J Parasitol* (2009) 39:163–73. doi:10.1016/j.ijpara.2008.09.013
28. Ejigiri I, Sinnis P. *Plasmodium* sporozoite–host interactions from the dermis to the hepatocyte. *Curr Opin Microbiol* (2009) 12:401–7. doi:10.1016/j.mib.2009.06.006
29. Yang ASP, O'Neill MT, Jennison C, Lopaticki S, Allison CC, Armistead JS, et al. Cell traversal activity is important for *Plasmodium falciparum* liver infection in humanized mice. *Cell Rep* (2017) 18:3105–16. doi:10.1016/j.celrep.2017.03.017
30. Mota MM. Migration of *Plasmodium* sporozoites through cells before infection. *Science* (2001) 291:141–4. doi:10.1126/science.291.5501.141
31. Carrolo M, Giordano S, Cabrita-Santos L, Corso S, Vigário AM, Silva S, et al. Hepatocyte growth factor and its receptor are required for malaria infection. *Nat Med* (2003) 9:1363–9. doi:10.1038/nm947
32. Organ SL, Tsao M-S. An overview of the c-MET signaling pathway. *Ther Adv Med Oncol* (2011) 3:S7–19. doi:10.1177/1758834011422556
33. Rodrigues V, Cordeiro-da-Silva A, Laforge M, Ouaissi A, Silvestre R, Estaquier J. Modulation of mammalian apoptotic pathways by intracellular protozoan parasites. *Cell Microbiol* (2012) 14:325–33. doi:10.1111/j.1462-5822.2011.01737.x
34. Leirião P, Albuquerque SS, Corso S, Van Gemert G-J, Sauerwein RW, Rodriguez A, et al. HGF/MET signalling protects *Plasmodium*-infected host cells from apoptosis. *Cell Microbiol* (2005) 7:603–9. doi:10.1111/j.1462-5822.2004.00490.x
35. Regad T. Targeting RTK signaling pathways in cancer. *Cancers (Basel)* (2015) 7:1758–84. doi:10.3390/cancers7030860
36. Koyama FC, Chakrabarti D, Garcia CRS. Molecular machinery of signal transduction and cell cycle regulation in *Plasmodium*. *Mol Biochem Parasitol* (2009) 165:1–7. doi:10.1016/j.molbiopara.2009.01.003
37. Ng S, March S, Galstian A, Hanson K, Carvalho T, Mota MM, et al. Hypoxia promotes liver-stage malaria infection in primary human hepatocytes in vitro. *Dis Model Mech* (2013) 7:215–24. doi:10.1242/dmm.013490
38. Navale R, Atul, Allanki AD, Sijwali PS. Characterization of the autophagy marker protein Atg8 reveals atypical features of autophagy in *Plasmodium falciparum*. *PLoS One* (2014) 9:e113220. doi:10.1371/journal.pone.0113220
39. Thieleke-Matos C, Lopes da Silva M, Cabrita-Santos L, Portal MD, Rodrigues IP, Zuzarte-Luis V, et al. Host cell autophagy contributes to *Plasmodium* liver development. *Cell Microbiol* (2015) 18:437–50. doi:10.1111/cmi.12524
40. Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med* (2010) 207:1807–17. doi:10.1084/jem.20101157
41. Philip N, Waters AP. Conditional degradation of *Plasmodium* calcineurin reveals functions in parasite colonization of both host and vector. *Cell Host Microbe* (2015) 18:122–31. doi:10.1016/j.chom.2015.05.018
42. Gao X, Gunalan K, Yap SSL, Preiser PR. Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum*. *Nat Commun* (2013) 4:2862. doi:10.1038/ncomms3862
43. Bargieri D, Lagal V, Andenmatten N, Tardieux I, Meissner M, Ménard R. Host cell invasion by apicomplexan parasites: the junction conundrum. *PLoS Pathog* (2014) 10:e1004273. doi:10.1371/journal.ppat.1004273
44. Baum J, Tonkin CJ, Paul AS, Rug M, Smith BJ, Gould SB, et al. A malaria parasite formin regulates actin polymerization and localizes to the

parasite-erythrocyte moving junction during invasion. *Cell Host Microbe* (2008) 3:188–98. doi:10.1016/j.chom.2008.02.006
45. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. *Cell* (2006) 124:755–66. doi:10.1016/j.cell.2006.02.006
46. Bargieri D, Lagal V, Tardieux I, Ménard R. Host cell invasion by apicomplexans: what do we know? *Trends Parasitol* (2012) 28:131–5. doi:10.1016/j.pt.2012.01.005
47. Koch M, Baum J. The mechanics of malaria parasite invasion of the human erythrocyte – towards a reassessment of the host cell contribution. *Cell Microbiol* (2016) 18:319–29. doi:10.1111/cmi.12557
48. Gazarini ML, Thomas AP, Pozzan T, Garcia CRS. Calcium signaling in a low calcium environment. *J Cell Biol* (2003) 161:103–10. doi:10.1083/jcb.200212130
49. Tripathi AK, Sha W, Shulaev V, Stins MF, Sullivan DJ. *Plasmodium falciparum*-infected erythrocytes induce NF-kappaB regulated inflammatory pathways in human cerebral endothelium. *Blood* (2009) 114:4243–52. doi:10.1182/blood-2009-06-226415
50. Cooke BM, Lingelbach K, Bannister LH, Tilley L. Protein trafficking in *Plasmodium falciparum*-infected red blood cells. *Trends Parasitol* (2004) 20:581–9. doi:10.1016/j.pt.2004.09.008
51. Marti M. Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* (2004) 306:1930–3. doi:10.1126/science.1102452
52. Boddey JA, Moritz RL, Simpson RJ, Cowman AF. Role of the *Plasmodium* export element in trafficking parasite proteins to the infected erythrocyte. *Traffic* (2009) 10:285–99. doi:10.1111/j.1600-0854.2008.00864.x
53. Heussler VT, Stanway RR. Cellular and molecular interactions between the apicomplexan parasites *Plasmodium* and *Theileria* and their host cells. *Parasite* (2008) 15:211–8. doi:10.1051/parasite/2008153211
54. Lee MCS, Fidock DA. Arresting malaria parasite egress from infected red blood cells. *Nat Chem Biol* (2008) 4:161–2. doi:10.1038/nchembio0308-161
55. Millholland MG, Chandramohanadas R, Pizzarro A, Wehr A, Shi H, Darling C, et al. The malaria parasite progressively dismantles the host erythrocyte cytoskeleton for efficient egress. *Mol Cell Proteomics* (2011) 10:M111.010678. doi:10.1074/mcp.m111.010678
56. Millholland MG, Mishra S, Dupont CD, Love MS, Patel B, Shilling D, et al. A host GPCR signaling network required for the cytolysis of infected cells facilitates release of apicomplexan parasites. *Cell Host Microbe* (2013) 13:15–28. doi:10.1016/j.chom.2012.12.001
57. Gosling R, von Seidlein L. The future of the RTS,S/AS01 malaria vaccine: an alternative development plan. *PLoS Med* (2016) 13:e1001994. doi:10.1371/journal.pmed.1001994
58. Leiby DA. Transfusion-transmitted *Babesia* spp.: bull's-eye on *Babesia microti*. *Clin Microbiol Rev* (2011) 24:14–28. doi:10.1128/cmr.00022-10
59. Marathe A, Tripathi J, Handa V, Date V. Human babesiosis – a case report. *Indian J Med Microbiol* (2005) 23:267–9.
60. Gohil S, Kats LM, Sturm A, Cooke BM. Recent insights into alteration of red blood cells by Babesia bovis: moovin' forward. *Trends Parasitol* (2010) 26:591–9. doi:10.1016/j.pt.2010.06.012
61. Cooke BM, Mohandas N, Cowman AF, Coppel RL. Cellular adhesive phenomena in apicomplexan parasites of red blood cells. *Vet Parasitol* (2005) 132:273–95. doi:10.1016/j.vetpar.2005.07.002
62. Chauvin A, Moreau E, Bonnet S, Plantard O, Malandrin L. *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet Res* (2009) 40:37. doi:10.1051/vetres/2009020
63. Mehlhorn H, Schein E. The piroplasms: life cycle and sexual stages. *Adv Parasitol* (1985) 23:37–103. doi:10.1016/s0065-308x(08)60285-7
64. Lobo CA, Rodriguez M, Cursino-Santos JR. *Babesia* and red cell invasion. *Curr Opin Hematol* (2012) 19:170–5. doi:10.1097/moh.0b013e328352245a
65. Allred DR. Molecular technology and antigenic variation among intraerythrocytic hemoparasites: do we see reality? *Vet Parasitol* (2001) 101:261–74. doi:10.1016/s0304-4017(01)00571-4
66. Hines S. Genetic and antigenic characterization of *Babesia bovis* merozoite spherical body protein Bb-1. *Mol Biochem Parasitol* (1995) 69:149–59. doi:10.1016/0166-6851(94)00200-7
67. Dowling SC, Perryman LE, Jasmer DP. A Babesia bovis 225-kilodalton spherical-body protein: localization to the cytoplasmic face of infected erythrocytes after merozoite invasion. *Infect Immun* (1996) 64:2618–26.
68. Bork S, Das S, Okubo K, Yokoyama N, Igarashi I. Effects of protein kinase inhibitors on the in vitro growth of Babesia bovis. *Parasitology* (2006) 132:775. doi:10.1017/s0031182006009917
69. Okubo K, Wilawan P, Bork S, Okamura M, Yokoyama N, Igarashi I. Calcium-ions are involved in erythrocyte invasion by equine *Babesia* parasites. *Parasitology* (2006) 133:289. doi:10.1017/s0031182006000436
70. Mossaad E, Asada M, Nakatani D, Inoue N, Yokoyama N, Kaneko O, et al. Calcium ions are involved in egress of *Babesia bovis* merozoites from bovine erythrocytes. *J Vet Med Sci* (2015) 77:53–8. doi:10.1292/jvms.14-0391
71. Agarwal S, Singh MK, Garg S, Chitnis CE, Singh S. Ca2+-mediated exocytosis of subtilisin-like protease 1: a key step in egress of *Plasmodium falciparum* merozoites. *Cell Microbiol* (2012) 15:910–21. doi:10.1111/cmi.12086
72. Baumgartner M. Constitutive exclusion of Csk from Hck-positive membrane microdomains permits Src kinase-dependent proliferation of *Theileria*-transformed B lymphocytes. *Blood* (2002) 101:1874–81. doi:10.1182/blood-2002-02-0456
73. Gonzalez V, Combe A, David V, Malmquist NA, Delorme V, Leroy C, et al. Host cell entry by apicomplexa parasites requires actin polymerization in the host cell. *Cell Host Microbe* (2009) 5:259–72. doi:10.1016/j.chom.2009.01.011
74. Chen XM, Splinter PL, Tietz PS, Huang BQ, Billadeau DD, LaRusso NF. Phosphatidylinositol 3-kinase and frabin mediate *Cryptosporidium parvum* cellular invasion via activation of Cdc42. *J Biol Chem* (2004) 279:31671–8. doi:10.1074/jbc.M401592200
75. Shaw MK. *Theileria parva* sporozoite entry into bovine lymphocytes involves both parasite and host cell signal transduction processes. *Exp Parasitol* (1996) 84:344–54. doi:10.1006/expr.1996.0123
76. Hashim A, Mulcahy G, Bourke B, Clyne M. Interaction of *Cryptosporidium hominis* and *Cryptosporidium parvum* with primary human and bovine intestinal cells. *Infect Immun* (2005) 74:99–107. doi:10.1128/iai.74.1.99-107.2006
77. Heussler VT, Küenzi P, Rottenberg S. Inhibition of apoptosis by intracellular protozoan parasites. *Int J Parasitol* (2001) 31:1166–76. doi:10.1016/s0020-7519(01)00271-5
78. Molestina RE, Sinai AP. Detection of a novel parasite kinase activity at the *Toxoplasma gondii* parasitophorous vacuole membrane capable of phosphorylating host IkappaBalpha. *Cell Microbiol* (2005) 7:351–62. doi:10.1111/j.1462-5822.2004.00463.x
79. Chen XM, Levine SA, Splinter PL, Tietz PS, Ganong AL, Jobin C, et al. *Cryptosporidium parvum* activates nuclear factor kappaB in biliary epithelia preventing epithelial cell apoptosis. *Gastroenterology* (2001) 120:1774–83. doi:10.1053/gast.2001.24850
80. Haidar M, Whitworth J, Noé G, Liu WQ, Vidal M, Langsley G. TGF-β2 induces Grb2 to recruit PI3-K to TGF-RII that activates JNK/AP-1-signaling and augments invasiveness of Theileria-transformed macrophages. *Sci Rep* (2015) 5:15688. doi:10.1038/srep15688
81. Kim L. *Toxoplasma gondii* triggers Gi-dependent PI 3-kinase signaling required for inhibition of host cell apoptosis. *J Cell Sci* (2006) 119:2119–26. doi:10.1242/jcs.02934
82. Dessauge F, Lizundia R, Langsley G. Constitutively activated CK2 potentially plays a pivotal role in *Theileria*-induced lymphocyte transformation. *Parasitology* (2005) 130:S37. doi:10.1017/s0031182005008140
83. Laliberté J, Carruthers VB. Host cell manipulation by the human pathogen *Toxoplasma gondii*. *Cell Mol Life Sci* (2008) 65:1900–15. doi:10.1007/s00018-008-7556-x
84. Olias P, Etheridge RD, Zhang Y, Holtzman MJ, Sibley LD. Toxoplasma effector recruits the Mi-2/NuRD complex to repress STAT1 transcription and block IFN-γ-dependent gene expression. *Cell Host Microbe* (2016) 20:72–82. doi:10.1016/j.chom.2016.06.006
85. Lean I-S, McDonald SAC, Bajaj-Elliott M, Pollok RCG, Farthing MJG, McDonald V. Interleukin-4 and transforming growth factor have opposing regulatory effects on gamma interferon-mediated inhibition of *Cryptosporidium parvum* reproduction. *Infect Immun* (2003) 71:4580–5. doi:10.1128/iai.71.8.4580-4585.2003
86. Haller D, Mackiewicz M, Gerber S, Beyer D, Kullmann B, Schneider I, et al. Cytoplasmic sequestration of p53 promotes survival in leukocytes transformed by *Theileria*. *Oncogene* (2010) 29:3079–86. doi:10.1038/onc.2010.61
87. Kaushansky A, Ye AS, Austin LS, Mikolajczak SA, Vaughan AM, Camargo N, et al. Suppression of host p53 is critical for *Plasmodium* liver-stage infection. *Cell Rep* (2013) 3:630–7. doi:10.1016/j.celrep.2013.02.010
88. Bougdour A, Durandau E, Brenier-Pinchart M-P, Ortet P, Barakat M, Kieffer S, et al. Host cell subversion by *Toxoplasma* GRA16, an Exported dense granule

protein that targets the host cell nucleus and alters gene expression. *Cell Host Microbe* (2013) 13:489–500. doi:10.1016/j.chom.2013.03.002
89. Lizundia R. c-Jun NH2-terminal Kinase/c-Jun signaling promotes survival and metastasis of B lymphocytes transformed by *Theileria*. *Cancer Res* (2006) 66:6105–10. doi:10.1158/0008-5472.can-05-3861
90. Liu J, Deng M, Lancto CA, Abrahamsen MS, Rutherford MS, Enomoto S. Biphasic modulation of apoptotic pathways in *Cryptosporidium parvum*-infected human intestinal epithelial cells. *Infect Immun* (2009) 77:837–49. doi:10.1128/IAI.00955-08
91. Brumlik MJ, Pandeswara S, Ludwig SM, Jeansonne DP, Lacey MR, Murthy K, et al. TgMAPK1 is a *Toxoplasma gondii* MAP kinase that hijacks host MKK3 signals to regulate virulence and interferon-γ-mediated nitric oxide production. *Exp Parasitol* (2013) 134:389–99. doi:10.1016/j.exppara.2013.03.016
92. Muñoz-Caro T, Lendner M, Daugschies A, Hermosilla C, Taubert A. NADPH oxidase, MPO, NE, ERK1/2, p38 MAPK and Ca^{2+} influx are essential for *Cryptosporidium parvum*-induced NET formation. *Dev Comp Immunol* (2015) 52:245–54. doi:10.1016/j.dci.2015.05.007
93. Li Z-Y, Wang Z-D, Huang S-Y, Zhu X-Q, Liu Q. TgERK7 is involved in the intracellular proliferation of *Toxoplasma gondii*. *Parasitol Res* (2016) 115:3419–24. doi:10.1007/s00436-016-5103-5
94. Duszenko M, Ginger ML, Brennand A, Gualdrón-López M, Colombo MI, Coombs GH, et al. Autophagy in protists. *Autophagy* (2011) 7:127–58. doi:10.4161/auto.7.2.13310
95. Muniz-Feliciano L, Van Grol J, Portillo J-AC, Liew L, Liu B, Carlin CR, et al. *Toxoplasma gondii*-induced activation of EGFR prevents autophagy protein-mediated killing of the parasite. *PLoS Pathog* (2013) 9:e1003809. doi:10.1371/journal.ppat.1003809
96. Metheni M, Echebli N, Chaussepied M, Ransy C, Chéreau C, Jensen K, et al. The level of H2O2type oxidative stress regulates virulence of *Theileria*-transformed leukocytes. *Cell Microbiol* (2013) 16:269–79. doi:10.1111/cmi.12218
97. Treeck M, Sanders JL, Gaji RY, LaFavers KA, Child MA, Arrizabalaga G, et al. The calcium-dependent protein kinase 3 of *Toxoplasma* influences basal calcium levels and functions beyond egress as revealed by quantitative phosphoproteome analysis. *PLoS Pathog* (2014) 10:e1004197. doi:10.1371/journal.ppat.1004197
98. Hong S, Kim J-H, Yoon S, Kim K, Sim S, Park WY, et al. Expression of *Cryptosporidium parvum* thioredoxin peroxidase in COS-7 cells confers radioprotection. *Exp Parasitol* (2016) 163:8–15. doi:10.1016/j.exppara.2016.01.012
99. Bosch SS, Kronenberger T, Meissner KA, Zimbres FM, Stegehake D, Izui NM, et al. Oxidative stress control by apicomplexan parasites. *Biomed Res Int* (2015) 2015:351289. doi:10.1155/2015/351289
100. Durrani Z, Weir W, Pillai S, Kinnaird J, Shiels B. Modulation of activation-associated host cell gene expression by the apicomplexan parasite *Theileria annulata*. *Cell Microbiol* (2012) 14:1434–54. doi:10.1111/j.1462-5822.2012.01809.x
101. Chiwakata CB, Hemmer CJ, Dietrich M. High levels of inducible nitric oxide synthase mRNA are associated with increased monocyte counts in blood and have a beneficial role in *Plasmodium falciparum* malaria. *Infect Immun* (2000) 68:394–9. doi:10.1128/IAI.68.1.394-399.2000
102. Goff WL, Johnson WC, Cluff CW. *Babesia bovis* immunity: *in vitro* and *in vivo* evidence for IL-10 regulation of IFN-γ and iNOS. *Ann N Y Acad Sci* (1998) 849:161–80. doi:10.1111/j.1749-6632.1998.tb11046.x
103. Metheni M, Lombès A, Bouillaud F, Batteux F, Langsley G. HIF-1α induction, proliferation and glycolysis of *Theileria*-infected leukocytes. *Cell Microbiol* (2015) 17:467–72. doi:10.1111/cmi.12421
104. Menendez MT, Teygong C, Wade K, Florimond C, Blader IJ. siRNA screening identifies the host hexokinase 2 (HK2) gene as an important hypoxia-inducible transcription factor 1 (HIF-1) target gene in *Toxoplasma gondii*-infected cells. *MBio* (2015) 6:e00462. doi:10.1128/mbio.00462-15
105. Chandramohanadas R, Davis PH, Beiting DP, Harbut MB, Darling C, Velmourougane G, et al. Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. *Science* (2009) 324:794–7. doi:10.1126/science.1171085
106. Jongejan F, Uilenberg G. The global importance of ticks. *Parisitology* (2004) 129:3–14. doi:10.1017/S0031182004005967
107. George N, Bhandari V, Reddy DP, Sharma P. Molecular and phylogenetic analysis revealed new genotypes of *Theileria annulata* parasites from India. *Parasit Vectors* (2015) 8:468. doi:10.1186/s13071-015-1075-z
108. Shaw MK. Cell invasion by *Theileria* sporozoites. *Trends Parasitol* (2003) 19:2–6. doi:10.1016/s1471-4922(02)00015-6
109. Shiels B, Langsley G, Weir W, Pain A, McKellar S, Dobbelaere D. Alteration of host cell phenotype by *Theileria annulata* and *Theileria parva*: mining for manipulators in the parasite genomes. *Int J Parasitol* (2006) 36:9–21. doi:10.1016/j.ijpara.2005.09.002
110. Mans BJ, Pienaar R, Latif AA. A review of *Theileria* diagnostics and epidemiology. *Int J Parasitol* (2015) 4:104–18. doi:10.1016/j.ijppaw.2014.12.006
111. Shaw MK, Tilney LG, Musoke AJ. The entry of *Theileria parva* sporozoites into bovine lymphocytes: evidence for MHC class I involvement. *J Cell Biol* (1991) 113:87–101. doi:10.1083/Jcb.113.1.87
112. Shaw MK. The same but different: the biology of *Theileria* sporozoite entry into bovine cells. *Int J Parasitol* (1997) 27:457–74. doi:10.1016/s0020-7519(97)00015-5
113. Dobbelaere DAE, Fernandez PC, Heussler VT. *Theileria parva*: taking control of host cell proliferation and survival mechanisms. *Cell Microbiol* (2000) 2:91–9. doi:10.1046/j.1462-5822.2000.00045.x
114. Dobbelaere DAE, Küenzi P. The strategies of the *Theileria* parasite: a new twist in host-pathogen interactions. *Curr Opin Immunol* (2004) 16:524–30. doi:10.1016/j.coi.2004.05.009
115. Dessauge F, Hilaly S, Baumgartner M, Blumen B, Werling D, Langsley G. c-Myc activation by *Theileria* parasites promotes survival of infected B-lymphocytes. *Oncogene* (2005) 24:1075–83. doi:10.1038/sj.onc.1208314
116. Tretina K, Gotia HT, Mann DJ, Silva JC. *Theileria*-transformed bovine leukocytes have cancer hallmarks. *Trends Parasitol* (2015) 31:306–14. doi:10.1016/j.pt.2015.04.001
117. Marsolier J, Perichon M, DeBarry JD, Villoutreix BO, Chluba J, Lopez T, et al. *Theileria* parasites secrete a prolyl isomerase to maintain host leukocyte transformation. *Nature* (2015) 520:378–82. doi:10.1038/nature14044
118. Wiens O, Xia D, von Schubert C, Wastling JM, Dobbelaere DAE, Heussler VT, et al. Cell cycle-dependent phosphorylation of *Theileria annulata* schizont surface proteins. *PLoS One* (2014) 9:e103821. doi:10.1371/journal.pone.0103821
119. Swan DG, Stern R, McKellar S, Phillips K, Oura CA, Karagenc TI, et al. Characterisation of a cluster of genes encoding *Theileria annulata* AT hook DNA-binding proteins and evidence for localisation to the host cell nucleus. *J Cell Sci* (2001) 114:2747–54.
120. Shiels BR, McKellar S, Katzer F, Lyons K, Kinnaird J, Ward C, et al. A Theileria annulata DNA binding protein localized to the host cell nucleus alters the phenotype of a bovine macrophage cell line. *Eukaryot Cell* (2004) 3:495–505. doi:10.1128/ec.3.2.495-505.2004
121. Marsolier J, Pineau S, Medjkane S, Perichon M, Yin Q, Flemington E, et al. OncomiR addiction is generated by a miR-155 feedback loop in *Theileria*-transformed leukocytes. *PLoS Pathog* (2013) 9:e1003222. doi:10.1371/journal.ppat.1003222
122. Dobbelaere D, Heussler V. Transformation of leukocytes by *Theileria parva* and *T. annulata*. *Annu Rev Microbiol* (1999) 53:1–42. doi:10.1146/annurev.micro.53.1.1
123. Ahmed JS, Schnittger L, Mehlhorn H. Review: *Theileria* schizonts induce fundamental alterations in their host cells. *Parasitol Res* (1999) 85:527–38. doi:10.1007/s004360050592
124. Frénal K, Soldati-Favre D. Role of the parasite and host cytoskeleton in apicomplexa parasitism. *Cell Host Microbe* (2009) 5:602–11. doi:10.1016/j.chom.2009.05.013
125. De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, et al. Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. *Nature* (2001) 414:308–13. doi:10.1038/35104560
126. Chaussepied M, Janski N, Baumgartner M, Lizundia R, Jensen K, Weir W, et al. TGF-b2 induction regulates invasiveness of *Theileria*-transformed leukocytes and disease susceptibility. *PLoS Pathog* (2010) 6:e1001197. doi:10.1371/journal.ppat.1001197
127. Haidar M, Echebli N, Ding Y, Kamau E, Langsley G. Transforming growth factor β2 promotes transcription of COX2 and EP4, leading to a prostaglandin E2-driven autostimulatory loop that enhances virulence of *Theileria annulata*-transformed macrophages. *Infect Immun* (2015) 83:1869–80. doi:10.1128/iai.02975-14
128. Botteron C, Dobbelaere D. AP-1 and ATF-2 are constitutively activated via the JNK pathway in *Theileria parva*-transformed T-cells. *Biochem Biophys Res Commun* (1998) 246:418–21. doi:10.1006/bbrc.1998.8635

129. Chaussepied M, Lallemand D, Moreau M-F, Adamson R, Hall R, Langsley G. Upregulation of Jun and Fos family members and permanent JNK activity lead to constitutive AP-1 activation in *Theileria*-transformed leukocytes. *Mol Biochem Parasitol* (1998) 94:215–26. doi:10.1016/s0166-6851(98)00070-x
130. Lizundia R, Chaussepied M, Naissant B, Masse GX, Quevillon E, Michel F, et al. The JNK/AP-1 pathway upregulates expression of the recycling endosome rab11a gene in B cells transformed by *Theileria*. *Cell Microbiol* (2007) 9:1936–45. doi:10.1111/j.1462-5822.2007.00925.x
131. Hakimi M, Cannella D. Apicomplexan parasites and subversion of the host cell microRNA pathway. *Trends Parasitol* (2011) 27:481–6. doi:10.1016/j.pt.2011.07.001
132. Baylis H. Infection with *Theileria annulata* induces expression of matrix metalloproteinase 9 and transcription factor AP-1 in bovine leucocytes. *Mol Biochem Parasitol* (1995) 69:211–22. doi:10.1016/0166-6851(94)00216-a
133. Adamson R, Logan M, Kinnaird J, Langsley G, Hall R. Loss of matrix metalloproteinase 9 activity in *Theileria annulata*-attenuated cells is at the transcriptional level and is associated with differentially expressed AP-1 species. *Mol Biochem Parasitol* (2000) 106:51–61. doi:10.1016/s0166-6851(99)00213-3
134. Guergnon J, Chaussepied M, Sopp P, Lizundia R, Moreau M-F, Blumen B, et al. A tumour necrosis factor alpha autocrine loop contributes to proliferation and nuclear factor-kappaB activation of *Theileria parva*-transformed B cells. *Cell Microbiol* (2003) 5:709–16. doi:10.1046/j.1462-5822.2003.00314.x
135. Heussler V, Sturm A, Langsley G. Regulation of host cell survival by intracellular *Plasmodium* and *Theileria* parasites. *Parasitology* (2006) 132:S49. doi:10.1017/s0031182006000850
136. Khwaja A. Akt is more than just a bad kinase. *Nature* (1999) 401:33–4. doi:10.1038/43354
137. Fujioka S, Niu J, Schmidt C, Sclabas GM, Peng B, Uwagawa T, et al. NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. *Mol Cell Biol* (2004) 24:7806–19. doi:10.1128/MCB.24.17.7806-7819.2004
138. Medjkane S, Perichon M, Marsolier J, Dairou J, Weitzman JB. *Theileria* induces oxidative stress and HIF1α activation that are essential for host leukocyte transformation. *Oncogene* (2013) 33:1809–17. doi:10.1038/onc.2013.134
139. Medjkane S, Weitzman JB. A reversible Warburg effect is induced by Theileria parasites to transform host leukocytes. *Cell Cycle* (2013) 12:2167–8. doi:10.4161/cc.25540
140. Vasudevan KM, Gurumurthy S, Rangnekar VM. Suppression of PTEN expression by NF-B prevents apoptosis. *Mol Cell Biol* (2004) 24:1007–21. doi:10.1128/mcb.24.3.1007-1021.2004
141. Xie Y, Naizabekov S, Chen Z, Tokay T. Power of PTEN/AKT: molecular switch between tumor suppressors and oncogenes (Review). *Oncol Lett* (2016) 12:375–8. doi:10.3892/ol.2016.4636
142. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* (2000) 30:1217–58. doi:10.1016/s0020-7519(00)00124-7
143. Vargas-Villavicencio JA, Besné-Mérida A, Correa D. Vertical transmission and fetal damage in animal models of congenital toxoplasmosis: a systematic review. *Vet Parasitol* (2016) 223:195–204. doi:10.1016/j.vetpar.2016.04.024
144. Berghold C, Herzog SA, Jakse H, Berghold A. Prevalence and incidence of toxoplasmosis: a retrospective analysis of mother-child examinations, Styria, Austria, 1995 to 2012. *Euro Surveill* (2016) 21:30317. doi:10.2807/1560-7917.es.2016.21.33.30317
145. Elsheikha HM, Büsselberg D, Zhu X-Q. The known and missing links between *Toxoplasma gondii* and schizophrenia. *Metab Brain Dis* (2016) 31:749–59. doi:10.1007/s11011-016-9822-1
146. Torgerson PR, Mastroiacovo P. The global burden of congenital toxoplasmosis: a systematic review. *Bull World Health Organ* (2013) 91:501–8. doi:10.2471/blt.12.111732
147. Furtado J, Smith J, Belfort R, Gattey D, Winthrop K. Toxoplasmosis: a global threat. *J Glob Infect Dis* (2011) 3:281. doi:10.4103/0974-777x.83536
148. Jones JL, Dubey JP. Foodborne toxoplasmosis. *Clin Infect Dis* (2012) 55:845–51. doi:10.1093/cid/cis508
149. Dubey JP. History of the discovery of the life cycle of *Toxoplasma gondii*. *Int J Parasitol* (2009) 39:877–82. doi:10.1016/j.ijpara.2009.01.005
150. Dubey JP. Toxoplasma gondii. *Medical Microbiology* (1996). p. 1–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21413265
151. Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. *Int J Parasitol* (1998) 28:1019–24. doi:10.1016/s0020-7519(98)00023-x
152. Dubey JP. Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet Parasitol* (2006) 140:69–75. doi:10.1016/j.vetpar.2006.03.018
153. Sibley LD. Intracellular parasite invasion strategies. *Science* (2004) 304:248–53. doi:10.1126/science.1094717
154. Pollard AM, Onatolu KN, Hiller L, Haldar K, Knoll LJ. Highly polymorphic family of glycosylphosphatidylinositol-anchored surface antigens with evidence of developmental regulation in *Toxoplasma gondii*. *Infect Immun* (2007) 76:103–10. doi:10.1128/iai.01170-07
155. Blader IJ, Saeij JP. Communication between *Toxoplasma gondii* and its host: impact on parasite growth, development, immune evasion, and virulence. *APMIS* (2009) 117:458–76. doi:10.1111/j.1600-0463.2009.02453.x
156. Lourido S, Tang K, Sibley LD. Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. *EMBO J* (2012) 31:4524–34. doi:10.1038/emboj.2012.299
157. Bonhomme A, Bouchot A, Pezzella N, Gomez J, Le Moal H, Pinon JM. Signaling during the invasion of host cells by *Toxoplasma gondii*. *FEMS Microbiol Rev* (1999) 23:551–61. doi:10.1016/S0168-6445(99)00021-2
158. Heintzelman MB. Gliding motility in apicomplexan parasites. *Semin Cell Dev Biol* (2015) 46:135–42. doi:10.1016/j.semcdb.2015.09.020
159. Alexander DL, Mital J, Ward GE, Bradley P, Boothroyd JC. Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. *PLoS Pathog* (2005) 1:e17. doi:10.1371/journal.ppat.0010017
160. Straub KW, Cheng SJ, Sohn CS, Bradley PJ. Novel components of the apicomplexan moving junction reveal conserved and coccidia-restricted elements. *Cell Microbiol* (2009) 11:590–603. doi:10.1111/j.1462-5822.2008.01276.x
161. Lamarque M, Besteiro S, Papoin J, Roques M, Vulliez-Le Normand B, Morlon-Guyot J, et al. The RON2-AMA1 interaction is a critical step in moving junction-dependent invasion by apicomplexan parasites. *PLoS Pathog* (2011) 7:e1001276. doi:10.1371/journal.ppat.1001276
162. Poukchanski A, Fritz HM, Tonkin ML, Treeck M, Boulanger MJ, Boothroyd JC. *Toxoplasma gondii* sporozoites invade host cells using two novel paralogues of RON2 and AMA1. *PLoS One* (2013) 8:e70637. doi:10.1371/journal.pone.0070637
163. Etheridge RD, Alaganan A, Tang K, Lou HJ, Turk BE, Sibley LD. The *Toxoplasma* pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host Microbe* (2014) 15:537–50. doi:10.1016/j.chom.2014.04.002
164. Hakimi M-A, Olias P, Sibley LD. *Toxoplasma* effectors targeting host signaling and transcription. *Clin Microbiol Rev* (2017) 30:615–45. doi:10.1128/CMR.00005-17
165. Blader IJ, Koshy AA. *Toxoplasma gondii* development of its replicative niche: in its host cell and beyond. *Eukaryot Cell* (2014) 13:965–76. doi:10.1128/EC.00081-14
166. Hwang I-Y, Quan JH, Ahn M-H, Hassan Ahmed HA, Cha G-H, Shin D-W, et al. *Toxoplasma gondii* infection inhibits the mitochondrial apoptosis through induction of Bcl-2 and HSP70. *Parasitol Res* (2010) 107:1313–21. doi:10.1007/s00436-010-1999-3
167. Wang Y, Weiss LM, Orlofsky A. Intracellular parasitism with *Toxoplasma gondii* stimulates mammalian-target-of-rapamycin-dependent host cell growth despite impaired signalling to S6K1 and 4E-BP1. *Cell Microbiol* (2009) 11:983–1000. doi:10.1111/j.1462-5822.2009.01305.x
168. Wang Y. *Modulation of Host Cell mTOR Signaling by Toxoplasma gondii*. New York: Yeshiva University, ProQuest Dissertations Publishing (2010). 3385052 p.
169. Rosowski EE, Lu D, Julien L, Rodda L, Gaiser RA, Jensen KDC, et al. Strain-specific activation of the NF-κB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *J Exp Med* (2011) 208:195–212. doi:10.1084/jem.20100717
170. Butcher BA, Fox BA, Rommereim LM, Kim SG, Maurer KJ, Yarovinsky F, et al. *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog* (2011) 7:e1002236. doi:10.1371/journal.ppat.1002236
171. Jensen KDC, Wang Y, Wojno EDT, Shastri AJ, Hu K, Cornel L, et al. *Toxoplasma* polymorphic effectors determine macrophage polarization and

intestinal inflammation. *Cell Host Microbe* (2011) 9:472–83. doi:10.1016/j.chom.2011.04.015
172. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* (1999) 13:2905–27. doi:10.1101/gad.13.22.2905
173. Gay G, Braun L, Brenier-Pinchart M-P, Vollaire J, Josserand V, Bertini R-L, et al. *Toxoplasma gondii* TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-γ–mediated host defenses. *J Exp Med* (2016) 213:1779–98. doi:10.1084/jem.20160340
174. Peng BW, Lin JY, Zhang T. *Toxoplasma gondii* induces prostaglandin E2 synthesis in macrophages via signal pathways for calcium-dependent arachidonic acid production and PKC-dependent induction of cyclooxygenase-2. *Parasitol Res* (2008) 102:1043–50. doi:10.1007/s00436-007-0873-4
175. Zare-Bidaki M, Assar S, Hakimi H, Abdollahi SH, Nosratabadi R, Kennedy D, et al. TGF-β in toxoplasmosis: friend or foe? *Cytokine* (2016) 86:29–35. doi:10.1016/j.cyto.2016.07.002
176. Wiley M, Sweeney KR, Chan DA, Brown KM, McMurtrey C, Howard EW, et al. *Toxoplasma gondii* activates hypoxia-inducible factor (HIF) by stabilizing the HIF-1α subunit via type I activin-like receptor kinase receptor signaling. *J Biol Chem* (2010) 285:26852–60. doi:10.1074/jbc.m110.147041
177. Blader IJ, Manger ID, Boothroyd JC. Microarray analysis reveals previously unknown changes in *Toxoplasma gondii*-infected human cells. *J Biol Chem* (2001) 276:24223–31. doi:10.1074/jbc.M100951200
178. Spear W, Chan D, Coppens I, Johnson RS, Giaccia A, Blader IJ. The host cell transcription factor hypoxia-inducible factor 1 is required for *Toxoplasma gondii* growth and survival at physiological oxygen levels. *Cell Microbiol* (2006) 8:339–52. doi:10.1111/j.1462-5822.2005.00628.x
179. Braun L, Brenier-Pinchart M-P, Yogavel M, Curt-Varesano A, Curt-Bertini R-L, Hussain T, et al. A *Toxoplasma* dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation. *J Exp Med* (2013) 210:2071–86. doi:10.1084/jem.20130103
180. Xiao J, Li Y, Prandovszky E, Karuppagounder SS, Talbot CC, Dawson VL, et al. MicroRNA-132 dysregulation in *Toxoplasma gondii* infection has implications for dopamine signaling pathway. *Neuroscience* (2014) 268:128–38. doi:10.1016/j.neuroscience.2014.03.015
181. Brooks JM, Carrillo GL, Su J, Lindsay DS, Fox MA, Blader IJ. *Toxoplasma gondii* infections alter GABAergic synapses and signaling in the central nervous system. *MBio* (2015) 6:e01428–15. doi:10.1128/mbio.01428-15
182. Roiko MS, Carruthers VB. New roles for perforins and proteases in apicomplexan egress. *Cell Microbiol* (2009) 11:1444–52. doi:10.1111/j.1462-5822.2009.01357.x
183. Laurent F, McCole D, Eckmann L, Kagnoff MF. Pathogenesis of *Cryptosporidium parvum* infection. *Microbes Infect* (1999) 1:141–8. doi:10.1016/s1286-4579(99)80005-7
184. Di Genova BM, Tonelli RR. Infection strategies of intestinal parasite pathogens and host cell responses. *Front Microbiol* (2016) 7:256. doi:10.3389/fmicb.2016.00256
185. Medema G, Teunis P, Blokker M, Deere D, Charles P, Loret J-F. *EHC Cryptosporidium Draft 2. 1. WHO Guidelines for Drinking Water Quality*. Geneva: World Health Organization Press (2006).
186. Rossle NF, Latif B. Cryptosporidiosis as threatening health problem: a review. *Asian Pac J Trop Biomed* (2013) 3:916–24. doi:10.1016/s2221-1691(13)60179-3
187. Ward H, Cevallos A. *Cryptosporidium*: molecular basis of host-parasite interaction. *Adv Parasitol* (1998) 40:151–85. doi:10.1016/s0065-308x(08)60120-7
188. Bouzid M, Hunter PR, Chalmers RM, Tyler KM. *Cryptosporidium* pathogenicity and virulence. *Clin Microbiol Rev* (2013) 26:115–34. doi:10.1128/cmr.00076-12
189. Forney JR, DeWald DB, Yang S, Speer CA, Healey MC. A role for host phosphoinositide 3-kinase and cytoskeletal remodeling during *Cryptosporidium parvum* infection. *Infect Immun* (1999) 67:844–52.
190. O'Hara SP, Small AJ, Chen XM, Larusso NF. Host cell actin remodeling in response to *Cryptosporidium*. *Subcell Biochem* (2008) 47:92–100. doi:10.1007/978-0-387-78267-6_7
191. Yao L, Yin J, Zhang X, Liu Q, Li J, Chen L, et al. *Cryptosporidium parvum:* identification of a new surface adhesion protein on sporozoite and oocyst by screening of a phage-display cDNA library. *Exp Parasitol* (2007) 115:333–8. doi:10.1016/j.exppara.2006.09.018
192. Singh P, Mirdha BR, Srinivasan A, Rukmangadachar LA, Singh S, Sharma P, et al. Identification of invasion proteins of *Cryptosporidium parvum*. *World J Microbiol Biotechnol* (2015) 31:1923–34. doi:10.1007/s11274-015-1936-9
193. Lendner M, Daugschies A. *Cryptosporidium* infections: molecular advances. *Parasitology* (2014) 141:1511–32. doi:10.1017/s0031182014000237
194. Varughese EA, Kasper S, Anneken EM, Yadav JS. SHP-2 mediates *Cryptosporidium parvum* infectivity in human intestinal epithelial cells. *PLoS One* (2015) 10:e0142219. doi:10.1371/journal.pone.0142219
195. Chen X-M, Huang BQ, Splinter PL, Cao H, Zhu G, Mcniven MA, et al. *Cryptosporidium parvum* invasion of biliary epithelia requires host cell tyrosine phosphorylation of cortactin via c-Src. *Gastroenterology* (2003) 125:216–28. doi:10.1016/s0016-5085(03)00662-0
196. Chen X-M, O'Hara SP, Huang BQ, Splinter PL, Nelson JB, LaRusso NF. Localized glucose and water influx facilitates *Cryptosporidium parvum* cellular invasion by means of modulation of host-cell membrane protrusion. *Proc Natl Acad Sci U S A* (2005) 102:6338–43. doi:10.1073/pnas.0408563102
197. Perez-Cordon G, Nie W, Schmidt D, Tzipori S, Feng H. Involvement of host calpain in the invasion of *Cryptosporidium parvum*. *Microbes Infect* (2011) 13:103–7. doi:10.1016/j.micinf.2010.10.007
198. Keyloun KR, Reid MC, Choi R, Song Y, Fox AMW, Hillesland HK, et al. The gatekeeper residue and beyond: homologous calcium-dependent protein kinases as drug development targets for veterinarian Apicomplexa parasites. *Parasitology* (2014) 141:1499–509. doi:10.1017/s0031182014000857
199. Manque PA, Woehlbier U, Lara AM, Tenjo F, Alves JM, Buck GA. Identification and characterization of a novel calcium-activated apyrase from *Cryptosporidium* parasites and its potential role in pathogenesis. *PLoS One* (2012) 7:e31030. doi:10.1371/journal.pone.0031030
200. Schaumburg F, Hippe D, Vutova P, Lüder CGK, Der CGKL. Pro- and anti-apoptotic activities of protozoan parasites pro-and anti-apoptotic activities of protozoan parasites. *Parasitology* (2006) 132:69–85. doi:10.1017/S0031182006000874
201. Chen XM, Gores GJ, Paya CV, LaRusso NF. *Cryptosporidium parvum* induces apoptosis in biliary epithelia by a Fas/Fas ligand-dependent mechanism. *Am J Physiol* (1999) 277:G599–608.
202. Liu J, Enomoto S, Lancto CA, Abrahamsen MS, Rutherford MS. Inhibition of apoptosis in *Cryptosporidium parvum*-infected intestinal epithelial cells is dependent on survivin. *Infect Immun* (2008) 76(8):3784–92. doi:10.1128/IAI.00308-08
203. Castellanos-Gonzalez A, Yancey LS, Wang H, Pantenburg B, Liscum KR, Lewis DE, et al. *Cryptosporidium* infection of human intestinal epithelial cells increases expression of osteoprotegerin: a novel mechanism for evasion of host defenses. *J Infect Dis* (2008) 197:916–23. doi:10.1086/528374
204. Choudhry N, Korbel DS, Edwards LA, Bajaj-Elliott M, McDonald V. Dysregulation of interferon-γ-mediated signalling pathway in intestinal epithelial cells by *Cryptosporidium parvum* infection. *Cell Microbiol* (2009) 11:1354–64. doi:10.1111/j.1462-5822.2009.01336.x
205. Robinson P, Okhuysen PC, Chappell CL, Lewis DE, Shahab I, Lahoti S, et al. Transforming growth factor beta 1 is expressed in the jejunum after experimental *Cryptosporidium parvum* infection in humans. *Infect Immun* (2000) 68:5405–7. doi:10.1128/iai.68.9.5405-5407.2000
206. Lean I-S, Lacroix-Lamande S, Laurent F, McDonald V. Role of tumor necrosis factor alpha in development of immunity against *Cryptosporidium parvum* infection. *Infect Immun* (2006) 74:4379–82. doi:10.1128/iai.00195-06

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REVIEW
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The Promise of Systems Biology Approaches for Revealing Host Pathogen Interactions in Malaria

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Despite global eradication efforts over the past century, malaria remains a devastating public health burden, causing almost half a million deaths annually (WHO, 2016). A detailed understanding of the mechanisms that control malaria infection has been hindered by technical challenges of studying a complex parasite life cycle in multiple hosts. While many interventions targeting the parasite have been implemented, the complex biology of *Plasmodium* poses a major challenge, and must be addressed to enable eradication. New approaches for elucidating key host-parasite interactions, and predicting how the parasite will respond in a variety of biological settings, could dramatically enhance the efficacy and longevity of intervention strategies. The field of systems biology has developed methodologies and principles that are well poised to meet these challenges. In this review, we focus our attention on the Liver Stage of the *Plasmodium* lifecycle and issue a "call to arms" for using systems biology approaches to forge a new era in malaria research. These approaches will reveal insights into the complex interplay between host and pathogen, and could ultimately lead to novel intervention strategies that contribute to malaria eradication.

Keywords: malaria, plasmodium, liver, systems biology, computational modeling, omics-technologies

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INTRODUCTION

Parasitic diseases infect over half a billion people worldwide, and are a tremendous public health burden. Malaria is the most lethal, causing infection and death primarily in young children in sub Saharan Africa (WHO, 2016). In humans, five *Plasmodium* species are known to cause disease, with the greatest burden arising from infection with *P. falciparum* and *P. vivax*. Despite multifaceted control efforts, the adaptive nature of the *Plasmodium* parasite has confounded vaccine development (Neafsey et al., 2015; Schats et al., 2015), and has contributed to the emergence of widespread drug resistance (reviewed in Blasco et al., 2017).

The life cycle of *Plasmodium* is complex. The parasite cycles between mosquito and mammalian hosts, with elaborate developmental and differentiation processes within each. Every transition represents an opportunity to arrest the parasite, and to stop subsequent life cycle progression. A systematic approach that identifies key components required by the parasite at each stage of its life cycle could ultimately elucidate fundamental pathogenesis strategies, which will

aid the development of cohesive intervention approaches. By contrast, any approach that reduces the biology of the parasite to a single antigen or drug target leaves open the possibility of parasite adaptation and, ultimately, intervention failure. Here, we propose a systems biology approach to interrogate the *Plasmodium* parasite that, although not without its challenges, will result in a global view of the host-parasite interactions during key transition states in the life cycle. This view could inform interventions that are not easily circumvented by the parasite and therefore contribute to malaria eradication.

PLASMODIUM PARASITES HAVE A COMPLEX LIFE CYCLE THAT ENGAGES MULTIPLE HOST ENVIRONMENTS

Plasmodium infection of mammals begins with injection of the sporozoite into the skin of the vertebrate host during the bite of a female *Anopheles* mosquito. After migration through the skin and entrance into a capillary, sporozoites travel through the blood stream to the liver. The parasite then traverses through the sinusoidal barrier to gain access to hepatocytes (Mota et al., 2001; Ishino et al., 2004; Tavares et al., 2013; Cha et al., 2016; Yang et al., 2017). Once within the liver parenchyma, sporozoites infect a host hepatocyte within which they will reside for the next 2–10 days (reviewed in Kaushansky and Kappe, 2015b; Vaughan and Kappe, 2017). Following liver stage development, parasites exit the liver, re-enter the blood stream and infect erythrocytes. During asexual blood stage infection, parasites undergo cycles of replication, followed by destruction of the host cell. It is this cycle that causes disease symptoms.

During the blood stage, a portion of parasites commit to sexual development (Coleman et al., 2014; Kafsack et al., 2014; Sinha et al., 2014; Poran et al., 2017) and initiate a differentiation process that occurs largely in the bone marrow (Joice et al., 2014). Once female and male forms have nearly completed maturation, they re-enter the blood stream and are transmitted to mosquitoes. In the mosquito midgut, fertilization occurs, generating a motile diploid (ookinete), which then replicates its DNA and develops into a stationary oocyst. Sporozoites then form within the midgut oocyst, become motile, and travel to the salivary glands. Once within the salivary glands, the parasite is transmitted to the next mammalian host during a blood meal. Each of these stage transitions is initiated by, and induces, broad, systematic changes that alter cellular behaviors (**Table 1**, **Figure 1**). Yet, these changes cannot be fully represented by any single transcript or individual cellular measurement. Rather, comprehensive changes within interconnected networks occur on multiple scales. This includes changes in gene regulatory networks, protein interactions with other biomolecules, and morphological variation of host and parasite subcellular structures. Together, these changes drive stage transitions. The goal must therefore be to establish a comprehensive picture of the host and parasite effector molecules and networks that are required to facilitate life cycle transitions.

TABLE 1 | Stage transitions in the *Plasmodium* life cycle.

Life cycle stage transition	System-level alteration reported	References
Development from midgut sporozoite to salivary gland sporozoite	Transcriptome changes	Matuschewski et al., 2002; Mikolajczak et al., 2008
Transmission between mosquito and mammalian host	Translational repression	Zhang et al., 2010; Gomes-Santos et al., 2011; Muller et al., 2011; Lindner et al., 2013; Silvie et al., 2014; Silva et al., 2016
Development through Liver Stage	Transcriptome and proteome changes	Tarun et al., 2008; Albuquerque et al., 2009; Vaughan et al., 2009
Exit from Liver Stage and Entry into Blood Stage	Transcriptome changes	Tarun et al., 2008
Differentiation into sexual forms	Epigenetic and Transcriptome changes	Coleman et al., 2014; Kafsack et al., 2014; Sinha et al., 2014; Poran et al., 2017
Transmission from mammalian to mosquito host	Translational repression	Mair et al., 2006; Guerreiro et al., 2014; Lasonder et al., 2016
Gametocyte to gamete transformation	Proteome changes	Khan et al., 2005

PLASMODIUM PARASITES SIGNIFICANTLY ALTER THE BIOLOGY OF THEIR HOSTS

To illustrate the need to comprehensively evaluate changes during the *Plasmodium* life cycle, we will consider one stage of the complex life cycle of the parasite in detail—the Liver Stage of infection. Once within the liver sinusoid, the parasite traverses through phagocytic Kupffer cells, liver-resident macrophages, and liver endothelial sinusoidal cells, to access hepatocytes, while avoiding phagocytosis (Mota et al., 2001; Ishino et al., 2004; Usynin et al., 2007; Tavares et al., 2013; Cha et al., 2016; Yang et al., 2017). Once in the liver parenchyma, the parasite continues to traverse through several hepatocytes before selecting a suitable host for invasion. While the precise properties that make one hepatocyte more hospitable than another remain unknown, altered levels of specific hepatocyte receptors dramatically alter infection rates (Silvie et al., 2003; Ishino et al., 2004; Rodrigues et al., 2008; Yalaoui et al., 2008a; Kaushansky et al., 2015).

Following establishment of an intracellular niche within the hepatocyte, *Plasmodium* replicates extensively, stretching the hepatocyte to 50–100 times its normal volume (Shortt and Garnham, 1948; Vaughan et al., 2012). This rapid expansion is surprising, given the cell's strict cell size regulations under normal conditions (Sinturel et al., 2017). This observation suggests that *Plasmodium* effectively overwrites the hepatocyte's hardwiring to exert massive influence over the host cell. *Plasmodium* likely disrupts a multitude of classical signaling pathways during infection, only a small

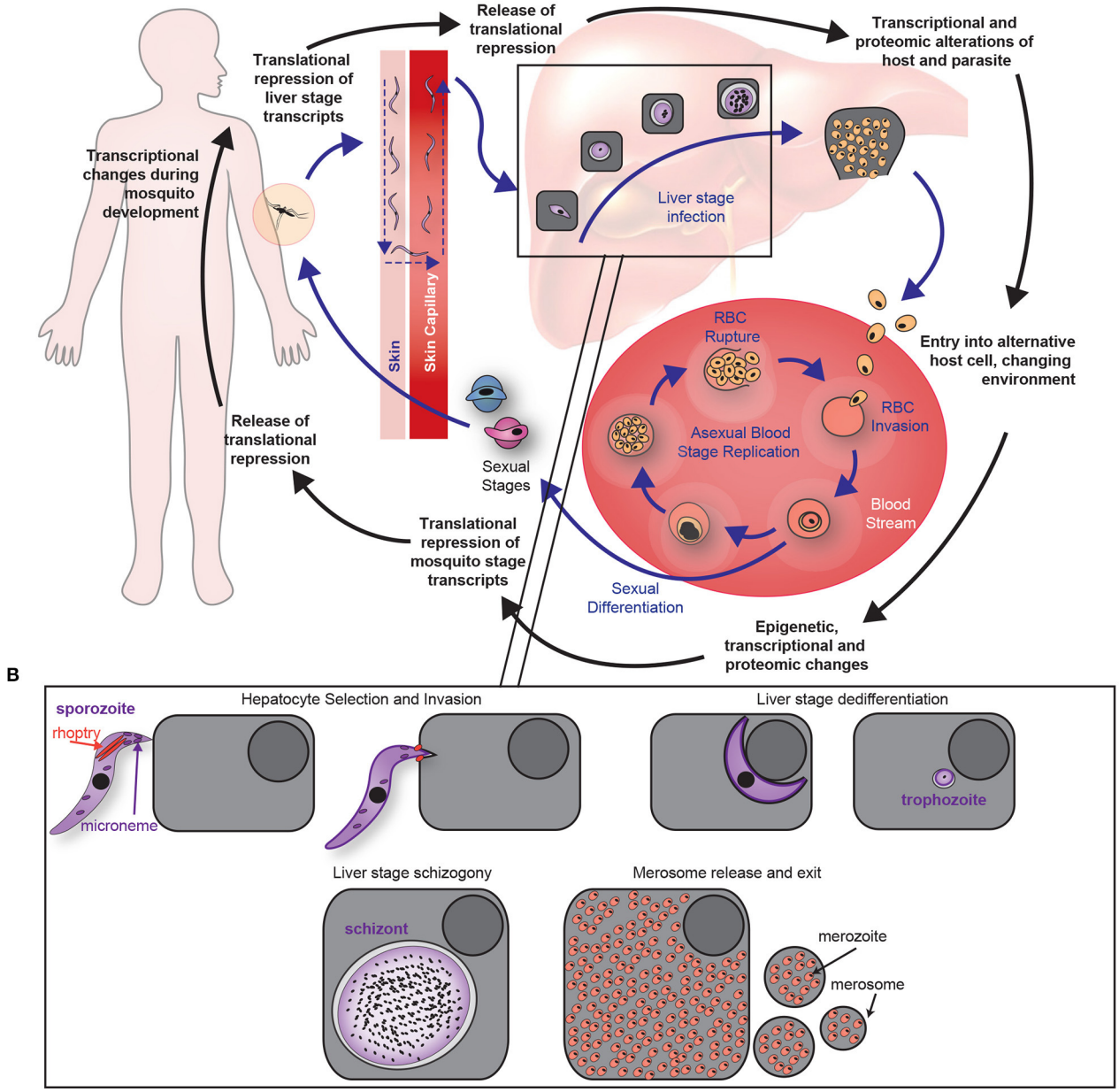

FIGURE 1 | *Plasmodium* life cycle. **(A)** Each stage of the malaria life cycle is accompanied by unique transcriptional or translational changes, which ultimately allow for successful transition to each stage of the life cycle. Red Blood Cell is abbreviated "RBC." **(B)** Liver stage infection of a hepatocyte is a unique microenvironment that allows the parasite to invad and differentiate into several forms to ensure growth, replication, and eventual egress from the hepatocyte. These key transitions occur in specific subcellular locations during liver stage infection.

fraction of which have been described (Kaushansky et al., 2013a,b; Ruivo et al., 2016). Interrogating single proteins in a pathway to determine functionality is limiting in this context, and ignores secondary effects within the complex cell system. Instead, it is critical to comprehensively and quantitatively evaluate changes that occur during infection to illuminate mechanisms of control employed by the parasite.

WHAT CAN SYSTEMS BIOLOGY DO FOR MALARIA?

Understanding biology is a systems-level problem. Interactions between components of a system lead to the emergence of properties that cannot be understood from the study of the components individually. The study of systems biology is predicated on two basic assumptions. First, that the whole

is far greater than the sum of its parts; and second, that a more comprehensive understanding of the components and their relationships within a system will allow for more accurate predictions of the system's behavior. It is through this lens that systems biology aims to determine the relationships and interactions of the components of a system. In practice, systems biology is a set of principles and processes by which we take complex systems apart and put them back together, with the aim of understanding the properties of the entire biological system. The approach generally starts with the systematic and comprehensive identification and quantification of molecules, called omics datasets, as a biological system transitions from one state to another. Initially, these data are evaluated using standard statistical tools, resulting in ordered lists, with significance values for each observed difference. These data provide the basis for the deployment of simple tools such as pathway analysis and clustering to interpret the data, or more complex analysis, such as regression or inference methods, to suggest causal or correlative relationships between components. These approaches can, and have, identified major molecular players at each stage, but fall short of a detailed and comprehensive understanding. Visualizing the data is also important for generating insights and predictive models that describe key determinants of the stage transition being interrogated (**Figure 2**). Predictions that are generated are then tested, often using "classic" or "reductionist" approaches. This process results in the refinement of both the model, and of our biological understanding. Molecular details are important, and systems biology must not ignore them. Examining the individual components of a system allows us to understand their molecular and physicochemical properties, as well as the function of the components in context of the entire system (Van Regenmortel, 2004). As the data that informs a model becomes more detailed, the predictions generated become more mechanistic. This level of insight is critical for rational intervention.

Modeling is not unique to systems biology. Indeed, all scientists generate "models," sometimes in the form of cartoons to aid in the design of the next line of inquiry. Systems biology models are often in the form of networks composed of balls and sticks, where balls (also called nodes) represent genes or proteins, and the sticks (or lines, also called edges) between them represent a relationship between molecular players. These simple visualizations can themselves facilitate the development of novel hypotheses. Many representations allow scientists to superimpose multiple types of data onto these networks (for example, molecule types, confidence of the interactions or subcellular organization). There are many popular and facile tools for these network visualizations (reviewed in Gehlenborg et al., 2010; Pavlopoulos et al., 2017). Once established, these networks can be mined to design subsequent experiments, and also used as a foundation for more complex models of the dynamics of molecular interactions and information flow. Depending on the complexity and the question to be addressed, models can take many forms. Some widely used approaches include Boolean networks, ordinary differential equations, and stochastic simulations. Different model classes involve different approximations, assumptions and levels of granularity. All models are best informed by quantitative, high quality, and biochemical data. While omics approaches can contribute to a more comprehensive view than possible with classical biochemical approaches, many models also incorporate data from rigorous reductionist approaches. Regardless of model class, the power of modeling lies in its capacity to capture insights that are difficult to reach through intuition alone.

Many of the technological and computational tools of systems biology are modular, and the resulting data can be integrated in different ways to inform the biological question (Danziger et al., 2014). Indeed, modeling biological data increasingly aims to incorporate a range of types of information, which monitor changes at different scales. This allows the researcher to determine what types of data are most informative when predicting a biological outcome of interest (Hwang et al., 2005a,b; Janes et al., 2005; Bonneau et al., 2007; AlQuraishi et al., 2014) and design subsequent experiments accordingly. Nevertheless, in most applications, applying computational analysis to quantitative datasets enables predictions (or new hypotheses) about how a perturbation, such as a gene deletion, drug treatment, or new environment, will influence the system as a whole.

The Role of Quantitative and Comprehensive Datasets in Malaria Research

In the case of malaria, numerous studies have generated omics data during life cycle transitions (**Table 1**). These include the cataloging of genes (genomics), mRNA transcripts (transcriptomics), translated protein (proteomics), metabolites (metabolomics), and translational repression/de-repression of transcripts as the parasite transitions through its life cycle stages. The genome of the *Plasmodium* parasite was initially published in 2002 (Gardner et al., 2002), and has been refined since. Initial transcriptomes and proteomes of *P. yoelii* and *P. berghei* Liver Stages have been generated, which have provided lists of the components involved in liver stage development, and further revealed the requirement of fatty acid synthesis from both the parasite and host during liver infection (Tarun et al., 2008; Albuquerque et al., 2009; Vaughan et al., 2009).

Insights that originate from transcriptomic analysis of Liver Stage infection reveal that Type I interferons and ER stress are systematically upregulated during liver stage infection and can modulate the level of liver stage infection (Liehl et al., 2014; Miller et al., 2014; Inacio et al., 2015; Kaushansky and Kappe, 2015a). Additional information can be obtained by monitoring changes in the parasite and host during infection under different environmental conditions. These, and related datasets can inform models that predict causality and cellular outcomes. The goal of this effort would be to identify networks of parasite and/or host factors that facilitate the development or demise of the parasite during its infection of the liver.

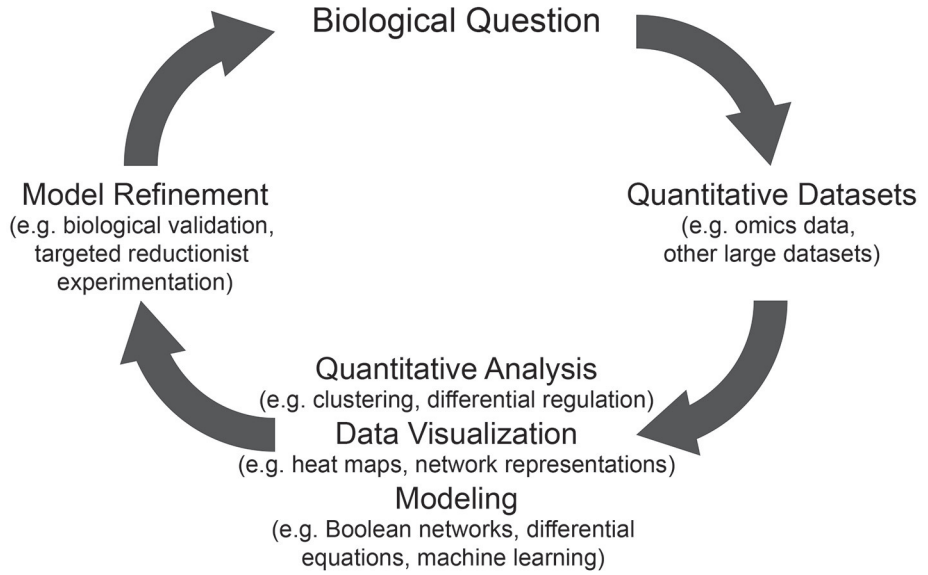

FIGURE 2 | The processes involved in generating systems biology-informed models. To inform the biological question, quantitative datasets are generated, which are then used in quantitative analysis, data visualization, and modeling to describe how the system behaves. These tools can be used interchangeably and/or in succession before further refining the model. Refinement of the model can then provide new biological insights.

Protein-Protein Interactions

A major goal of host-pathogen studies is to elucidate specific interactions that dictate success or failure of the pathogen. While transcriptomics and proteomics can catalog changes that occur in the infected cell, a list of alterations alone does not provide mechanistic insight, and is unsatisfying to most cell biologists. Databases of protein interactions in many organisms, including humans, are becoming highly populated (Hein et al., 2015; Huttlin et al., 2015). Yet, many immunopurification—mass-spectrometry (IP-MS) based approaches to study protein-protein interactions do not meet the standard of quantitative and comprehensive. As datasets become larger, statistical tools can be used to predict which interactions are more likely to be specific, compared to commonly identified (abundant or promiscuous) proteins (Mellacheruvu et al., 2013). The pitfalls of qualitative and low throughput data have been partially overcome in model organisms such as yeast, where whole-genome GFP tag libraries have been generated and used in IP-MS experiments, although even these datasets remain incomplete, and are error prone (Ghaemmaghami et al., 2003; Huh et al., 2003; Mellacheruvu et al., 2013).

More sophisticated approaches designed to distinguish between bona fide and spurious interactions are being developed and applied. For example, Isotopic Differentiation of Interactions as Random or Targeted (I-DIRT) and variants (Tackett et al., 2005; Selbach and Mann, 2006; Trinkle-Mulcahy et al., 2008; Byrum et al., 2012; Trinkle-Mulcahy, 2012) exploit isotopic labeling and immunopurification to distinguish between interactions that occur before cell lysis, from those interactions that are introduced during the purification process. While these approaches improve confidence in interactions, they are neither widely adopted, nor have they been applied in genome scale studies.

The Role of Imaging in Defining Quantitative Stage Transitions

Since the initial discovery of liver stage parasites by microscopy in 1948 (Shortt and Garnham, 1948), imaging has been an invaluable tool of malaria research. However, most common imaging methods are neither quantitative nor comprehensive, limiting their capacity to inform modeling approaches. This is particularly troubling for applying a systems biology approach, as cellular outcomes are what we aim to predict, but are often poorly defined. Traditional imaging also falls short of reaching the temporal resolution necessary to elucidate the dynamic cellular processes during invasion and throughout liver stage infection.

A number of new imaging modalities enhance our ability to increase resolution, quantification and throughput. A comprehensive review of the advances made in increasing throughput, quantification and resolution in the imaging field is outside the scope of this review, we will highlight some examples that are particularly relevant to malaria research. One example, correlated light microscopy and electron microscopy (CLEM) combines fluorescence microscopy with electron microscopy, thereby increasing the throughput of monitoring rare events like liver stage infection at EM-level resolution (van Rijnsoever et al., 2008), and has already been applied to monitor liver stage development (Grutzke et al., 2014). Intravital imaging (IVM) has been adapted for malaria research and facilitates analysis of live tissue with microscopic resolution to reveal cellular responses that closely mimic *in vivo* infection, both spatially and temporally

(Pittet and Weissleder, 2011; De Niz et al., 2017). Additional instrumentation, such as the Lattice Light Sheet Microscope (Betzig et al., 2006), enhances temporal and spatial resolution, with applications in both *in vivo* and *in vitro* systems, which could enable a more quantitative assessment of cellular outcomes.

The Power of New Genetic Tools and Screens in Determining Function

An essential component of systems biology is the experimental testing of predictions made by modeling efforts. This testing is greatly assisted by the capacity to perform genetic perturbations. Indeed, one of the major shortfalls of employing systems biology is that testing predictions is largely performed by single candidate-based approaches, and thus often fails to recapitulate the complexity of the system. In many cases, it remains difficult to determine if the model is incorrect, or if reductionist approaches cannot fully capture the emergent properties associated with a complex system. New genome-editing approaches, like CRISPR/Cas, can assess multiple perturbations in combination, in both mammalian and parasite genomes, which will facilitate testing more complex models (Cong et al., 2013; Mali et al., 2013; Ghorbal et al., 2014; Wagner et al., 2014; Lu et al., 2016).

In addition to evaluating individual or groups of gene candidates for function, new genome-editing approaches also have the ability to globally evaluate both host and parasite genes. Whole genome CRISPR/Cas9 knockout screens are now common in mammalian cells (Cong et al., 2013; Mali et al., 2013) and can be adapted to the *Plasmodium* genome. The *Plasmodium* Genetic Modification Project (PlasmoGEM), a new community resource from the Wellcome Trust Sanger Institute, aims to produce new tools for the genetic modification of malaria parasites at genome scale. This resource has already demonstrated that two-thirds of *P. berghei* genes contribute to normal blood stage development (Bushell et al., 2017). Subsequent studies should not only focus on the role of parasite genes in other life cycle stages, but also interrogate the role of host genes during each stage of parasite development.

KEY QUESTIONS AND FINDINGS IN MALARIA LIVER STAGE BIOLOGY

The existing literature provides a basis upon which global experiments can be designed and modeled, and also highlights the most critical questions that remain. Given the potential and increasing power of systems biology, the challenge lies in how to use this approach to bolster the rich collection of findings that have been amassed by the *Plasmodium* research community, and address hurdles that have been unattainable by more traditional approaches. In this next section, we focus on some of the key findings on liver stage malaria with an emphasis on questions that remain.

Hepatocyte Invasion

During hepatocyte invasion, the parasite attaches to the host cell, at least partially through circumsporozoite protein (CSP), which interacts directly with highly sulfated proteoglycans (HSPGs) on the cell surface to trigger CSP cleavage, inducing the sporozoite to switch to an invasive state (**Table 2A**) (Coppi et al., 2007, 2011). Thrombospondin-related anonymous protein (TRAP) is also involved in this process (Kappe et al., 1999; Matuschewski et al., 2002; Morahan et al., 2009). Additionally, *Plasmodium* proteins P36 and P52 play a role in invasion, parasitophorous vacuole membrane (PVM) formation, and protecting the host against apoptosis (Ishino et al., 2005; van Dijk et al., 2005; Ploemen et al., 2012). How each of these factors works in concert to facilitate productive invasion of the hepatocyte remains unknown.

A collection of host factors have also been described to impact parasite infection (**Table 2B**). Scavenger Receptor B1 (SRB1) and the tetraspanin CD81 both play roles in cholesterol-rich microdomain formation and are critical for hepatocyte invasion (Silvie et al., 2003; Rodrigues et al., 2008; Yalaoui et al., 2008a; Valacchi et al., 2011). More recently, it has been described that CD81 and SRB1 are involved in invasion in different species; CD81 is required for *P. yoelii* and *P. falciparum* infection, but appears to be dispensable for *P. berghei* and *P. vivax* infection. SRB1 plays a more substantial role in *P. vivax* and *P. berghei* infections (Silvie et al., 2003; Manzoni et al., 2017). It remains unknown if either protein makes contact with the sporozoite, although it has been suggested that SRB1 might directly engage the parasite, whereas CD81 indirectly impacts infection (Yalaoui et al., 2008b; Manzoni et al., 2017). The receptor tyrosine kinase EphA2 is also critical for hepatocyte infection, at least in part by engaging the parasite protein P36 (Kaushansky et al., 2015). While each of these factors contributes to the infection process, how they work in concert, and how changes in one invasion factor impacts another remain unknown. New approaches that integrate biochemical information and omics datasets are well-suited to merge with existing candidate-based research to create a more comprehensive view of the molecular components required for hepatocyte invasion (AlQuraishi et al., 2014; Gujral et al., 2014). The capacity to integrate biochemical data into a more global framework also paves the way for the identification of molecules or networks that could be targeted for intervention.

Liver Stage Development

Once the parasite has taken up residence in the hepatocyte, the sporozoite dedifferentiates over the course of 12 h in rodents, or 2–3 days in humans. This process results in a rounded trophozoite, which is characterized by dramatic changes in the parasite including the disassembly of molecular and cellular structures and the expulsion of invasion machinery (Bano et al., 2007) (reviewed in Kaushansky and Kappe, 2015b; Vaughan and Kappe, 2017). Following dedifferentiation, schizogony begins, which involves the massive replication the genome, and takes place over the course of 2–10 days, depending on the *Plasmodium* species. During this time, cellular structures including lysosomes and late endosomes sequester around the parasitophorous vacuole membrane and associate with the tubovesicular network (Lopes da Silva et al., 2012; Grutzke et al., 2014). The unfolded protein response is triggered, which promotes endoplasmic reticulum stress and the survival of the Liver Stage parasite (Inacio et al., 2015; Kaushansky and Kappe, 2015a). The most

TABLE 2 | Determinants of hepatocyte liver stage infection: **(A)** *Plasmodium* determinants of infection and **(B)** Host determinants of infection.

Host/Parasite factor	Stage of infection	Main findings	References
(A)			
SPECT	Traversal	Essential for cell traversal	Ishino et al., 2004, 2005
PLP1 (SPECT2)		Essential for cell traversal	Ishino et al., 2004, 2005
CelTOS		Hypothesized to play a role in the exit step of traversal	Kariu et al., 2006
TRAP-like protein (TLP)		TLP-deficient sporozoites show a diminished ability to traverse	Moreira et al., 2008
PL (UIS10)	Hepatocyte Invasion	PL-deficient sporozoites show reduction in Liver Stage burden	Bhanot et al., 2005
Circumsporozoite protein (CSP)		Multiple roles in motility and invasion, including transition from traversing state to invasive state	Coppi et al., 2007
P36		Contributes to PVM formation	Ishino et al., 2005; Labaied et al., 2007
P52/P36p		Contributes to PVM formation	Ishino et al., 2005; Labaied et al., 2007
Cysteine proteases		Inhibition of sporozoite cysteine proteases completely inhibits infectivity	Coppi et al., 2005
Calcium Dependent Protein Kinase-6 (CDPK-6)		Sporozoites from CDPK-6-deficient parasites show decrease in invasion and CSP cleavage	Coppi et al., 2007
TRAP		Direct role in invasion through attachment with cytoplasmic tail	Kappe et al., 1999; Matuschewski et al., 2002; Morahan et al., 2009
Upregulated in Sporozoite 4 (UIS4)	Liver stage Development	UIS4-deficient *P. berghei* parasites severely impaired in Liver Stage development	Mueller et al., 2005
Upregulated in Sporozoite (UIS3)		UIS3-deficient parasites severely impaired in Liver Stage development. UIS3 has been hypothesized to play a role in fatty acid uptake	Mikolajczak et al., 2007
EXP1		Interacts with host Apolipoprotein H to promote liver stage development	Sa et al., 2017
LISP2		Hypothesized to be involved in merozoite formation and exported to host cytosol	Orito et al., 2013
B9		P9 mutants show liver stage growth arrest	Annoura et al., 2014
Sequestrin		Mutants lacking sequestrin show a reduction in liver stage development	Annoura et al., 2014
MSP1		Conditional mutagenesis of MSP1 in sporozoites impaired merozoite formation	Combe et al., 2009
LISP1	Hepatocyte Exit	In *P. berghei*, LISP1 is required for lysis of the PVM prior to egress	Ishino et al., 2009
SUB1		SUB1-deficient *P. berghei* parasites fail to rupture the PVM prior to egress	Tawk et al., 2013
(B)			
CD68	Traversal	Putative receptor of Kupffer cells, gateway for liver stage infection	Cha et al., 2016
Hepatocyte Growth Factor	Hepatocyte Invasion	Secretion of HGF renders *P. berghei* host hepatocytes susceptible to infection	Carrolo et al., 2003
CD81		Required on hepatocytes for *P. yoelii* invasion with PVM formation	Silvie et al., 2003
Cholesterol		Involved in assembly of CD81 microdomains on the cell surface	Silvie et al., 2003, 2006, 2007
HSPGs		Binds CSP, increased sulfation on HSPGs triggers invasion of migrating sporozoite	Frevert et al., 1993; Coppi et al., 2007
EphA2		Engages parasite protein P36 to facilitate hepatocyte invasion	Kaushansky et al., 2015
Scavenger Receptor B1		Required for CD81 microdomain formation, additional roles independent of CD81 for *P. berghei* and *P. vivax*	Rodrigues et al., 2008; Yalaoui et al., 2008a; Manzoni et al., 2017
HGF/MET signaling	Liver Stage Development	Prevents the apoptosis of *P. berghei* infected cells, promoting successful infection	Leiriao et al., 2005
Endosomes and lysosomes		Endosomes and lysosomes are localized around the PVM during development	Lopes da Silva et al., 2012; Grutzke et al., 2014

(Continued)

TABLE 2 | Continued

Host/Parasite factor	Stage of infection	Main findings	References
Phosphatidylcholine		Required for correct localization of proteins within the PVM; important for parasite survival	Itoe et al., 2014
P53		Decreased levels of P53 are important for successful Liver Stage infection.	Kaushansky et al., 2013b
Apolipoprotein H		Interacts with parasite protein EXP1 to promote successful Liver Stage infection	Sa et al., 2017
ALK4		Knockdown reduces Liver Stage infection	Arang et al., 2017
CAMKK2		Knockdown reduces Liver Stage infection	Arang et al., 2017
CSK		Knockdown reduces Liver Stage infection	Arang et al., 2017
FGFR4		Knockdown reduces Liver Stage infection	Arang et al., 2017
FLT1		Knockdown reduces Liver Stage infection	Arang et al., 2017
FLT3		Knockdown reduces Liver Stage infection	Arang et al., 2017
IKBKB		Knockdown reduces Liver Stage infection	Arang et al., 2017
IRAK1		Knockdown reduces Liver Stage infection	Arang et al., 2017
MAPK1		Knockdown reduces Liver Stage infection	Arang et al., 2017
MAPKAPK2		Knockdown reduces Liver Stage infection	Arang et al., 2017
MARK2		Knockdown reduces Liver Stage infection	Prudencio et al., 2008; Arang et al., 2017
MARK4		Knockdown reduces Liver Stage infection	Arang et al., 2017
MET		Knockdown reduces Liver Stage infection	Prudencio et al., 2008; Arang et al., 2017
PKCζ		Knockdown reduces Liver Stage infection	Prudencio et al., 2008; Arang et al., 2017
PRKWNK1		Knockdown reduces Liver Stage infection	Prudencio et al., 2008
SGK2		Knockdown reduces Liver Stage infection	Prudencio et al., 2008
STK35		Knockdown reduces Liver Stage infection	Prudencio et al., 2008
TGFBR1		Knockdown reduces Liver Stage infection	Arang et al., 2017
TYRO3		Knockdown reduces Liver Stage infection	Arang et al., 2017
ULK1		Knockdown reduces Liver Stage infection	Arang et al., 2017
WEE1		Knockdown reduces Liver Stage infection	Arang et al., 2017

dramatic change, however, is the replication of the liver stage schizont, which produces tens of thousands of merozoites (membrane-bound, haploid, red blood cell invasive forms) that eventually invade erythrocytes during blood stage development. During this process, some parasites survive, and re-wire their host cells to resist certain types of apoptotic stimuli, while others succumb to host cell apoptosis or alternative cell death stimuli (Leiriao et al., 2005; van de Sand et al., 2005; Kaushansky et al., 2013a,b; Douglass et al., 2015). While these dramatic cellular changes have been qualitatively observed, they are rarely monitored quantitatively.

Some molecular determinants have been linked to Liver Stage survival and development. For example, *Plasmodium* proteins Upregulated in Infectious Sporozoites (UIS) UIS3 and UIS4 have been hypothesized to play an active role in host nutrient acquisition, in part because of the demonstration that UIS3 associates with the host Liver Fatty Acid Binding Protein (L-FABP) (Mikolajczak et al., 2007; Blume et al., 2011; Slavic et al., 2011; Favretto et al., 2013) and localizes both proteins to the PVM (Mueller et al., 2005). Fatty acids of both host and parasite origin, including host phosphatidylcholine, have been demonstrated to be required for optimal liver stage development (Mazumdar et al., 2006; Vaughan et al., 2009; Itoe et al., 2014). How each of these components specifically contributes to the observed cellular changes, and how each factor co-opts host defenses remains unknown. A more quantitative assessment of the cellular changes that occur, matched to molecular information, will enable the development of models that describe networks of host-parasite interactions required for development of the liver stage parasite.

Liver Stage Exit

Intracellular pathogens must exit their host cell in order to propagate and survive. The precise strategies they use directly impact their ability to disseminate within a host, transmit to new hosts, and engage or avoid host immune responses. Despite these important functions of exit, detailed investigations into the mechanisms governing *Plasmodium* exit have been lacking. This process is important not only for our basic understanding of liver stage development, but also for immunity. This is illustrated by the finding that the most potent stimulus of the immune system is elicited by parasites that develop through the liver stage and exit, but cannot undergo replication within the blood stage (Bijker et al., 2013).

Egress from hepatocytes occurs through the rupture of the PVM, followed by destabilization of the actin cytoskeleton, to allow the budding of merozoites from the host cell through the formation of merozoite-filled vesicles (merosomes). These structures are surrounded by a membrane of host origin (Graewe et al., 2011), and have been hypothesized to shuttle merozoites into the bloodstream to begin blood stage infection (Burda et al., 2017). This process inhibits exposure of phosphatidylserine (PS) on the outer surface of the cell, thereby simultaneously ensuring migration of parasites to the bloodstream and protection from host immune responses (Sturm et al., 2006; Tarun et al., 2006; Baer et al., 2007). Recent methodological advances have developed a platform for quantifying exit events (Stanway et al., 2009). This quantification, and a global assessment of molecular changes that occur during exit, will drive the development of models that describe networks of host-parasite interactions that underlie the exit process. Importantly, these networks could then be used to predict host and parasite determinants of dissemination to the blood stream, and the ability to engage or avoid host immune responses.

CONCLUSIONS AND NEW DIRECTIONS

Parasites must successfully navigate a wide variety of different environmental milieus, and each alternative setting presents challenges for the parasite, as well as opportunities for intervention. Here, we have described how the tools and approaches of systems biology can be deployed to more comprehensively characterize the complex interaction between parasite and host. This will inform our understanding of how the parasite and the host interact, and also facilitate future strategies to combat the parasite. Interventions that have been designed and employed without a comprehensive understanding of the complex dynamic between the *Plasmodium* parasite and its host have only partially controlled malaria in the field.

Despite the challenges, many influential leaders have called for malaria eradication in recent years (Gates, 2007; WHO, 2017). This goal is most likely to be realized if control strategies are deployed rationally with the capacity to predict how a given treatment will impact systematic changes in the parasite and host alike, to facilitate readiness for these changes. The integration of systems biology could evaluate the capacity of the parasite to circumvent new interventions, and in doing so, contribute to the success of eradication efforts. While references to the principles of systems biology first occurred decades ago, the field was established in earnest ∼15 years ago with the completion of the human genome. Since then, most systems biology studies have steered clear of the complexity that is introduced when multiple genomes collide, as is the case during infection. Pathogens and their host cells have coevolved, introducing alterations to both genomes along the way (Miller et al., 1976; Zimmerman et al., 2013). What has resulted is the capacity of a pathogen to fundamentally alter the biology of its host, by changing the size, shape, composition and function of the cell. Intracellular pathogens thus are expert cell biologists, controlling the host cell to their own advantage. As such, the study of host-pathogen interactions presents an unmatched opportunity for the field of systems biology, just as the approach of systems biology presents an unmatched opportunity for the eradication of malaria.

AUTHOR CONTRIBUTIONS

MZ, LSA, SAD, JDA, and AK were involved in the conception of the article. MZ, JDA, and AK wrote the article with assistance from LSA and SAD.

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REFERENCES

Albuquerque, S. S. Carret, C., Grosso, A. R., Tarun, A. S., Peng, X., Kappe, S. H., et al. (2009). Host cell transcriptional profiling during malaria liver stage infection reveals a coordinated and sequential set of biological events. *BMC Genomics* 10:270. doi: 10.1186/1471-2164-10-270

AlQuraishi, M., Koytiger, G., Jenney, A., MacBeath, G., and Sorger, P. K. (2014). A multiscale statistical mechanical framework integrates biophysical and genomic data to assemble cancer networks. *Nat. Genet.* 46, 1363–1371. doi: 10.1038/ng.3138

Annoura, T., van Schaijk, B. C., Ploemen, I. H., Sajid, M., Lin, J. W., Vos, M. W., et al. (2014). Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development. *FASEB J.* 28, 2158–2170. doi: 10.1096/fj.13-241570

Arang, N., Kain, H. S., Glennon, E. K., Bello, T., Dudgen, D. R., Walter, E. N. F., et al. (2017). Identifying host regulators and inhibitors of liver stage malaria infection using kinase activity profiles. *Nat. Commun.* 8:1232. doi: 10.1038/s41467-017-01345-2

Baer, K., Klotz, C., Kappe, S. H., Schnieder, T., and Frevert, U. (2007). Release of hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. *PLoS Pathog.* 3:e171. doi: 10.1371/journal.ppat.0030171

Bano, N., Romano, J. D., Jayabalasingham, B., and Coppens, I. (2007). Cellular interactions of Plasmodium liver stage with its host mammalian cell. *Int. J. Parasitol.* 37, 1329–1341. doi: 10.1016/j.ijpara.2007.04.005

Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., et al. (2006). Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313, 1642–1645. doi: 10.1126/science.1127344

Bhanot, P., Schauer, K., Coppens, I., and Nussenzweig, V. (2005). A surface phospholipase is involved in the migration of plasmodium sporozoites through cells. *J Biol Chem.* 280, 6752–6760. doi: 10.1074/jbc.M411465200

Bijker, E. M., Bastiaens, G. J., Teirlinck, A. C., van Gemert, G. J., Graumans, W., M. van de Vegte-Bolmer, et al. (2013). Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7862–7867. doi: 10.1073/pnas.1220360110

Blasco, B., Leroy, D., and Fidock, D. A. (2017). Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. *Nat. Med.* 23, 917–928. doi: 10.1038/nm.4381

Blume, M., Hliscs, M., Rodriguez-Contreras, D., Sanchez, M., Landfear, S., Lucius, R., et al. (2011). A constitutive pan-hexose permease for the Plasmodium life cycle and transgenic models for screening of antimalarial sugar analogs. *FASEB J.* 25, 1218–1229. doi: 10.1096/fj.10-173278

Bonneau, R., Facciotti, M. T., Reiss, D. J., Schmid, A. K., Pan, M., Kaur, A., et al. (2007). A predictive model for transcriptional control of physiology in a free living cell. *Cell* 131, 1354–1365. doi: 10.1016/j.cell.2007.10.053

Burda, P. C., Caldelari, R., and Heussler, V. T. (2017). Manipulation of the host cell membrane during plasmodium liver stage egress. *MBio*. 8:17. doi: 10.1128/mBio.00139-17

Bushell, E., Gomes, A. R., Sanderson, T., Anar, B., Girling, G., Herd, C., et al. (2017). Functional profiling of a plasmodium genome reveals an abundance of essential genes. *Cell* 170, 260–272 e8. doi: 10.1016/j.cell.2017.06.030

Byrum, S., Smart, S. K., Larson, S., and Tackett, A. J. (2012). Analysis of stable and transient protein-protein interactions. *Methods Mol. Biol.* 833, 143–152. doi: 10.1007/978-1-61779-477-3_10

Carrolo, M., Giordano, S., Cabrita-Santos, L., Corso, S., Vigario, A. M., Silva, S., et al. (2003). Hepatocyte growth factor and its receptor are required for malaria infection. *Nat. Med.* 9, 1363–1369. doi: 10.1038/nm947

Cha, S. J., Kim, M. S., Pandey, A., and Jacobs-Lorena, M. (2016). Identification of GAPDH on the surface of Plasmodium sporozoites as a new candidate for targeting malaria liver invasion. *J. Exp. Med.* 213, 2099–2112. doi: 10.1084/jem.20160059

Coleman, B. I., Skillman, K. M., Jiang, R. H. Y., Childs, L. M., Altenhofen, L. M., Ganter, M., et al. (2014). A *Plasmodium falciparum* histone deacetylase regulates antigenic variation and gametocyte conversion. *Cell Host Microbe* 16, 177–186. doi: 10.1016/j.chom.2014.06.014

Combe, A., Giovannini, D., Carvalho, T. G., Spath, S., Boisson, B., Loussert, C., et al. (2009). Clonal conditional mutagenesis in malaria parasites. *Cell Host Microbe* 5, 386–396. doi: 10.1016/j.chom.2009.03.008

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. doi: 10.1126/science.1231143

Coppi, A., Natarajan, R., Pradel, G., Bennett, B. L., James, E. R., Roggero, M. A., et al. (2011). The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *J. Exp. Med.* 208, 341–356. doi: 10.1084/jem.20101488

Coppi, A., Pinzon-Ortiz, C., Hutter, C., and Sinnis, P. (2005). The Plasmodium circumsporozoite protein is proteolytically processed during cell invasion. *J. Exp. Med.* 201, 27–33. doi: 10.1084/jem.20040989

Coppi, A., Tewari, R., Bishop, J. R., Bennett, B. L., Lawrence, R., Esko, J. D., et al. (2007). Heparan sulfate proteoglycans provide a signal to Plasmodium sporozoites to stop migrating and productively invade host cells. *Cell Host Microbe* 2, 316–327. doi: 10.1016/j.chom.2007.10.002

Danziger, S. A. Ratushny, A. V., Smith, J. J., Saleem, R. A., Wan, Y., Arens, C. E., et al. (2014). Molecular mechanisms of system responses to novel stimuli are predictable from public data. *Nucleic Acids Res.* 42, 1442–1460. doi: 10.1093/nar/gkt938

De Niz, M., Burda, P. C., Kaiser, G., Del Portillo, H. A., Spielmann, T., Frischknecht, F., et al. (2017). Progress in imaging methods: insights gained into Plasmodium biology. *Nat. Rev. Microbiol.* 15, 37–54. doi: 10.1038/nrmicro.2016.158

Douglass, A. N., Kain, H. S., Abdullahi, M., Arang, N., Austin, L. S., Mikolajczak, S. A., et al. (2015). Host-based prophylaxis successfully targets liver stage malaria parasites. *Mol. Ther.* 23, 857–865. doi: 10.1038/mt.2015.18

Favretto, F., Assfalg, M., Molinari, H., and D'Onofrio, M. (2013). Evidence from NMR interaction studies challenges the hypothesis of direct lipid transfer from L-FABP to malaria sporozoite protein UIS3. *Protein Sci.* 22, 133–138. doi: 10.1002/pro.2194

Frevert, U., Sinnis, P., Cerami, C., Shreffler, W., Takacs, B., and Nussenzweig, V. (1993). Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J. Exp. Med.* 177, 1287–1298. doi: 10.1084/jem.177.5.1287

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., and Hyman, R. W. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498–511. doi: 10.1038/nature01097

Gates, M. (2007). *Malaria Forum Keynote Address*. Available online at: https://www.gatesfoundation.org/media-center/speeches/2007/10/melinda-french-gates-malaria-forum

Gehlenborg, N., O'Donoghue, S. I., Baliga, N. S., Goesmann, A., Hibbs, M. A., Kitano, H., et al. (2010). Visualization of omics data for systems biology. *Nat. Methods* 7, S56–S68. doi: 10.1038/nmeth.1436

Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., et al. (2003). Global analysis of protein expression in yeast. *Nature* 425, 737–741. doi: 10.1038/nature02046

Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., Scherf, A., and Lopez-Rubio, J. J. (2014). Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nat. Biotechnol.* 32, 819–821. doi: 10.1038/nbt.2925

Gomes-Santos, C. S., Braks, J., Prudencio, M., Carret, C., Gomes, A. R., Pain, A., et al. (2011). Transition of Plasmodium sporozoites into liver stage-like forms is regulated by the RNA binding protein Pumilio. *PLoS Pathog.* 7:e1002046. doi: 10.1371/journal.ppat.1002046

Graewe, S., Rankin, K. E., Lehmann, C., Deschermeier, C., Hecht, L., Froehlke, U., et al. (2011). Hostile takeover by Plasmodium: reorganization of parasite and host cell membranes during liver stage egress. *PLoS Pathog.* 7:e1002224. doi: 10.1371/journal.ppat.1002224

Grutzke, J., Rindte, K., Goosmann, C., Silvie, O., Rauch, C., Heuer, D., et al. (2014). The spatiotemporal dynamics and membranous features of the Plasmodium liver stage tubovesicular network. *Traffic* 15, 362–382. doi: 10.1111/tra.12151

Guerreiro, A., Deligianni, E., Santos, J. M., Silva, P. A., Louis, C., Pain, A., et al. (2014). Genome-wide RIP-Chip analysis of translational repressor-bound mRNAs in the Plasmodium gametocyte. *Genome Biol.* 15:493. doi: 10.1186/s13059-014-0493-0

Gujral, T. S., Peshkin, L., and Kirschner, M. W. (2014). Exploiting polypharmacology for drug target deconvolution. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5048–5053. doi: 10.1073/pnas.1403080111

Hein, M. Y., Hubner, N. C., Poser, I., Cox, J., Nagaraj, N., Toyoda, Y., et al. (2015). A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell* 163, 712–723. doi: 10.1016/j.cell.2015.09.053

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., et al. (2003). Global analysis of protein localization in budding yeast. *Nature* 425, 686–691. doi: 10.1038/nature02026

Huttlin, E. L., Ting, L., Bruckner, R. J., Gebreab, F., Gygi, M. P., Szpyt, J., et al. (2015). The BioPlex network: a systematic exploration of the human interactome. *Cell* 162, 425–440. doi: 10.1016/j.cell.2015.06.043

Hwang, D., Rust, A. G., Ramsey, S., Smith, J. J., Leslie, D. M., Weston, A. D., et al. (2005a). A data integration methodology for systems biology. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17296–17301. doi: 10.1073/pnas.0508647102

Hwang, D., Smith, J. J., Leslie, D. M., Weston, A. D., Rust, A. G., Ramsey, S., et al. (2005b). A data integration methodology for systems biology: experimental verification. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17302–17307. doi: 10.1073/pnas.0508649102

Inacio, P., Zuzarte-Luis, V., Ruivo, M. T., Falkard, B., Nagaraj, N., Rooijers, K., et al. (2015). Parasite-induced ER stress response in hepatocytes facilitates Plasmodium liver stage infection. *EMBO Rep.* 16, 955–964. doi: 10.15252/embr.201439979

Ishino, T., Boisson, B., Orito, Y., Lacroix, C., Bischoff, E., Loussert, C., et al. (2009). LISP1 is important for the egress of *Plasmodium berghei* parasites from liver cells. *Cell. Microbiol.* 11, 1329–1339. doi: 10.1111/j.1462-5822.2009.01333.x

Ishino, T., Chinzei, Y., and Yuda, M. (2005). Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Mol. Microbiol.* 58, 1264–1275. doi: 10.1111/j.1365-2958.2005.04801.x

Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2004). Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biol.* 2:E4. doi: 10.1371/journal.pbio.0020004

Itoe, M. A., Sampaio, J. L., Cabal, G. G., Real, E., Zuzarte-Luis, V., March, S., et al. (2014). Host cell phosphatidylcholine is a key mediator of malaria parasite survival during liver stage infection. *Cell Host Microbe* 16, 778–786. doi: 10.1016/j.chom.2014.11.006

Janes, K. A., Albeck, J. G., Gaudet, S., Sorger, P. K., Lauffenburger, D. A., and Yaffe, M. B. (2005). A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis. *Science* 310, 1646–1653. doi: 10.1126/science.1116598

Joice, R., Nilsson, S. K., Montgomery, J., Dankwa, S., Egan, E., Morahan, B., et al. (2014). *Plasmodium falciparum* transmission stages accumulate in the human bone marrow. *Sci. Transl. Med.* 6:244re5. doi: 10.1126/scitranslmed.3008882

Kafsack, B. F., Rovira-Graells, N., Clark, T. G., Bancells, C., Crowley, V. M., Campino, S. G., et al. (2014). A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature* 507, 248–252. doi: 10.1038/nature12920

Kappe, S., Bruderer, T., Gantt, S., Fujioka, H., Nussenzweig, V., and Menard, R. (1999). Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. *J. Cell Biol.* 147, 937–944. doi: 10.1083/jcb.147.5.937

Kariu, T., Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2006). CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Mol. Microbiol.* 59, 1369–1379. doi: 10.1111/j.1365-2958.2005.05024.x

Kaushansky, A., Douglass, A. N., Arang, N., Vigdorovich, V., Dambrauskas, N., Kain, H. S., et al. (2015). Malaria parasites target the hepatocyte receptor EphA2 for successful host infection. *Science* 350, 1089–1092. doi: 10.1126/science.aad3318

Kaushansky, A., and Kappe, S. H. (2015a). Host ER stress during malaria parasite infection. *EMBO Rep.* 16, 883–884. doi: 10.15252/embr.201540792

Kaushansky, A., and Kappe, S. H. (2015b). Selection and refinement: the malaria parasite's infection and exploitation of host hepatocytes. *Curr. Opin. Microbiol.* 26, 71–78. doi: 10.1016/j.mib.2015.05.013

Kaushansky, A., Metzger, P. G., Douglass, A. N., Mikolajczak, S. A., Lakshmanan, V., Kain, H. S., et al. (2013a). Malaria parasite liver stages render host hepatocytes susceptible to mitochondria-initiated apoptosis. *Cell Death Dis.* 4:e762. doi: 10.1038/cddis.2013.286

Kaushansky, A., Ye, A. S., Austin, L. S., Mikolajczak, S. A., Vaughan, A. M., Camargo, N., et al. (2013b). Suppression of host p53 is critical for Plasmodium liver-stage infection. *Cell Rep.* 3, 630–637. doi: 10.1016/j.celrep.2013.02.010

Khan, S. M., Franke-Fayard, B., Mair, G. R., Lasonder, E., Janse, C. J., Mann, M., et al. (2005). Proteome analysis of separated male and female gametocytes reveals novel sex-specific Plasmodium biology. *Cell* 121, 675–687. doi: 10.1016/j.cell.2005.03.027

Labaied, M., Harupa, A., Dumpit, R. F., Coppens, I., Mikolajczak, S. A., and Kappe, S. H. (2007). Plasmodium yoelii sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. *Infect. Immun.* 75, 3758–3768. doi: 10.1128/IAI.00225-07

Lasonder, E., Rijpma, S. R., van Schaijk, B. C., Hoeijmakers, W. A., Kensche, P. R., Gresnigt, M. S., et al. (2016). Integrated transcriptomic and proteomic analyses of *P. falciparum* gametocytes: molecular insight into sex-specific processes and translational repression. *Nucleic Acids Res.* 44, 6087–6101. doi: 10.1093/nar/gkw536

Leiriao, P., Albuquerque, S. S., Corso, S., van Gemert, G. J., Sauerwein, R. W., Rodriguez, A., et al. (2005). HGF/MET signalling protects Plasmodium-infected host cells from apoptosis. *Cell. Microbiol.* 7, 603–609. doi: 10.1111/j.1462-5822.2004.00490.x

Liehl, P., Zuzarte-Luis, V., Chan, J., Zillinger, T., Baptista, F., Carapau, D., et al. (2014). Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection. *Nat. Med.* 20, 47–53. doi: 10.1038/nm.3424

Lindner, S. E., Mikolajczak, S. A., Vaughan, A. M., Moon, W., Joyce, B. R., Sullivan W. J. Jr., et al. (2013). Perturbations of Plasmodium Puf2 expression and RNA-seq of Puf2-deficient sporozoites reveal a critical role in maintaining RNA homeostasis and parasite transmissibility. *Cell. Microbiol.* 15, 1266–1283. doi: 10.1111/cmi.12116

Lopes da Silva, M., Thieleke-Matos, C., Cabrita-Santos, L., Ramalho, J. S., Wavre-Shapton, S. T., Futter, C. E., et al. (2012). The host endocytic pathway is essential for *Plasmodium berghei* late liver stage development. *Traffic* 13, 1351–1363. doi: 10.1111/j.1600-0854.2012.01398.x

Lu, J., Tong, Y., Pan, J., Yang, Y., Liu, Q., Tan, X., et al. (2016). A redesigned CRISPR/Cas9 system for marker-free genome editing in *Plasmodium falciparum*. *Parasit. Vectors* 9:198. doi: 10.1186/s13071-016-1487-4

Mair, G. R., Braks, J. A., Garver, L. S., Wiegant, J. C., Hall, N., Dirks, R. W., et al. (2006). Regulation of sexual development of Plasmodium by translational repression. *Science* 313, 667–669. doi: 10.1126/science.1125129

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., et al. (2013). RNA-guided human genome engineering via Cas9. *Science* 339, 823–826. doi: 10.1126/science.1232033

Manzoni, G., Marinach, C., Topcu, S., Briquet, S., Grand, M., Tolle, M., et al. (2017). Plasmodium P36 determines host cell receptor usage during sporozoite invasion. *Elife* 6:e25903. doi: 10.7554/eLife.25903

Matuschewski, K., Ross, J., Brown, S. M., Kaiser, K., Nussenzweig, V., and Kappe, S. H. (2002). Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. *J. Biol. Chem.* 277, 41948–41953. doi: 10.1074/jbc.M207315200

Mazumdar, J., Emma, H. W., Masek, K., Hunter, A. C., and Striepen, B. (2006). Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13192–13197. doi: 10.1073/pnas.0603391103

Mellacheruvu, D., Wright, Z., Couzens, A. L., Lambert, J. P., St-Denis, N. A., Li, T., et al. (2013). The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nat. Methods* 10, 730–736. doi: 10.1038/nmeth.2557

Mikolajczak, S. A., Jacobs-Lorena, V., MacKellar, D. C., Camargo, N., and Kappe, S. H. (2007). L-FABP is a critical host factor for successful malaria liver stage development. *Int. J. Parasitol.* 37, 483–489. doi: 10.1016/j.ijpara.2007.01.002

Mikolajczak, S. A., Silva-Rivera, H., Peng, X., Tarun, A. S., Camargo, N., Jacobs-Lorena, V., et al. (2008). Distinct malaria parasite sporozoites reveal transcriptional changes that cause differential tissue infection competence in the mosquito vector and mammalian host. *Mol. Cell. Biol.* 28, 6196–6207. doi: 10.1128/MCB.00553-08

Miller, J. L., Sack, B. K., Baldwin, M., Vaughan, A. M., and Kappe, S. H. (2014). Interferon-mediated innate immune responses against malaria parasite liver stages. *Cell Rep.* 7, 436–447. doi: 10.1016/j.celrep.2014.03.018

Miller, L. H., Mason, S. J., Clyde, D. F., and McGinniss, M. H. (1976). The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N. Engl. J. Med.* 295, 302–304.

Morahan, B. J., Wang, L., and Coppel, R. L. (2009). No TRAP, no invasion. *Trends Parasitol.* 25, 77–84. doi: 10.1016/j.pt.2008.11.004

Moreira, C. K., Templeton, T. J., Lavazec, C., Hayward, R. E., Hobbs, C. V., Kroeze, H., et al. (2008). The Plasmodium TRAP/MIC2 family member, TRAP-Like Protein (TLP), is involved in tissue traversal by sporozoites. *Cell. Microbiol.* 10, 1505–1516. doi: 10.1111/j.1462-5822.2008.01143.x

Mota, M. M., Pradel, G., Vanderberg, J. P., Hafalla, J. C., Frevert, U., Nussenzweig, R. S., et al. (2001). Migration of Plasmodium sporozoites through cells before infection. *Science* 291, 141–144. doi: 10.1126/science.291.5501.141

Mueller, A. K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., et al. (2005). Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3022–3027. doi: 10.1073/pnas.0408442102

Muller, K., Matuschewski, K., and Silvie, O. (2011). The Puf-family RNA-binding protein Puf2 controls sporozoite conversion to liver stages in the malaria parasite. *PLoS ONE* 6:e19860. doi: 10.1371/journal.pone.0019860

Neafsey, D. E., Juraska, M., Bedford, T., Benkeser, D., Valim, C., Griggs, A., et al. (2015). Genetic diversity and protective efficacy of the RTS,S/AS01 malaria vaccine. *N. Engl. J. Med.* 373, 2025–2037. doi: 10.1056/NEJMoa1505819

Orito, Y., Ishino, T., Iwanaga, S., Kaneko, I., Kato, T., Menard, R., et al. (2013). Liver-specific protein 2: a Plasmodium protein exported to the hepatocyte cytoplasm and required for merozoite formation. *Mol. Microbiol.* 87, 66–79. doi: 10.1111/mmi.12083

Pavlopoulos, G. A., Paez-Espino, D., Kyrpides, N. C., and Iliopoulos, I. (2017). Empirical comparison of visualization tools for larger-scale network analysis. *Adv. Bioinformatics* 2017:1278932. doi: 10.1155/2017/1278932

Pittet, M. J., and Weissleder, R. (2011). Intravital imaging. *Cell* 147, 983–991. doi: 10.1016/j.cell.2011.11.004

Ploemen, I. H., Croes, H. J., van Gemert, G. J., Wijers-Rouw, M., Hermsen, C. C., and Sauerwein, R. W. (2012). *Plasmodium berghei Δp52&p36* parasites develop independent of a parasitophorous vacuole membrane in Huh-7 liver cells. *PLoS ONE* 7:e50772. doi: 10.1371/journal.pone.0050772

Poran, A., Nötzel, C., Aly, O., Mencia-Trinchant, N., Harris, C. T., Guzman, M. L., et al. (2017). Single-Cell RNA sequencing reveals a signature of sexual commitment in malaria parasites. *Nature.* 551, 95–99. doi: 10.1038/nature24280

Prudencio, M., Rodrigues, C. D., Hannus, M., Martin, C., Real, E., Goncalves, L. A., et al. (2008). Kinome-wide RNAi screen implicates at least 5 host hepatocyte kinases in Plasmodium sporozoite infection. *PLoS Pathog.* 4:e1000201. doi: 10.1371/journal.ppat.1000201

Rodrigues, C. D., Hannus, M., Prudencio, M., Martin, C., Goncalves, L. A., Portugal, S., et al. (2008). Host scavenger receptor SR-BI plays a dual role in the establishment of malaria parasite liver infection. *Cell Host Microbe* 4, 271–282. doi: 10.1016/j.chom.2008.07.012

Ruivo, M. T., Vera, I. M., Sales-Dias, J., Meireles, P., Gural, N., Bhatia, S. N., et al. (2016). Host AMPK Is a modulator of plasmodium liver infection. *Cell Rep.* 16, 2539–2545. doi: 10.1016/j.celrep.2016.08.001

Sa, E. C. C., Nyboer, B., Heiss, K., Sanches-Vaz, M., Fontinha, D., Wiedtke, E., et al. (2017). *Plasmodium berghei* EXP-1 interacts with host Apolipoprotein H during Plasmodium liver-stage development. *Proc. Natl. Acad. Sci. U.S.A.* 114, E1138–E1147. doi: 10.1073/pnas.1606419114

Schats, R., Bijker, E. M., van Gemert, G. J., Graumans, W., van de Vegte-Bolmer, M., van Lieshout, L., et al. (2015). Heterologous protection against malaria after immunization with *Plasmodium falciparum* sporozoites. *PLoS ONE* 10:e0124243. doi: 10.1371/journal.pone.0124243

Selbach, M., and Mann, M. (2006). Protein interaction screening by quantitative immunoprecipitation combined with knockdown (QUICK). *Nat. Methods*. 3, 981–983. doi: 10.1038/nmeth972

Shortt, H. E., and Garnham, P. C. (1948). Pre-erythrocytic stage in mammalian malaria parasites. *Nature* 161:126. doi: 10.1038/161126a0

Silva, P. A., Guerreiro, A., Santos, J. M., Braks, J. A., Janse, C. J., and Mair, G. R. (2016). Translational control of UIS4 protein of the host-parasite interface is mediated by the RNA binding protein Puf2 in *Plasmodium berghei* sporozoites. *PLoS ONE* 11:e0147940. doi: 10.1371/journal.pone.0147940

Silvie, O., Briquet, S., Muller, K., Manzoni, G., and Matuschewski, K. (2014). Post-transcriptional silencing of UIS4 in *Plasmodium berghei* sporozoites is important for host switch. *Mol. Microbiol.* 91, 1200–1213. doi: 10.1111/mmi.12528

Silvie, O., Charrin, S., Billard, M., Franetich, J. F., Clark, K. L., van Gemert, G. J., et al. (2006). Cholesterol contributes to the organization of tetraspanin-enriched microdomains and to CD81-dependent infection by malaria sporozoites. *J. Cell Sci.* 119(Pt 10), 1992–2002. doi: 10.1242/jcs.02911

Silvie, O., Franetich, J. F., Boucheix, C., Rubinstein, E., and Mazier, D. (2007). Alternative invasion pathways for *Plasmodium berghei* sporozoites. *Int. J. Parasitol.* 37, 173–182. doi: 10.1016/j.ijpara.2006.10.005

Silvie, O., Rubinstein, E., Franetich, J. F., Prenant, M., Belnoue, E., Renia, L., et al. (2003). Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nat. Med.* 9, 93–96. doi: 10.1038/nm808

Sinha, A., Hughes, K. R., Modrzynska, K. K., Otto, T. D., Pfander, C., Dickens, N. J., et al. (2014). A cascade of DNA-binding proteins for sexual commitment and development in Plasmodium. *Nature* 507, 253–257. doi: 10.1038/nature12970

Sinturel, F., Gerber, A., Mauvoisin, D., Wang, J., Gatfield, D., Stubblefield, J. J., et al. (2017). Diurnal oscillations in liver mass and cell size accompany ribosome assembly cycles. *Cell* 169, 651–663.e14. doi: 10.1016/j.cell.2017.04.015

Slavic, K., Delves, M. J., Prudencio, M., Talman, A. M., Straschil, U., Derbyshire, E. T., et al. (2011). Use of a selective inhibitor to define the chemotherapeutic potential of the plasmodial hexose transporter in different stages of the parasite's life cycle. *Antimicrob. Agents Chemother.* 55, 2824–2830. doi: 10.1128/AAC.01739-10

Stanway, R. R., Graewe, S., Rennenberg, A., Helm, S., and Heussler, V. T. (2009). Highly efficient subcloning of rodent malaria parasites by injection of single merosomes or detached cells. *Nat. Protoc.* 4, 1433–1439. doi: 10.1038/nprot.2009.172

Sturm, A., Amino, R., C. van de Sand, Regen, T., Retzlaff, S., Rennenberg, A., et al. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* 313, 1287–1290. doi: 10.1126/science.1129720

Tackett, A. J., DeGrasse, J. A., Sekedat, M. D., Oeffinger, M., Rout, M. P., and Chait, B. T. (2005). I-DIRT, a general method for distinguishing between specific and nonspecific protein interactions. *J. Proteome Res.* 4, 1752–1756. doi: 10.1021/pr050225e

Tarun, A. S., Baer, K., Dumpit, R. F., Gray, S., Lejarcegui, N., Frevert, U., et al. (2006). Quantitative isolation and *in vivo* imaging of malaria parasite liver stages. *Int. J. Parasitol.* 36, 1283–1293. doi: 10.1016/j.ijpara.2006.06.009

Tarun, A. S., Peng, X., Dumpit, R. F., Ogata, Y., Silva-Rivera, H., Camargo, N., et al. (2008). A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc. Natl. Acad. Sci. U.S.A.* 105, 305–310. doi: 10.1073/pnas.0710780104

Tavares, J., Formaglio, P., Thiberge, S., Mordelet, E., Van Rooijen, N., Medvinsky, A., et al. (2013). Role of host cell traversal by the malaria sporozoite during liver infection. *J. Exp. Med.* 210, 905–915. doi: 10.1084/jem.20121130

Tawk, L., Lacroix, C., Gueirard, P., Kent, R., Gorgette, O., Thiberge, S., et al. (2013). A key role for Plasmodium subtilisin-like SUB1 protease in egress of malaria parasites from host hepatocytes. *J. Biol. Chem.* 288, 33336–33346. doi: 10.1074/jbc.M113.513234

Trinkle-Mulcahy, L. (2012). Resolving protein interactions and complexes by affinity purification followed by label-based quantitative mass spectrometry. *Proteomics* 12, 1623–1638. doi: 10.1002/pmic.201100438

Trinkle-Mulcahy, L., Boulon, S., Lam, Y. W., Urcia, R., Boisvert, F. M., Vandermoere, F., et al. (2008). Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *J. Cell Biol.* 183, 223–239. doi: 10.1083/jcb.200805092

Usynin, I., Klotz, C., and Frevert, U. (2007). Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells. *Cell. Microbiol.* 9, 2610–2628. doi: 10.1111/j.1462-5822.2007.00982.x

Valacchi, G., Sticozzi, C., Lim, Y., and Pecorelli, A., (2011). Scavenger receptor class B type I: a multifunctional receptor. *Ann. N.Y. Acad. Sci.* 1229, E1–E7. doi: 10.1111/j.1749-6632.2011.06205.x

van de Sand, C., Horstmann, S., Schmidt, A., Sturm, A., Bolte, S., Krueger, A., et al. (2005). The liver stage of *Plasmodium berghei* inhibits host cell apoptosis. *Mol. Microbiol.* 58, 731–742. doi: 10.1111/j.1365-2958.2005.04888.x

van Dijk, M. R., Douradinha, B., Franke-Fayard, B., Heussler, V., van Dooren, M. W., van Schaijk, B., et al. (2005). Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12194–12199. doi: 10.1073/pnas.0500925102

Van Regenmortel, M. H. (2004). Reductionism and complexity in molecular biology. Scientists now have the tools to unravel biological and overcome the limitations of reductionism. *EMBO Rep.* 5, 1016–1020. doi: 10.1038/sj.embor.7400284

van Rijnsoever, C., Oorschot, V., and Klumperman, J. (2008). Correlative light-electron microscopy (CLEM) combining live-cell imaging and immunolabeling of ultrathin cryosections. *Nat. Methods* 5, 973–980. doi: 10.1038/nmeth.1263

Vaughan, A. M., Mikolajczak, S. A., Wilson, E. M., Grompe, M., Kaushansky, A., Camargo, N., et al. (2012). Complete *Plasmodium falciparum* liver-stage development in liver-chimeric mice. *J. Clin. Invest.* 122, 3618–3628. doi: 10.1172/JCI62684

Vaughan, A. M., O'Neill, M. T., Tarun, A. S., Camargo, N., Phuong, T. M., Aly, A. S., et al. (2009). Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. *Cell. Microbiol.* 11, 506–520. doi: 10.1111/j.1462-5822.2008.01270.x

Vaughan, A. M., and Kappe, S. H. I. (2017). Malaria parasite liver infection and exoerythrocytic biology. *Cold Spring Harb. Perspect. Med.* 7:a025486. doi: 10.1101/cshperspect.a025486

Wagner, J. C., Platt, R. J., Goldfless, S. J., Zhang, F., and Niles, J. C. (2014). Efficient CRISPR-Cas9-mediated genome editing in *Plasmodium falciparum*. *Nat. Methods* 11, 915–918. doi: 10.1038/nmeth.3063

WHO (2016). *World Malaria Report*. Geneva: World Health Organization.

WHO (2017). *Overview of Malaria Elimination*. Geneva: World Health Organization.

Yalaoui, S., Huby, T., Franetich, J. F., Gego, A., Rametti, A., Moreau, M., et al. (2008a). Scavenger receptor BI boosts hepatocyte permissiveness to Plasmodium infection. *Cell Host Microbe* 4, 283–292. doi: 10.1016/j.chom.2008.07.013

Yalaoui, S., Zougbede, S., Charrin, S., Silvie, O., Arduise, C., Farhati, K., et al. (2008b). Hepatocyte permissiveness to Plasmodium infection is conveyed by a short and structurally conserved region of the CD81 large extracellular domain. *PLoS Pathog.* 4:e1000010. doi: 10.1371/journal.ppat.1000010

Yang, A. S., O'Neill, M. T., Jennison, C., Lopaticki, S., Allison, C. C., Armistead, J. S., et al. (2017). Cell traversal activity is important for *Plasmodium falciparum* liver infection in humanized mice. *Cell Rep.* 18, 3105–3116. doi: 10.1016/j.celrep.2017.03.017

Zhang, M., Fennell, C., Ranford-Cartwright, L., Sakthivel, R., Gueirard, P., Meister, S., et al. (2010). The Plasmodium eukaryotic initiation factor-2alpha kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. *J. Exp. Med.* 207, 1465–1474. doi: 10.1084/jem.20091975

Zimmerman, P. A., Ferreira, M. U., Howes, R. E., and Mercereau-Puijalon, O. (2013). Red blood cell polymorphism and susceptibility to *Plasmodium vivax*. *Adv Parasitol.* 81, 27–76. doi: 10.1016/B978-0-12-407826-0.00002-3

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Integrative Approaches to Understand the Mastery in Manipulation of Host Cytokine Networks by Protozoan Parasites with Emphasis on *Plasmodium* and *Leishmania* Species

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Diseases by protozoan pathogens pose a significant public health concern, particularly in tropical and subtropical countries, where these are responsible for significant morbidity and mortality. Protozoan pathogens tend to establish chronic infections underscoring their competence at subversion of host immune processes, an important component of disease pathogenesis and of their virulence. Modulation of cytokine and chemokine levels, their crosstalks and downstream signaling pathways, and thereby influencing recruitment and activation of immune cells is crucial to immune evasion and subversion. Many protozoans are now known to secrete effector molecules that actively modulate host immune transcriptome and bring about alterations in host epigenome to alter cytokine levels and signaling. The complexity of multi-dimensional events during interaction of hosts and protozoan parasites ranges from microscopic molecular levels to macroscopic ecological and epidemiological levels that includes disrupting metabolic pathways, cell cycle (*Toxoplasma* and *Theileria* sp.), respiratory burst, and antigen presentation (*Leishmania* spp.) to manipulation of signaling hubs. This requires an integrative systems biology approach to combine the knowledge from all these levels to identify the complex mechanisms of protozoan evolution *via* immune escape during host–parasite coevolution. Considering the diversity of protozoan parasites, in this review, we have focused on *Leishmania* and *Plasmodium* infections. Along with the biological understanding, we further elucidate the current efforts in generating, integrating, and modeling of multi-dimensional data to explain the modulation of cytokine networks by these two protozoan parasites to achieve their persistence in host *via* immune escape during host–parasite coevolution.

Keywords: cytokine networks, manipulation, *Plasmodium*, *Leishmania*, *inflammation*, signalling hubs, cross regulation, system biology

INTRODUCTION

Parasitic protozoa are responsible for some of the major diseases of humans affecting several million people each year resulting in significant morbidity and mortality and loss of economic activity. There have been some gains in reducing the incidence of these diseases owing to better intervention strategies, but in absence of effective vaccines, diseases like malaria, leishmaniasis, trypanosomiasis still pose a major public health problem. These protozoans typically establish chronic infections validating their success in evasion and manipulation of host defense and of metabolic processes for their survival, proliferation, and transmission. Many of these pathogenic protozoa have adapted to intracellular habitat as seen in infections by *Plasmodium* spp., *Leishmania* spp., and others. The intracellular niche makes them vulnerable to lysosomal enzymes, reactive oxygen intermediates, and detection by cytosolic sensors of infection, but also offers some protection from adaptive immunity (1). This dynamic host–pathogen interaction, leads to the activation of a series of intracellular and intercellular biochemical signaling processes leading to synthesis of diffusible effector molecules that includes cytokines and reactive oxygen species. "The earliest stages of infection are a parasite's first opportunity to establish itself within its host and conversely, it is also the host's chance to mount a rapid and effective response to clear, or at least control the infection" (2). Recent studies demonstrate that pathogens including protozoa modulate the host cell environment by manipulating the host transcriptome by epigenetic modifications besides targeting the major signaling hubs of metabolic, immune, and cell cycle processes to promote their growth, multiplication and survival (3–9). Many protozoans secrete effector molecules that actively modulate host immune transcriptome to alter cytokine levels and signaling either to escape immune processes as in liver stages of *P. falciparum* or to drive their growth as seen in the blood stages of this pathogen.

Considering the diversity of protozoan pathogenesis, this review will focus on manipulation and hijacking of cytokine networks by *Leishmania* and *Plasmodium* spp. for their survival in human host. We will highlight few recently published representative omics and systems biology based studies on *Leishmania* and *Plasmodium* parasites, toward understanding modulation of cytokine and chemokine networks in the host by the parasite to achieve their persistence in host *via* immune escape.

CYTOKINES AND CYTOKINE REGULATION

Cytokines are small molecules of the immune system, synthesized by various cell types that by virtue of binding to their receptors present on a multitude of cells mediate immune cell activation, differentiation, and cross talk to maintain immune homeostasis (10, 11). Synthesis and regulation of cytokine expression depends on the type of stimulus, cell type, and its state of activation (12–14). Expression of cytokine genes is also regulated by epigenetic modifications that include DNA methylation, histone modifications, and higher order chromatin interactions (15, 16) and posttranscriptional regulation by micro RNA-mediated mechanisms (16–19). Differentiation of immune cells as in T cell subpopulations and macrophage phenotypes is determined and regulated by cytokine environment (4, 16, 20, 21) and epigenetic modifications at cytokine gene loci (22, 23). Cytokine crosstalk between IFNα/β and TNF-α was noted to be at level of chromatin wherein IFNs in addition to regulating interferon signaling genes, also potentiated the TNF genes (4). Similarly, emerging data suggest extensive crosstalk between NLR family proteins of inflammation complex for IL-1β and IL-18 secretion and other cytokines integrated signalosome facilitating integration of diverse pathways for optimal immune response (24). H3K27, methyltransferase enhancer of zeste homolog 1 is reported to promote TLR-triggered inflammatory cytokine production by suppressing the TLR negative regulator toll-interacting protein, thereby contributing to the full activation of the innate immune response against invading pathogens (25).

CYTOKINE SIGNALING MANIPULATION BY PROTOZOAN PATHOGENS

Intracellular protozoa modulate cytokine gene expression and signaling by some common themes that include targeting of transcription factors (15, 23) phosphorylation status of signaling molecules like STATs, immune check point molecules like CTLA-4 and PD-1 to drive regulatory pathways (26) as well as kinases (5, 6, 27). The pathways usually targeted by pathogens include NF-κB, cell cycle, interferons, MAP Kinase JAK–STAT and pathways mediated by TLR and NLR receptors because of their wide range of functionality and core association with the host genome (28–30).

Toxoplasma spp. secrete dense granular protein (GRA) and Rhoptry proteins that activate host kinases and possess kinase activity, respectively, into host cell, which by phosphorylating STAT3 and STAT6, nuclear translocation of NF-κB or activation status of MAPK pathways modulate the levels of IL-4, IL-6, IL-12, and IFN-g (31–35). "*T. gondii* inhibitor of STAT1 transcriptional is another secretory protein that recruits the host nucleosome remodeling and deaceytlase complex to block STAT1-mediated gene transcription" (36). *Trypanasoma cruzi* modulates NF-κB pathway by TLR and NLR mediated signaling for favorable cytokine environment (37–39) However, the protozoa is also reported to manipulate TGF β pathway (40) and also induces the production of IL-10 (40, 41) and arginase for its survival and replication.

PLASMODIUM AND HOST INFLAMMATORY RESPONSE

Malaria, caused by *Plasmodium* spp. of Apicomplexa phylum, has been the strongest evolutionary selective force in recent human history and has shaped human genome (42) and is one of the major causes of mortality of children below 5 years of age particularly in WHO African region, taking the life of a child every 2 min (43). The life cycle of the parasite is complex and completed in multiple stages in the human and in the mosquito (female *Anopheles* spp.) hosts with stage specific gene and protein signatures (44). Briefly, sporozoites inoculated into human

host by bite of infected mosquito travel to liver to mature into merozoites that infect RBCs to continue asexual cycle and also develop into gametocytes which, after fertilization in mosquito gut, develop and mature into sporozoites.

During the liver stages of the parasite, the host immune response tends to be tolerogenic and circumsporozoite protein was seen to inhibit NADPH oxidase and IL-12 and suppressed IL-6 and TNF-α secretion with simultaneous increase of IL-10 levels, allowing parasite to escape detection by immune system (45, 46).

Inflammation is recognized as pivotal feature of immune response to blood stages of *Plasmodium* infection (47). Notably, clinical manifestations of the disease are related to erythrocytic stage of infection. An early and finely balanced inflammatory response with increase in levels of pro-inflammatory IL-12, IFN-γ, TNF-α, IL-1β, and IL-6 and of anti-inflammatory IL-10 and TGF-β is essential for resolution of parasitemia and of disease (48–52). However, pathological activation of exaggerated levels of the very same pro-inflammatory cytokines (cytokine storm) concomitant with lower levels of regulatory mechanisms has been attributed to severe and cerebral malaria syndromes (14, 53–57). A recent study examined the levels of different biomarkers of immune response and found high concentrations of sCDI63 and Fractalkine, which are involved in immune response downregulation and modulation of anti-inflammatory responses in asymptomatic malaria (58). These authors also reported high levels of Neopterin, which is related to increased cell-mediated immune responses and macrophage activation in severe and cerebral malaria patients, indicating an overall sustained state of inflammation supporting the hypothesis of intense and prolonged inflammatory response in severe and in cerebral malaria patients.

The question then arises is that why and how would the parasite drive intense inflammatory response that has the potential to be fatal which could limit parasite transmission and hence not be in interest of the pathogen? The answer appears to lie in (a) enhanced expression of adhesion molecules on endothelial cells by pro-inflammatory cytokines (IFNγ and TNFα) (59) and (b) by requirement for endothelial adhesion mediated by *P. falciparum* membrane protein 1 (PfEMP1) with CD36 and endothelial protein C receptor (EPCR) (60, 61). From the parasite view, endothelial sequestration is essential to escape clearance in spleen and to facilitate *falciparum* merozoite maturation. The highly diverse PfEMP1 proteins encoded by parasite *var* genes contain a Duffy-binding like and cysteine-rich interdomain region (CIDR) domains. Most CIDRα1 domains bind to EPCR and CIDRα2–6 bind CD36 (60, 61). Notably, interaction of EPCR with its ligand the activated protein C (APC) has a role in anti-inflammatory, coagulation homeostasis, and endothelial barrier protection functions (62) and its blockade of these functions by PfEMP1–EPCR interaction that is postulated to contribute to cerebral malaria pathology (59, 61). Interestingly, Smith et al. (61) found increased association of severe malaria with EPCR binding CIDRα1domain containing isolates supporting the contention. Interactions with CD36 are also reported to inhibit IL-12 synthesis and suppressing dendritic cell (DC) maturation and T cell activation.

It is, therefore, not unimaginable that parasite manipulates NF-κB and Type 1 interferon pathway to drive inflammation. *Plasmodium*-derived PAMPs that include GPI anchors, CpG motifs, AT-rich motifs, and haemazoin are sensed by PRRs of host that include TLRs, NLRs, and AIM2 on cells of monocyte/macrophage lineage and on DCs (61, 63–65). These ligand–receptor interactions initiate MyD88 and STING-IRF3 mediated downstream signaling leading to activation of NF-κB and IRF3 pathways and synthesis of pro-inflammatory cytokines and interferon α/β (55, 65–68). It is the exaggerated activation of these pathways "mediated by IFN-γ pro-inflammatory priming with extreme levels of pro-inflammatory mediators" with concomitant loss of regulatory cytokines that drives malaria pathogenesis (46, 57, 68). It has also been proposed that in addition to driving inflammation, *P. falciparum* by downregulating GATA3 expression suppresses IL-10 and SOCS3 that are necessary to control inflammation, possibly by exploiting the IFNα/β pathway as summarized in **Figure 1**.

LEISHMANIA: T CELL DIFFERENTIATION AND CROSS REGULATION OF CYTOKINE SIGNALING

Leishmaniasis caused by *Leishmania* spp. is a public health problem with 1.3 million reported Leishmaniasis cases worldwide which is intensified by availability of few effective drugs (70) and vaccine (71, 72). Being an intracellular parasite, it needs to overcome host-resistance mechanisms and exploit host environment for survival. From the parasite context, metabolism of *Leishmania* possesses a unique metabolic organization that can re-route metabolites, the uptake of which is constrained in different host environments toward synthesis of specific biomass metabolites; thereby providing novel mechanisms for metabolic adaptations (73, 74). From the host context, the contribution of specific virulence factors in immune suppression or the inability of the host to generate a sufficient immune response against the parasite, which promotes infection. Survival strategy of *Leishmania* is to modulate the signaling pathways of the macrophages after entering the phagolysosome. Depending on the type of infection and the parasite burden, either Th-1 healing or the Th-2 non-healing immune responses are generated, but detailed mechanism is poorly explored. This can be largely understood with respect to the interaction of parasite molecules with the host signaling pathways to suppress host immunity against infection (71).

During invasion, the surface molecules of *Leishmania* interact with the toll-like-receptor proteins present on the macrophages membrane (75). The activation of the TLRs triggers the downstream signaling pathways such as the RAS–RAF-mediated MAPK pathway, canonical and non-canonical NF-κB pathway, JAK–STAT pathway, PI3K–PLC Gamma pathway, and the JNK pathway (76). Subsequently several transcription factors, such as ERK1/2, NF-κB, NFAT, AP1, STAT3, are activated that initiate the synthesis and secretion of several cytokines, growth factors, chemokines and antimicrobicidal molecules which are responsible for the host immune responses during the infection (77).

However, during chronic infection (**Figure 2**), the antigenic molecules of the *Leishmania* parasite activate the phosphatase proteins in the macrophage, e.g., SHP-1 and PTP1B, which leads

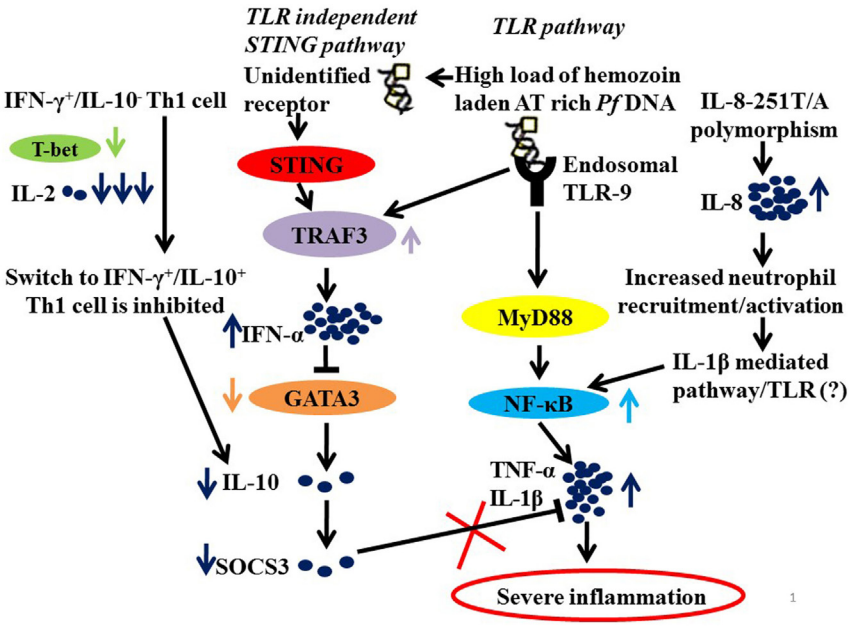

FIGURE 1 | A hypothetical model summarizing the probable mechanisms of severe inflammation in malaria. Parasite molecules like Haemazoin, Pf AT-rich DNA recruited by TLR and TLR independent (STING) pathways (63, 69). High load of Pf AT rich DNA would lead to increased levels of TRAF3 and of IFN-α. And IFN-α, in turn, suppresses GATA3 expression in Th2 cells resulting in low levels of IL-10 and hence down regulated SOCS3 (68). In addition, low levels of IL-2 and T-bet fail to mediate switch from IFN-γ+/IL-10– to IFN-γ+/IL-10+ Th1 cells that requires T-bet and IL-2 levels, also explain low levels of IL-10. Finally, downregulated SOCS3, which is known to mediate the anti-inflammatory functions of IL-10, fails to regulate an exaggerated proinflammatory response. Another contributory role to severe inflammation in malaria is the high prevalence of IL-8-251T/A, which increases IL-8 expression for enhanced recruitment and activation of inflammatory cells neutrophils resulting in increased activation of NF-κB *via* IL-1β-mediated pathway.

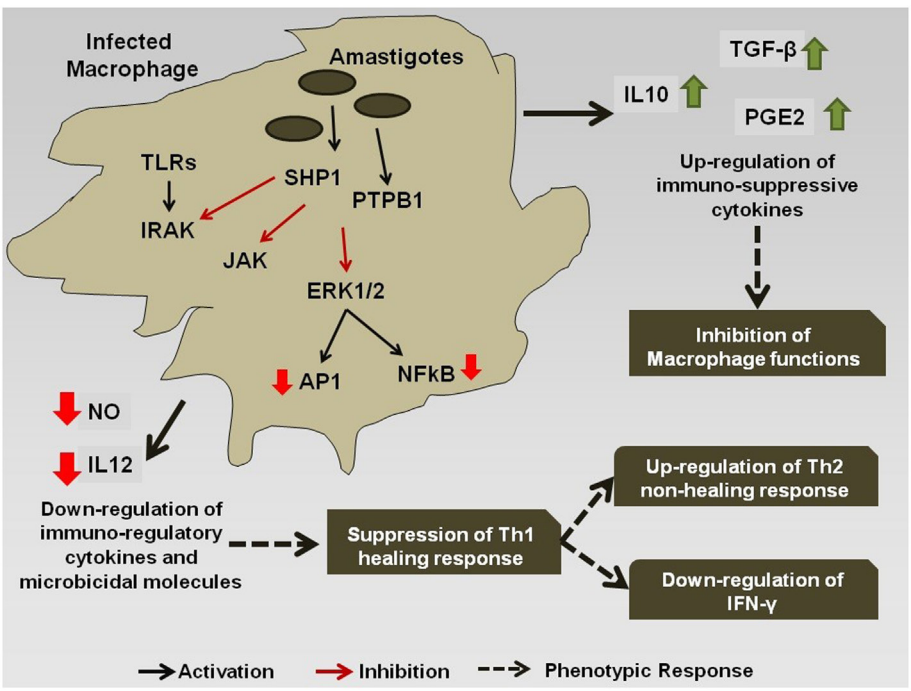

FIGURE 2 | Immuno-modulation by *Leishmania* parasite: *Leishmania* antigens interfere with the signaling cascade of the macrophage and promote the Th-2 non-healing response that helps in the survival of the parasite inside the host.

to the dephosphorylation and deactivation of selected signaling pathways (78). This leads to downregulation of expression of iNOS and nitric oxide in the infected macrophages, thereby compromising microbicidal functions of the cell and creating an immune-suppressed condition, which is favorable for the continued survival of the pathogen inside APC. Simultaneously, the production of the cytokines, such as IL-12 and TNF-α, gets severely reduced. Such changes in the cytokine expression pattern of the antigen-presenting cells leads to the alteration of the phenotypic responses of the T-cells that now start showing a bias toward the non-healing Th-2 immune response that is characterized by an increased production of IL-4, IL-10, IL-13, and TGF-β cytokines (79), and the suppression of IFN-γ that regulates the healing Th-1 response (71). The transcription factors T-bet and GATA3 play a pivotal role in the regulation of the Th-1/Th-2 ratio during the infection (80). *Leishmania* also inhibits the ability of the host cell for antigen presentation to other immune cells, by repressing the MHC class II gene expression (81) and by modulating the interaction of the co-stimulatory molecules B7-1/CD28 (82) and CD40/CD40L (83).

The difference in the antigenic challenge posed to the host gives rise to differences in expression of the macrophage proteins, as seen in visceral versus the cutaneous infections (84). The difference in macrophage protein expression profile, as exemplified by increased production of COX2 and PGE2 production in case of *L. donovani* infection (as opposed to *L. major*) (85) indicates different *Leishmania* species selectively activate or inhibits different host pathways due to differences in the antigenic challenge. Also, it has been observed in a study that *L. donovani*, which is known to cause visceral leishmaniasis, may in rare cases give rise to cutaneous leishmaniasis (86). This behavior of *L. donovani* infection may be attributed to host's resistance to the disease which restricts the spread of the infection to the visceral organs and keeps it localized to cutaneous regions (86).

The CD4+ CD25+ regulatory T cells also play a major role in regulating the persistence of the parasite *L. major* inside the host. Inhibition of the T-reg promoting cytokines such as IL-10 leads to the clearance of the pathogen from the host (87). However, during Leishmaniasis the low production of the IFN-γ and IL-12 cytokines leads to the increased proliferation of the T-reg cells that leads to the re-activation of the *Leishmania* parasites inside the host (87).

SYSTEMS BIOLOGY BASED INTEGRATIVE APPROACHES FOR UNDERSTANDING THE HOST–PARASITE INTERACTION AND CO-EVOLUTIONARY PATTERNS IN PROTOZOAN DISEASES

During the interaction of hosts and protozoan parasites, both employ mutual selective pressures on each other, which may facilitate rapid reciprocal adaptation. Different stages of the parasite life cycle introduce another layer of complexity (88). Significant amount of molecular, omics, clinical, epidemiological as well as ecological data has been generated at *in vitro* and *in vivo* levels using various pathogens and respective diseases. Integrative analysis of such discretely generated and located data from the host and protozoan parasite variants, in laboratory as well as natural populations is the most essential necessity to identify the complex mechanisms of protozoan evolution *via* immune escape during host–parasite coevolution. Public resources such as EuPathDB (89), Pathogen–Host Interactions (90), ProtozoaDB (91), together with protozoan species-specific databases are tremendously useful to collect useful information for initiating systems based integrative analysis. The key steps in such integrative approach involves data generation/data collection, data organization, data integration, integrative network construction, network analyses, and finally computer-based mathematical simulation and predictive modeling (92). As an example, using a reconstructed genome scale metabolic model of *Leishmania infantum* adaptations, (73) have identified the robustness of the parasite metabolic network against accidental errors and demonstrated the wide array of choices for the parasite to achieve optimal survival (73).

Recent advancement in RNA-Seq based techniques has facilitated the simultaneous sequencing of both host and parasite (including non-model parasites) transcriptomes (93). In a first of its kind RNA-seq experiment in control human neutrophils during priming with pro-inflammatory cytokines (TNF-α and GM-CSF), Wright et al. have shown the rapid expression of a common set of transcripts for cytokines, chemokines, and cell surface receptors (CXCL1, CXCL2, IL1A, IL1B, IL1RA, ICAM1) (94). They have demonstrated the utility of this approach to define functional changes in neutrophils following cytokine exposure. During a mega scale analysis of 116 malaria patients and infecting *P. falciparum* parasite, Yamagishi et al. have identified variable behaviors of the field malaria parasites, which were far more complex than those observed under laboratory conditions (95). Pittman et al. have generated a large scale *T. gondii*–host interactome, using dual transcriptional profiling of mice and parasite during acute and chronic infection (96) to demonstrate the influence of parasite development on host gene transcription as well as the epigenetic influence of the host environment on parasite gene transcription. Various systems-wide studies on malaria parasites have reported posttranscriptional (97) and translational (98) control at various points of the parasite lifecycle. One of such controlling mechanism is translational delay, by which protein expression in parasite is actively suspended for expressed mRNA transcripts. It was shown in *P. falciparum* that by suppressing more than 30% of its genes, the parasite rapidly adapts to new environments within the host by remaining undetected to the host immune system and undergo developmental switching in order to survive (99).

CONCLUSION AND FUTURE PERSPECTIVES

There is large apparent heterogeneity in offense strategies employed by the protozoan pathogen in human infections. In contrast to this, there appears to be a broad consensus on the major signaling hubs manipulated by the pathogens. It would

be worthwhile to dissect the host–pathogen interactions at cellular, molecular, and systems level to discriminate between infections that are virulent with potential for fatal outcomes from asymptomatic or uncomplicated infections with limited morbidity. It may be hypothesized that immuno regulatory mechanisms that confer disease tolerance are distinct from immune and metabolic responses to severe diseases and demand to be determined by large global studies employing different protozoan pathogen systems. However, despite the availability of huge amount of multi-dimensional data in host–protozoan interaction, functional characterization, and annotation of parasite genomes is severely limited by lack of both genetic tools and resources in protozoa. Given the size, heterogeneity and complexity of the host–parasite interaction data, development of new computational tools and user-friendly methods for integrating heterogeneous "Big Data" will facilitate to fill up the missing links. This will be beneficial for better understanding of the evolutionary arm race between the host and the parasite, and finally for the efficient management and control of the protozoan diseases in humans.

AUTHOR CONTRIBUTIONS

SB: manuscript design and contributed the introduction and sections on cytokines, malaria and *Toxoplasma* and future perspectives, PB: contributed the section on systems biology-based integrative approaches to understand host–parasite interaction and future perspectives, RRS: contributed in the sections on Leishmaniasis and *Trypanasoma*, AM: contributed to the section on malaria, NS: contributed to the section on cytokines and cytokine regulation, SP: contributed to the section on *Toxoplasma*, MB: contributed to the section on *Toxoplasma*, and PG: contributed in the sections on Leishmaniasis and *Trypanasoma*. SP and MB: contributed to section on Cytokine Signalling Manipulation by Protozoan parasites.

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REFERENCES

1. Sacks D, Sher A. Evasion of innate immunity by parasitic protozoa. *Nat Immunol* (2002) 3:1041–7. doi:10.1038/ni1102-1041
2. McGovern KE, Wilson EH. Dark side illuminated: imaging of *Toxoplasma gondii* through the decades. *Parasit Vectors* (2013) 6:334. doi:10.1186/1756-3305-6-334
3. de Monerri NCS, Kim K. Pathogens hijack the epigenome A new twist on host-pathogen interactions. *Am J Pathol* (2014) 184:897–911. doi:10.1016/j.ajpath.2013.12.022
4. Park SH, Kang K, Giannopoulou E, Qiao Y, Kang K, Kim G, et al. Type I interferons and the cytokine TNF cooperatively reprogram the macrophage epigenome to promote inflammatory activation. *Nat Immunol* (2017) 18:1104. doi:10.1038/ni.3818
5. Bougdour A, Tardieux I, Hakimi MA. *Toxoplasma* exports dense granule proteins beyond the vacuole to the host cell nucleus and rewires the host genome expression. *Cell Microbiol* (2014) 16:334–43. doi:10.1111/cmi.12255
6. Schneider AG, Abdallah DSA, Butcher BA, Denkers EY. toxoplasma gondii triggers phosphorylation and nuclear translocation of dendritic cell STAT1 while simultaneously blocking IFN gamma-induced STAT1 transcriptional activity. *PLoS One* (2013) 8. doi:10.1371/journal.pone.0060215
7. Escoll P, Mondino S, Rolando M, Buchrieser C. Targeting of host organelles by pathogenic bacteria: a sophisticated subversion strategy. *Nat Rev Microbiol* (2016) 14:5–19. doi:10.1038/nrmicro.2015.1
8. Hanein D. *Toxoplasma gondii* targets the host actin cytoskeleton during invasion, GO figure. *Biophys J* (2013) 104:140a. doi:10.1016/j.bpj.2012.11.796
9. Lee SH, Lee BY, Min DY, Kim JM, Ahn MH. Role of cytoskeleton in host cell invasion by intracellular protozoa *Toxoplasma gondii*. *J Microbiol Biotechnol* (2002) 12:628–34.
10. Jones MJ, Fejes AP, Kobor MS. DNA methylation, genotype and gene expression: who is driving and who is along for the ride? *Genome Biol* (2013) 14:126. doi:10.1186/gb-2013-14-7-126
11. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science* (2010) 327:291–5. doi:10.1126/science.1183021
12. Mayer-Barber KD, Yan B. Clash of the cytokine titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses. *Cell Mol Immunol* (2017) 14:22–35. doi:10.1038/cmi.2016.25
13. Kubo M, Motomura Y. Transcriptional regulation of the anti-inflammatory cytokine IL-10 in acquired immune cells. *Front Immunol* (2012) 3:275. doi:10.3389/fimmu.2012.00275
14. Freitas do Rosario AP, Lamb T, Spence P, Stephens R, Lang A, Roers A, et al. IL-27 promotes IL-10 production by effector Th1 CD4+ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. *J Immunol* (2012) 188:1178–90. doi:10.4049/jimmunol.1102755
15. Falvo JV, Jasenosky LD, Kruidenier L, Goldfeld AE. Epigenetic control of cytokine gene expression: regulation of the TNF/LT locus and T helper cell differentiation. *Adv Immunol* (2013) 118:37–128. doi:10.1016/B978-0-12-407708-9.00002-9
16. Mia S, Warnecke A, Zhang XM, Malmstrom V, Harris RA. An optimized protocol for human M2 macrophages using M-CSF and IL-4/IL-10/TGF-beta yields a dominant immunosuppressive phenotype. *Scand J Immunol* (2014) 79:305–14. doi:10.1111/sji.12162
17. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell* (2009) 136:26–36. doi:10.1016/j.cell.2008.12.027
18. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol* (2008) 9:839–45. doi:10.1038/ni.f.209
19. Asirvatham AJ, Magner WJ, Tomasi TB. miRNA regulation of cytokine genes. *Cytokine* (2009) 45:58–69. doi:10.1016/j.cyto.2008.11.010
20. McGovern KE, Wilson EH. Role of chemokines and trafficking of immune cells in parasitic infections. *Curr Immunol Rev* (2013) 9:157–68. doi:10.2174/1573395509666131217000000
21. Diaz-Gandarilla JA, Osorio-Trujillo C, Hernandez-Ramirez VI, Talamas-Rohana P. PPAR activation induces M1 macrophage polarization via cPLA(2)-COX-2 inhibition, activating ROS production against *Leishmania mexicana*. *Biomed Res Int* (2013) 2013:215283. doi:10.1155/2013/215283

22. Lee CG, Sahoo A, Im SH. Epigenetic regulation of cytokine gene expression in T lymphocytes. *Yonsei Med J* (2009) 50:322–30. doi:10.3349/ymj.2009.50.3.322
23. Zhu JF, Jankovic D, Oler AJ, Wei G, Sharma S, Hu GQ, et al. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* (2012) 37:660–73. doi:10.1016/j.immuni.2012.09.007
24. Barker BR, Taxman DJ, Ting JP. Cross-regulation between the IL-1beta/IL-18 processing inflammasome and other inflammatory cytokines. *Curr Opin Immunol* (2011) 23:591–7. doi:10.1016/j.coi.2011.07.005
25. Liu Y, Zhang Q, Ding Y, Li X, Zhao D, Zhao K, et al. Histone lysine methyltransferase Ezh1 promotes TLR-triggered inflammatory cytokine production by suppressing Tollip. *J Immunol* (2015) 194:2838–46. doi:10.4049/jimmunol.1402087
26. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. *Nat Rev Immunol* (2018) 18:91–104. doi:10.1038/nri.2017.112
27. Cheeseman K, Weitzman JB. Host-parasite interactions: an intimate epigenetic relationship. *Cell Microbiol* (2015) 17:1121–32. doi:10.1111/cmi.12471
28. Ghosh D, Stumhofer JS. Do you see what I see: recognition of protozoan parasites by toll-like receptors. *Curr Immunol Rev* (2013) 9:129–40. doi:10.2174/1573395509666131203225929
29. Sibley LD. Invasion and intracellular survival by protozoan parasites. *Immunol Rev* (2011) 240:72–91. doi:10.1111/j.1600-065X.2010.00990.x
30. Kim BH, Shenoy AR, Kumar P, Bradfield CJ, MacMicking JD. IFN-inducible GTPases in host cell defense. *Cell Host Microbe* (2012) 12:432–44. doi:10.1016/j.chom.2012.09.007
31. Du J, An R, Chen L, Shen Y, Chen Y, Cheng L, et al. *Toxoplasma gondii* virulence factor ROP18 inhibits the host NF-kappaB pathway by promoting p65 degradation. *J Biol Chem* (2014) 289:12578–92. doi:10.1074/jbc.M113.544718
32. Saeij JPJ, Coller S, Boyle JP, Jerome ME, White MW, Boothroyd JC. *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* (2007) 445:324–7. doi:10.1038/nature05395
33. Rosowski EE, Lu D, Julien L, Rodda L, Gaiser RA, Jensen KD, et al. Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *J Exp Med* (2011) 208:195–212. doi:10.1084/jem.20100717
34. Franco M, Shastri AJ, Boothroyd JC. Infection by *Toxoplasma gondii* specifically induces host c-Myc and the genes this pivotal transcription factor regulates. *Eukaryot Cell* (2014) 13:483–93. doi:10.1128/EC.00316-13
35. Peixoto L, Chen F, Harb OS, Davis PH, Beiting DP, Brownback CS, et al. Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. *Cell Host Microbe* (2010) 8:208–18. doi:10.1016/j.chom.2010.07.004
36. Bougdour A, Durandau E, Brenier-Pinchart MP, Ortet P, Barakat M, Kieffer S, et al. Host cell subversion by *Toxoplasma* GRA16, an exported dense granule protein that targets the host cell nucleus and alters gene expression. *Cell Host Microbe* (2013) 13:489–500. doi:10.1016/j.chom.2013.03.002
37. Cardoso MS, Reis-Cunha JL, Bartholomeu DC. Evasion of the immune response by *Trypanosoma cruzi* during acute infection. *Front Immunol* (2015) 6:659. doi:10.3389/fimmu.2015.00659
38. Rodrigues MM, Oliveira AC, Bellio M. The immune response to *Trypanosoma cruzi*: role of toll-like receptors and perspectives for vaccine development. *J Parasitol Res* (2012) 2012:507874. doi:10.1155/2012/507874
39. Silva GK, Gutierrez FR, Guedes PM, Horta CV, Cunha LD, Mineo TW, et al. Cutting edge: nucleotide-binding oligomerization domain 1-dependent responses account for murine resistance against *Trypanosoma cruzi* infection. *J Immunol* (2010) 184:1148–52. doi:10.4049/jimmunol.0902254
40. DosReis GA. Evasion of immune responses by *Trypanosoma cruzi*, the etiological agent of Chagas disease. *Braz J Med Biol Res* (2011) 44:84–90. doi:10.1590/S0100-879X2011007500005
41. Cribb P, Perdomo V, Alonso VL, Manarin R, Barrios-Payan J, Marquina-Castillo B, et al. *Trypanosoma cruzi* high mobility group B (TcHMGB) can act as an inflammatory mediator on mammalian cells. *Plos Negl Trop Dis* (2017) 11:e0005350. doi:10.1371/journal.pntd.0005350
42. Hedrick PW. Population genetics of malaria resistance in humans. *Heredity* (2011) 107:283–304. doi:10.1038/hdy.2011.16
43. World Health Organization. *Malaria Fact Sheet No 94*. Available from www.who.int/mediacentre/factsheets/fs375/en/
44. Joice R, Narasimhan V, Montgomery J, Sidhu AB, Oh K, Meyer E, et al. Inferring developmental stage composition from gene expression in human malaria. *PLoS Comput Biol* (2013) 9:e1003392. doi:10.1371/journal.pcbi.1003392
45. Zheng H, Tan Z, Xu W. Immune evasion strategies of pre-erythrocytic malaria parasites. *Mediators Inflamm* (2014) 2014:362605. doi:10.1155/2014/362605
46. Bertolino P, Bowen DG. Malaria and the liver: immunological hide-and-seek or subversion of immunity from within? *Front Microbiol* (2015) 6:41. doi:10.3389/fmicb.2015.00041
47. Clark IA, Budd AC, Alleva LM, Cowden WB. Human malarial disease: a consequence of inflammatory cytokine release. *Malar J* (2006) 5:85. doi:10.1186/1475-2875-5-85
48. Rovira-Vallbona E, Moncunill G, Bassat Q, Aguilar R, Machevo S, Puyol L, et al. Low antibodies against *Plasmodium falciparum* and imbalanced pro-inflammatory cytokines are associated with severe malaria in Mozambican children: a case-control study. *Malar J* (2012) 11:181. doi:10.1186/1475-2875-11-181
49. Anyona SB, Kempaiah P, Raballah E, Ouma C, Were T, Davenport GC, et al. Functional promoter haplotypes of interleukin-18 condition susceptibility to severe malarial anemia and childhood mortality. *Infect Immun* (2011) 79:4923–32. doi:10.1128/IAI.05601-11
50. Ayimba E, Hegewald J, Segbena AY, Gantin RG, Lechner CJ, Agosssou A, et al. Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe *Plasmodium falciparum* malaria. *Clin Exp Immunol* (2011) 166:218–26. doi:10.1111/j.1365-2249.2011.04474.x
51. Boeuf PS, Loizon S, Awandare GA, Tetteh JKA, Addae MM, Adjei GO, et al. Insights into deregulated TNF and IL-10 production in malaria: implications for understanding severe malarial anaemia. *Malar J* (2012) 11:253. doi:10.1186/1475-2875-11-253
52. Mendonca VRR, Queiroz ATL, Lopes FM, Andrade BB, Barral-Netto M. Networking the host immune response in *Plasmodium vivax* malaria. *Malar J* (2013) 12:69. doi:10.1186/1475-2875-12-69
53. Ockenhouse CF, Hu WC, Kester KE, Cummings JF, Stewart A, Heppner DG, et al. Common and divergent immune response signaling pathways discovered in peripheral blood mononuclear cell gene expression patterns in presymptomatic and clinically apparent malaria. *Infect Immun* (2006) 74:5561–73. doi:10.1128/IAI.00408-06
54. Sharma S, DeOliveira RB, Kalantari P, Parroche P, Goutagny N, Jiang Z, et al. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. *Immunity* (2011) 35:194–207. doi:10.1016/j.immuni.2011.05.016
55. Mahanta A, Kar SK, Kakati S, Baruah S. Heightened inflammation in severe malaria is associated with decreased IL-10 expression levels and neutrophils. *Innate Immun* (2015) 21:546–52. doi:10.1177/1753425914561277
56. Storm J, Craig AG. Pathogenesis of cerebral malaria-inflammation and cytoadherence. *Front Cell Infect Microbiol* (2014) 4:100. doi:10.3389/fcimb.2014.00100
57. Lourembam SD, Sawian CE, Baruah S. Dysregulation of cytokines expression in complicated *falciparum* malaria with increased TGF-beta and IFN-gamma and decreased IL-2 and IL-12. *Cytokine* (2013) 64:503–8. doi:10.1016/j.cyto.2013.08.007
58. Tahar R, Albergaria C, Zeghidour N, Ngane VF, Basco LK, Roussilhon C. Plasma levels of eight different mediators and their potential as biomarkers of various clinical malaria conditions in African children. *Malar J* (2016) 15:337. doi:10.1186/s12936-016-1378-3
59. Engwerda CR, Mynott TL, Sawhney S, De Souza JB, Bickle QD, Kaye PM. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. *J Exp Med* (2002) 195:1371–7. doi:10.1084/jem.20020128
60. Hsieh FL, Turner L, Bolla JR, Robinson CV, Lavstsen T, Higgins MK. The structural basis for CD36 binding by the malaria parasite. *Nat Commun* (2016) 7:12837. doi:10.1038/ncomms12837
61. Bernabeu M, Danziger SA, Avril M, Vaz M, Babar PH, Brazier AJ, et al. Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass. *Proc Natl Acad Sci U S A* (2016) 113:E3270–9. doi:10.1073/pnas.1524294113
62. Sarangi PP, Lee HW, Kim M. Activated protein C action in inflammation. *Br J Haematol* (2010) 148:817–33. doi:10.1111/j.1365-2141.2009.08020.x

63. Gazzinelli RT, Kalantari P, Fitzgerald KA, Golenbock DT. Innate sensing of malaria parasites. *Nat Rev Immunol* (2014) 14:744–57. doi:10.1038/nri3742
64. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* (2012) 13:325–32. doi:10.1038/ni.2231
65. Kalantari P, DeOliveira RB, Chan J, Corbett Y, Rathinam V, Stutz A, et al. Dual engagement of the NLRP3 and AIM2 inflammasomes by *Plasmodium*-derived hemozoin and DNA during malaria. *Cell Rep* (2014) 6:196–210. doi:10.1016/j.celrep.2013.12.014
66. Durai P, Govindaraj RG, Choi S. Structure and dynamic behavior of toll-like receptor 2 subfamily triggered by malarial glycosylphosphatidylinositols of *Plasmodium falciparum*. *FEBS J* (2013) 280:6196–212. doi:10.1111/febs.12541
67. Pichyangkul S, Yongvanitchit K, Kum-Arb U, Hemmi H, Akira S, Krieg AM, et al. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a toll-like receptor 9-dependent pathway. *J Immunol* (2004) 172:4926–33. doi:10.4049/jimmunol.172.8.4926
68. Mahanta A, Baruah S. Lower expression of GATA3 and T-bet correlates with downregulated IL-10 in severe *falciparum* malaria. *Clin Transl Immunology* (2015) 4:e49. doi:10.1038/cti.2015.30
69. Shaham SH. *IL-10 Regulation in Complicated Malaria*. [dissertation/master's thesis], Tezpur University.
70. Croft SL, Seifert K, Yardley V. Current scenario of drug development for leishmaniasis. *Indian J Med Res* (2006) 123:399–410.
71. Ganguli P, Chowdhury S, Sarkar RR. Identification of Th1/Th2 regulatory switch to promote healing response during leishmaniasis: a computational approach. *EURASIP J Bioinform Syst Biol* (2015) 2015(1):13. doi:10.1186/s13637-015-0032-7
72. Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. *Clin Transl Immunology* (2014) 3:e13. doi:10.1038/cti.2014.4
73. Subramanian A, Sarkar RR. Revealing the mystery of metabolic adaptations using a genome scale model of *Leishmania infantum*. *Sci Rep* (2017) 7:10262. doi:10.1038/s41598-017-10743-x
74. Saunders EC, Ng WW, Kloehn J, Chambers JM, Ng M, McConville MJ. Induction of a stringent metabolic response in intracellular stages of *Leishmania mexicana* leads to increased dependence on mitochondrial metabolism. *PLoS Pathog* (2014) 10:e1003888. doi:10.1371/journal.ppat.1003888
75. Tuon FF, Amato VS, Bacha HA, Almusawi T, Duarte MI, Amato Neto V. Toll-like receptors and leishmaniasis. *Infect Immun* (2008) 76:866–72. doi:10.1128/IAI.01090-07
76. Faria MS, Reis FC, Lima AP. Toll-like receptors in *Leishmania* infections: guardians or promoters? *J Parasitol Res* (2012) 2012:930257. doi:10.1155/2012/930257
77. Bhardwaj S, Srivastava N, Sudan R, Saha B. *Leishmani*a interferes with host cell signaling to devise a survival strategy. *J Biomed Biotechnol* (2010) 2010:109189. doi:10.1155/2010/109189
78. Shio MT, Hassani K, Isnard A, Ralph B, Contreras I, Gomez MA, et al. Host cell signalling and leishmania mechanisms of evasion. *J Trop Med* (2012) 2012:819512. doi:10.1155/2012/819512
79. Cummings HE, Tuladhar R, Satoskar AR. Cytokines and their STATs in cutaneous and visceral leishmaniasis. *J Biomed Biotechnol* (2010) 2010:294389. doi:10.1155/2010/294389
80. Diaz YR, Rojas R, Valderrama L, Saravia NG. T-bet, GATA-3, and Foxp3 expression and Th1/Th2 cytokine production in the clinical outcome of human infection with *Leishmania* (Viannia) species. *J Infect Dis* (2010) 202:406–15. doi:10.1086/653829
81. Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clin Microbiol Rev* (2005) 18:293. doi:10.1128/CMR.18.2.293-305.2005
82. Kaye PM, Rogers NJ, Curry AJ, Scott JC. Deficient expression of co-stimulatory molecules on *Leishmania*-infected macrophages. *Eur J Immunol* (1994) 24:2850–4. doi:10.1002/eji.1830241140
83. Soong L, Xu JC, Grewal IS, Kima P, Sun J, Longley BJ Jr., et al. Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to *Leishmania amazonensis* infection. *Immunity* (1996) 4:263–73. doi:10.1016/S1074-7613(00)80434-3
84. McMahon-Pratt D, Alexander J. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol Rev* (2004) 201:206–24. doi:10.1111/j.0105-2896.2004.00190.x
85. Gregory DJ, Sladek R, Olivier M, Matlashewski G. Comparison of the effects of *Leishmania major* or *Leishmania donovani* infection on macrophage gene expression. *Infect Immun* (2008) 76:1186–92. doi:10.1128/IAI.01320-07
86. McCall LI, Zhang WW, Matlashewski G. Determinants for the development of visceral leishmaniasis disease. *PLoS Pathog* (2013) 9:e1003053. doi:10.1371/journal.ppat.1003053
87. Mendez S, Reckling SK, Piccirillo CA, Sacks D, Belkaid Y. Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. *J Exp Med* (2004) 200:201–10. doi:10.1084/jem.20040298
88. Hill AV, Jepson A, Plebanski M, Gilbert SC. Genetic analysis of host-parasite coevolution in human malaria. *Philos Trans R Soc Lond B Biol Sci* (1997) 352:1317–25. doi:10.1098/rstb.1997.0116
89. Aurrecoechea C, Barreto A, Basenko EY, Brestelli J, Brunk BP, Cade S, et al. EuPathDB: the eukaryotic pathogen genomics database resource. *Nucleic Acids Res* (2017) 45:D581–91. doi:10.1093/nar/gkw1105
90. Urban M, Pant R, Raghunath A, Irvine AG, Pedro H, Hammond-Kosack KE. The pathogen-host interactions database (PHI-base): additions and future developments. *Nucleic Acids Res* (2015) 43:D645–55. doi:10.1093/nar/gku1165
91. Davila AM, Mendes PN, Wagner G, Tschoeke DA, Cuadrat RR, Liberman F, et al. ProtozoaDB: dynamic visualization and exploration of protozoan genomes. *Nucleic Acids Res* (2008) 36:D547–52. doi:10.1093/nar/gkm820
92. Swann J, Jamshidi N, Lewis NE, Winzeler EA. Systems analysis of host-parasite interactions. *Wiley interdiscip Rev Syst Biol Med* (2015) 7:381–400. doi:10.1002/wsbm.1311
93. Westermann AJ, Gorski SA, Vogel J. Dual RNA-seq of pathogen and host. *Nat Rev Microbiol* (2012) 10:618–30. doi:10.1038/nrmicro2852
94. Wright HL, Thomas HB, Moots RJ, Edwards SW. RNA-Seq reveals activation of both common and cytokine-specific pathways following neutrophil priming. *PLoS One* (2013) 8:e58598. doi:10.1371/journal.pone.0058598
95. Yamagishi J, Natori A, Tolba MEM, Mongan AE, Sugimoto C, Katayama T, et al. Interactive transcriptome analysis of malaria patients and infecting *Plasmodium falciparum*. *Genome Res* (2014) 24:1433–44. doi:10.1101/gr.158980.113
96. Pittman KJ, Aliota MT, Knoll LJ. Dual transcriptional profiling of mice and *Toxoplasma gondii* during acute and chronic infection. *BMC Genomics* (2014) 15:806. doi:10.1186/1471-2164-15-806
97. Doerig C, Rayner JC, Scherf A, Tobin AB. Post-translational protein modifications in malaria parasites. *Nat Rev Microbiol* (2015) 13:160–72. doi:10.1038/nrmicro3402
98. Cui LW, Lindner S, Miao J. Translational regulation during stage transitions in malaria parasites. *Ann N Y Acad Sci* (2015) 1342:1–9. doi:10.1111/nyas.12573
99. Bunnik EM, Chung DWD, Hamilton M, Ponts N, Saraf A, Prudhomme J, et al. Polysome profiling reveals translational control of gene expression in the human malaria parasite *Plasmodium falciparum*. *Genome Biol* (2013) 14:R128. doi:10.1186/gb-2013-14-11-r128

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Circulating Plasma MicroRNA-208a as Potential Biomarker of Chronic Indeterminate Phase of Chagas Disease

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Chagas cardiomyopathy is the most severe clinical manifestation of chronic Chagas disease. The disease affects most of the Latin American countries, being considered one of the leading causes of morbidity and death in the continent. The pathogenesis of Chagas cardiomyopathy is very complex, with mechanisms involving parasite-dependent cytopathy, immune-mediated myocardial damage and neurogenic disturbances. These pathological changes eventually result in cardiac myocyte hypertrophy, arrhythmias, congestive heart failure and stroke during chronic infection phase. Herein, we show that miR-208a, a microRNA that is a key factor in promoting cardiovascular dysfunction during cardiac hypertrophy processes of heart failure, has its circulating levels increased during chronic indeterminate phase when compared to cardiac (CARD) clinical forms in patients with Chagas disease. In contrast, we have not found altered serum levels of miR-34a, a microRNA known to promote pro-apoptotic role in myocardial infarction during degenerative process of cardiac injuries thus indicating intrinsic differences in the nature of the mechanisms underlying the heart failure triggered by *Trypanosoma cruzi* infection. Our findings support that the chronic indeterminate phase is a progressive phase involved in the genesis of chagasic cardiopathy and point out the use of plasma levels of miR-208a as candidate biomarker in risk-prediction score for the clinical prognosis of Chagas disease.

Keywords: Chagas disease, *Trypanosoma cruzi*, infectious heart disease, microRNA, disease biomarkers

INTRODUCTION

Chagas disease or American trypanosomiasis is a tropical parasitic disease caused by the protozoan *Trypanosoma cruzi* and transmitted mainly by insects of the subfamily Triatominae (Rassi et al., 2010). It is estimated that more than seven million people, especially in Mexico, Central America and South America, have Chagas disease. This has resulted in about 12,500 deaths per year since 2006. Large-scale population movements have expanded the areas where cases of Chagas disease are found, including now many countries in Europe and North America (World Health Organization, 2017). In the endemic areas, the vector consists the main mechanism of human transmission through host contact with feces and urine of infected vectors, mainly of the genera *Triatoma*, *Rhodnius,* and *Panstrongylus*. The insect vector acquires *Trypanosoma cruzi* parasite by feeding blood from infected animals or humans (Noireau et al., 2009).

Within the invertebrate vector, the parasite multiplies under epimastigote forms that later differentiate into trypomastigotes in the digestive tract of triatomine. The trypomastigote forms are the infecting stages of vertebrate host, being released in the feces of triatomine during bloodfeeding, and transmitted when host scratches contaminated feces into the wound (Kollien and Schaub, 2000). Once inside the host, the trypomastigote invades the cells near the inoculation, in the first line resident macrophages, where they differentiate into intracellular amastigotes (Burleigh and Andrews, 1995). The amastigotes multiply by binary division and differentiate into trypomastigotes, which are released into the bloodstream, infecting cells of various tissues, and this cycle is repeated several times, due to its ability to persist prolonged periods in the host tissues mainly as dividing amastigote forms inside tissue cells and pseudocysts by subverting humoral and cell-mediated immunity. The clinical manifestations in humans are the result of this infectious cycle and may include, at early stages of infection, symptoms such as fever, lymphadenitis, or local swelling of the biting site (Burleigh and Andrews, 1995; Kollien and Schaub, 2000; Andrade and Andrews, 2005).

After an asymptomatic acute phase in 90% of infected individuals, the infection persists and enters its chronic indeterminate phase, in which 60–80% of patients will never develop clinical symptoms, although parasitism remains in balance with host immune responses. During the intermediate phase there is a cumulative progression to diffuse myocardial damage in about 20–40% of the chronic patients developing cardiac and/or digestive problems (Teixeira et al., 2011). The pathogenesis of Chagas disease is due to an intense inflammatory lesion and fibrosis induced to the tissues and organs involved in the infectious cycle of the parasite (Machado et al., 2000; Lepletier et al., 2014). If left untreated, Chagas disease can be fatal, in most cases by damaging cardiac muscle tissue (Marin-Neto et al., 2007). The cytopathic effects induced by the parasite and the host immune responses against the intracellular amastigote harm the intramural neurons of the autonomic nervous system of the gut and heart, leading to megacolon and cardiac aneurysms, respectively (Zhang and Tarleton, 1999; Campos et al., 2016).

The nature of Chagas disease lesions induced by parasitism is intrinsically related to the perpetuation of the parasite in the affected tissues (Rassi et al., 2009). The chronicity of infection is associated with profound changes in the molecular levels of the affected organs, as it has been shown that myocardial gene expression patterns are altered in advanced forms of chronic disease (Ferreira et al., 2014). Although the molecular mechanisms underlying the myocardial gene expression are not well elucidated, studies have shown that microRNAs involved in the genetic regulation process of heart development and cardiovascular disorders have their expression disregulated in advanced forms of chagasic chronic heart disease (Eulalio et al., 2012). These studies were performed in patients with more advanced forms of chagasic heart disease and their comparisons with normal healthy individuals (Eulalio et al., 2012). However, studies in the earliest forms of chronic infection, using patients in the indeterminate phase of the disease, would allow us to explore the use of cardiac microRNAs as a potential biomarker to predict/diagnose, in the indeterminate phase of disease, the patients that will develop cardiomyopathy (20–40%).

Along these lines, we investigated herein the modulation of potential heart disease-specific miRNA biomarkers in chagasic patients at early chronic infection to be used as a risk predictor for clinical prognosis in *Trypanosoma cruzi*-induced cardiomyopathy. Our research include analysis of cross-sectional studies from chronic chagasic patients at the indeterminate (IND) as compared to cardiac (CARD) clinical forms of Chagas disease, or non-infected control individuals to profile the plasma expression of cardiac miRNA biomarkers of two extreme processes involved in the cardiovascular dysfunction. In degenerative cardiovascular diseases, microRNAs (miRNAs) are crucial regulators of cardiac function (Filipowicz et al., 2008; Boon et al., 2013). Endogenous miRNAs were shown to regulate gene expression at the post-transcriptional level either through translation repression or mRNA degradation affecting independent processes resulting in degeneration of the cardiac tissue (Callis et al., 2009).

MiR-208a is one of the most important heart-specific miRNA playing a critical role in the heart failure and has been considered as a potential biomarker of myocardial injuries (Cunha-Neto et al., 2005; Wang et al., 2013; Doka et al., 2015; Shyu et al., 2015; Zhang et al., 2017). The miR-208a is encoded by an intronic region of the alpha-cardiac muscle myosin heavy chain gene (Myh6) regulating the myosin heavy chain isoform switch (Cunha-Neto et al., 2005). Under pathophysiologic conditions, miR-208a is critical to induce arrhythmias, cardiac remodeling, expression of hypertrophy pathway components and the cardiac conduction system (Cunha-Neto et al., 2005). Studies on mice lacking miR-208a have indicated that this microRNA is required for expression of the homeodomain-only protein (HOP), which is required to modulate cardiac growth and development, in addition to GATA4 and the Gap junction alpha-5 protein (GJA5), also known as connexin 40 (Cx40). These factors are determinants in the induction of heart injuries associated with cardiac conduction (Shyu et al., 2015).

In a different aspect of the mechanisms capable of promoting heart injury, miR-34a is shown to contribute to the age-dependent deterioration of cardiac function, contributing to cardiomyocyte death following acute myocardial infarction (Filipowicz et al., 2008). Studies have identified the PPP1R10 gene (also known as PNUTS) as a direct miR-34a target, whose inhibition promotes telomere shortening and cardiomyocyte apoptosis. The anti-apoptotic role of PNUTS and its counter-regulation by age-induced expression of miR-34a has a direct effect on the processes that regulate cardiac contractile function and functional cardiac recovery after acute myocardial infarction (Filipowicz et al., 2008). In the present study we attempted to clarify whether changes in the circulating levels of miR-208a and miR-34a in the blood is associated with the development of severe cardiac clinical form of human chronic Chagas disease.

MATERIALS AND METHODS

Ethics Statement

All the protocols for animal (IMPPG038-05/16) and human (CAAE No. 46502615.1.0000.5257) studies were approved by the Research Ethics Committee of Federal University of Rio de Janeiro. Experimental mice infections and assay procedures with *T. cruzi* were performed in biosafety area recommended for activities with infective stages of the parasites, in accordance with the terms of the Brazilian and international guidelines for the welfare regulations.

Study Population

Healthy volunteers and *T. cruzi* chronic chagasic patients were recruited from Chagas Cardiomyopathy clinic of Hospital Universitário Clementino Fraga Filho of Universidade Federal do Rio de Janeiro, HUCFF-UFRJ, Brazil. All individuals analyzed in this study, including chronic infected patients and non-infected individuals with ages ranging from 30 to 64 years, confirmed the diagnosis for *T. cruzi* infection in serological tests. The selected individuals included 10 seropositive cases of cardiac chronic-infected patients showed dilated cardiomyopathy diagnosed based in a detailed clinical examination, electrocardiography (ECG), and unidimensional/bidimensional echocardiography with Doppler (ECHO). Additionally, we included 10 patients in the indeterminate forms of Chagas disease, without any cardiac alterations detected. Twenty donors, age and sex matched-non-infected controls were included in the study. The research was approved by the Research Ethics Committee (CEP) and all patients signed a free and informed consent form in accordance with current legislation and the regulations of the HUCFF-UFRJ (CAAE No. 46502615.1.0000.5257).

Quantification of Human Plasma Levels of microRNA

Human plasma samples were collected using heparin-coated tubes and frozen at −80°C. RNA from 200 μl of human plasma were isolated with miRNeasy Serum/Plasma Kit (Qiagen) according to manufacturer instructions. Synthetic *C. elegans* miR-39 mimic (Qiagen) was spiked-in to all samples before RNA isolation to control for proper isolation. We performed miRNA expression profiling using the Taqman miRNA Assays for miR-34a (hsa-miR-34a-5p; ID number 000426), miR-208a (hsa-miR-208a-3p; ID number 000511), miR-221 (hsa-miR-221-3p; ID number 000524), miR-484 (hsa-miR-484; ID number 001821), and miR-39 (cel-miR-39; ID number 000200) in technical triplicate and the Taqman miRNA Reverse Transcription kit and Taqman Universal Master Mix II, no UNG according to the manufacturer's instructions (ThermoFisher) in a Step ONE Plus Fast Real Time PCR System (Applied Biosystems). The data were analyzed by fitting four-parameter sigmoid curves to the Rn data using the qPCR library (Ritz and Spiess, 2008) for the R statistical package version 2.922. The gene-stability measurement was determined by the optimal number of control genes required for normalization using average pairwise variation ($V_{n/n+1}$) analysis between the normalization factors NF_n and NF_{n+1} by geNorm method (Vandesompele et al., 2002). An adaptation of this method was used to assay the cel-miR-39 (spike-in) for the average expression stability of reference genes, beside the non-related hsa-miR-221-3p and hsa-miR-484 endogenous genes. Based on our analysis we selected hsa-miR-221-3p and hsa-miR-484 as most stable miRNA in our samples, since they are below the recommended threshold for pairwise variation V of 0.15 (between the normalization factors NF_n and NF_{n+1}) and used them for normalization. The comparison of the means of the normalized gene expression values between the groups was performed by a non-parametric one-way ANOVA with 1,000 unrestricted permutations, followed by *post-hoc* pair-wise comparisons with a Bonferroni adjustment using a non-parametric *t*-test, also with 1,000 permutations (Basso et al., 2009).

Animals, Infection, Parasite Load, Detection of Creatine Kinase Cardiac Isoenzyme and Intra-Cardiac Levels of miR-34a

Male C57BL/6 mice, aged 4–8 weeks, were housed and maintained in IMPPG-Federal University of Rio de Janeiro animal facility for experimental infection experiments. Chronic *T. cruzi* infection was performed by inoculating the 6-week-old mice intraperitoneally with 2×10^3 blood-derived trypomastigote forms of Y strain of *T. cruzi*. The parasitemia was monitored by counting blood parasites using Neubauer's chambers, and animals were bled and sacrificed at 30 days post-infection (DPI). To test the creatine kinase cardiac isoenzyme (CK-MB), plasma samples were collected at 14 DPI from *T. cruzi* infected and non-infected mice, and the CK-MB activity was measured by the commercial kit CK-MB Liquiform (Labtest) as described by the manufacturer. The assays were read in a microplate spectrophotometer (SpectraMax® Plus 384 Microplate Reader, Molecular devices) allowing the analysis of small quantities of mouse plasma according to manufacturer's recommendation. The optical density at 340 nm was recorded every 1 min (A_1) at 37°C, for 5 min (A_2). Calibration and quality control of the equipment were performed according to the recommended protocol. To assess the intracardiac parasite load, hearts were collected at

14 DPI, minced and 100 mg of tissue were homogenized in 1 mL of TRIzol® (Invitrogen) for DNA extraction, following manufacturer's instructions. The concentrations of extracted total DNA were measured using NanoDrop-1000 (Thermo Scientific). Each PCR reaction contained 50 ng of the genomic DNA, 0.25mM *T. cruzi* 195-bp repeat DNA-specific primers: TCZ-F: 5′-GCTCTTGCCCACAAGGGTGC-3′, and TCZ-R: 5′-CCAAGCAGCGGATAGTTCAGG-3′, amplified with SYBR Green® PCR Master Mix (Qiagen) and PCR-grade H_2O (Qiagen) on a Step One Real Time PCR System (Applied Biosystems). For loading controls, we used reactions containing 50 ng of the genomic DNA, 0.25 mM of murine-specific tumor necrosis factor-a (TNF-a) gene primers TNF-α-F: 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ and TNF-α-R: 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′. To determine the intracardiac levels of mir-34a expression, high-purity miRNA was obtained from heart tissue by using standard protocol (Faragó et al., 2011). Quantitative real-time PCR analysis were performed using miScript SYBR Green PCR Kit for mouse mir-34a (Quiagen) at the same conditions as described above.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 5 software. Statistical differences between groups were compared by a non-parametrical test (Mann–Whitney Rank Sum Test). Results were expressed as mean ± standard deviation (S.D.), and differences between control and treated group were considered statistically significant when $p \leq 0.05$.

RESULTS

To evaluate whether changes in the plasma-circulating levels of cardiac-specific miRNAs is correlated with Chagas heart disease severity, we first aimed at normalizing the gene-expression levels by selected stable internal control gene in order to remove any non-specific variation of the target miRNAs in our analysis. To validate the genetic expression analyzes of the RT-qPCR data we should normalize the gene expression profiles of the cardiac-specific miRNA genes that are supposed to be modulated in the process of infectious cardiopathy with respect to stably expressed endogenous reference genes not related to the disease. However, it has been demonstrated that stable microRNAs derived from plasma/serum are likely disease-specific, and miRNA control genes for Chagas disease have not yet been identified. To address this question, beside using a spike with a micro RNA control which only controls for proper isolation and processing of miRNA samples we also used irrelevant microRNA genes known to be highly regulated in our donor sample collection study, as reference genes.

To this end, the expression ratios of both cardiac-specific miR-208a and miR-34a were assessed as the pairwise variation with the non-related internal hsa-mir-221-3p genes and hsa-miR-484 used as endogenous controls for other infectious diseases such as Hepatitis B and breast cancer were defined from internal control gene-stability measure analysis (Hu et al., 2012; Li et al., 2015). We first performed the expression stability of two internal control non-related genes in all samples (**Figure 1A**) and the algorithm for the normalization of the endogenous microRNAs was more stable than using the cel-miR-39 spike-in control (**Figure 1B**). Based on our findings, we decided to normalize the expression profile of cardiac-miRNA genes using a geometric mean of miR-221 and miR-484 expression as described in the methods section. In fact normalyzation analysis of cardiac-specific miR-208a and miR-34a related to spike-in control did not have significant differences among the groups as determined to access proper sample isolation for comparison between samples (**Figure 2**).

Using this averaging of the control genes in order to measure expression levels accurately, we next investigate whether the myocardial damage induced by *T. cruzi* infection is associated with the expression of miR-208a, a key factor in promoting cardiac hypertrophy during cardiovascular dysfunction. Based on our results, we found significant increased levels of circulating plasma miR-208a in cross-sectional studies (**Table 1**) of chronic chagasic patients at the indeterminate (IND) as compared

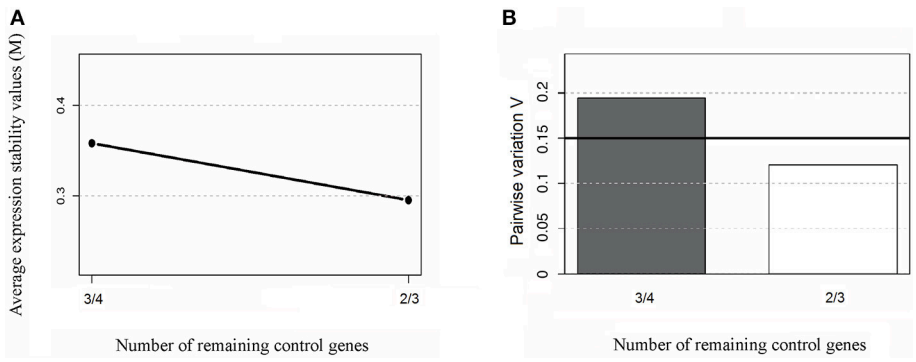

FIGURE 1 | Average expression stability of endogenous miRNAs. Ranking of the gene expression stability (M) performed in all samples analyzed in the study during stepwise exclusion **(A)**. A lower *M*-value indicates more stable expression. The least stable genes are on the left using the spike-in cel-miR-39 control gene; and the most stable genes are represented on the right using the expression of endogenous hsa-miR-221-3p and hsa-miR-484 as references. The non-related microRNAs hsa-mir-221-3p and hsa-miR-484 were used for relative normalization as endogenous controls. The optimal number of control genes was determined for normalization by Pairwise variation **(B)**, as described in section Materials and Methods.

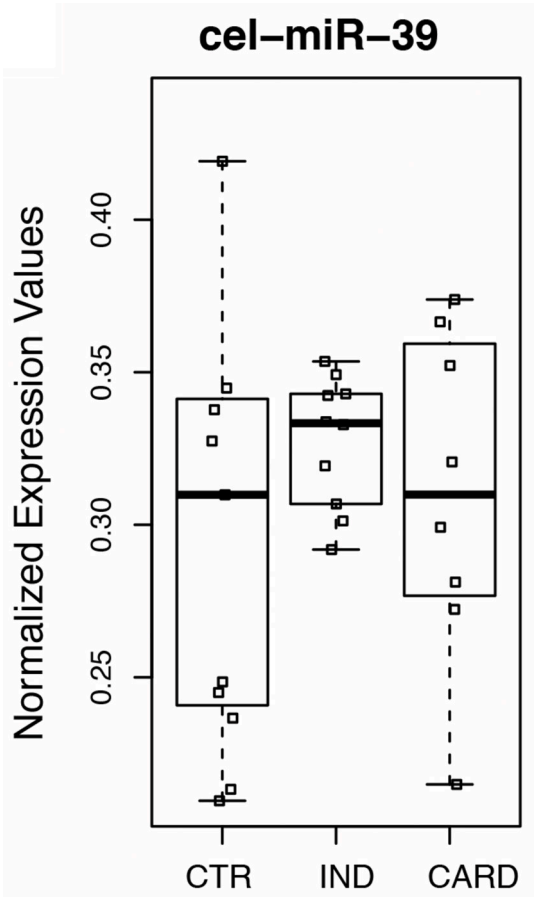

FIGURE 2 | Normalyzation analysis of cardiac-specific miR-208a and miR-34a related to cel-miR-39 spike-in gene. Spike-in control using external control miRNA gene expressed in *C. elegans* (cel-miR-39) was determined to access proper sample isolation for accurate comparison between samples. Real-time quantitative PCR was performed on total miRNA purified from plasma of non-infected individuals (CTR), patients at the indeterminate (IND), and cardiac (CARD) clinical forms of Chagas disease. Twenty donors, age, and sex matched-individuals were included for each group. Groups were compared by Mann-Whitney non-parametric test.

to cardiac (CARD) clinical forms of Chagas disease or non-infected control individuals (**Figure 3A**). However, we did not find any significant difference across the clinical groups in the expression levels of circulating miR-34a, a key regulator promoting cardiomyocyte cell death associated to heart failure (**Figure 3B**).

Although our goal is to investigate the plasma levels of cardiac miRNAs as potential candidate biomarkers of risk-predicting for patients in clinical prognosis, in fact some tissue-specific miRNA do not present satisfactory circulating levels in biomarkers analyzes.

We next ruled out the possibility that the absence of this modulation is due to an intrinsic characteristic of miR-34a being differentially expressed in cardiac tissues. We then measured the levels of intracardiac expression of this miRNA using an experimental Chagas' infection model. The murine models of heart disease share at different levels some of the pathophysiological changes seen in patients with chronic heart disease, such as extra-cellular deposition of fibronectin in the heart tissue and severe alterations of the heart's electrical activity (Pereira et al., 2014). These parameters are characteristic of more advanced forms of chronic human disease, so the murine model is not useful for studying the potential pathophisiological changes observed in the human chronic indeterminate phase of Chagas disease.

Therefore, using the murine experimental model of *Trypanosoma cruzi* infection-induced cardiomyopathy, by intraperitoneally injecting C57BL/6 mice with 2×10^3 bloodstream trypomastigotes of Y strain parasites, we observed a precocious blood parasitemia at day 8 post-infection (**Supplementary Figure 1A**), which further induced severe parasitism in the heart tissues as the infection continued (**Supplementary Figure 1B**). The heart parasitism intensity in this model is associated with cardiac muscle damage and can be monitored by measuring the release of CK-MB isoform of creatine kinase by heart muscle. This cardiac marker resides in the cytosol and facilitates the movement of high energy phosphates required for mitochondrial physiology.

Since CK-MB isoform has a short duration, its plasma levels can be used to access the level of heart tissue damage during the cardiopathy process (Adams et al., 1993; Pereira et al., 2014). As expected, we found a marked increase in the levels of CK-MB present in the peripheral blood circulation of *T. cruzi*-infected mice at 14 DPI (**Supplementary Figure 1C**). Our data indicated that this level of myocardial damage induced by *T. cruzi* infection is not associated with differences in the intracardiac expression of miR-34a as compared to non-infected controls (**Supplementary Figure 1D**), further indicating that the heart injury induced in the context of *T. cruzi* infection is not associated with upregulation of miR-34a pathway-dependent decline in cardiac activity. This finding corroborates the previous results showing unalterated plasma levels of miR-34a in the patients groups thus indicating that pro-apoptotic role of this cardiac miRNA is not involved in the heart injuries associated to chagas cardiomyopathy.

DISCUSSION

Chagas cardiomyopathy is the most severe and life-threatening manifestation of human disease caused by the protozoan parasite *Trypanosoma cruzi* (Marin-Neto et al., 2007). The knowledge of pathological changes in Chagas heart disease is largely derived from necropsy studies and endomyocardial biopsy of humans and observations in several experimental models that reasonably reproduce the various stages of the disease (Higuchi Mde et al., 1986; de Souza et al., 2001). The clinical manifestations and the tissue damage itself are closely associated with parasite multiplication and the consequent immunological reaction triggered in the parasitized myocardium (Marin-Neto et al., 2007). Thus, it is plausible to speculate that even if the parasitism is low-level, it may represent a mechanism of permanent antigenic activation and may constitute an

TABLE 1 | Circulating plasma miRNA in cross-sectional studies of chronic chagasic patients.

	Global *p*-value	log2(1ND/CTR)	*p*-value	log2(CARDC/CTR)	*p*-value	log2(CARDC/IND)	*p*-value
hsa-miR-208a-3p	0.008	0.942057053	0.005982038	0.1998333	0.923464	−0.742223753	0.18242931
hsa-miR-34a-5p	0.083	0.848200376	0.095676453	0.663184964	0.32519	−0.185015411	0.967330108

CTR, control group; IND, indeterminate group; CARD, cardiac clinical form of Chagas disease group.

essential pathogenic factor of immunological alteration in the chronic phase of Chagas disease (Rossi et al., 2010; Tarleton, 2015).

With the remission of parasitemia and systemic inflammatory reactions, it is believed that during the indeterminate stage the process of focal active myocarditis is more frequent, leading to the cumulative destruction of myocardial fibers, associated with microvascular disorders, intense neuronal depopulation, and the establishment of a gradual process of reparative fibrosis of cardiac tissue (Marin-Neto et al., 2007; Machado et al., 2012). Several studies using human and experimental models have shed light on the cellular and molecular mechanisms underlined in the pathogenesis of chronic manifestation of Chagas diseases. The heart infection by *T. cruzi* causes an intense inflammatory reaction resulting in leukocyte infiltration of the organ with an severely altered pattern of inflammatory-mediated factors in the infected tissues as seen by the increased expression levels of pro-inflammatory cytokines, chemokines, nitric oxide and vasoactive mediators in the cardiovascular system, with the predominance of CD8 T cells among lymphocyte populations in the chronic chagasic cardiomyopathy (Machado et al., 2012; Tarleton, 2015).

At the cellular and tissue level, the inflammatory injury of the cardiac tissue is characterized by the presence of parasitic pseudocysts associated with myonecrosis and vasculitis, with an intense accumulation of extracellular collagen affecting the function of cardiac muscle fibers (Machado et al., 2012). It has been shown that transforming growth factor-beta (TGF-β) participates in the fibrosis and heart remodeling during infection influencing the development of myocardiopathy in Chagas disease (Araújo-Jorge et al., 2002). Activation of fibrosis processes occurs through assessment of TGF-β-induced Smad2/3 and p38/ERK signaling (Zhang et al., 2006; Kolosova et al., 2011). The chagasic myocardial damage is disseminated throughout the heart, leading to electrocardiogram (ECG) abnormalities due to arrhythmias, conduction disturbances and also repolarization changes. Those alterations are representative of the widespread cardiac tissue damage (Elizari and Chiale, 1993; Machado et al., 2012). The nature of this injury process is considered an important feature of the mechanisms associated with pathogenesis of Chagasic cardiovascular disease (Machado et al., 2012).

In the present study we demonstrated that the human chronic indeterminate phase of Chagas disease results in increased levels of circulating miR-208a, an essential regulator of the genes involved in cardiac hypertrophy and fibrosis (Cunha-Neto et al., 2005; Wang et al., 2013; Doka et al., 2015; Shyu et al., 2015; Zhang et al., 2017). MiR-208a is encoded by an intronic region of the Myh6 gene that encodes α-myosin heavy chain, the

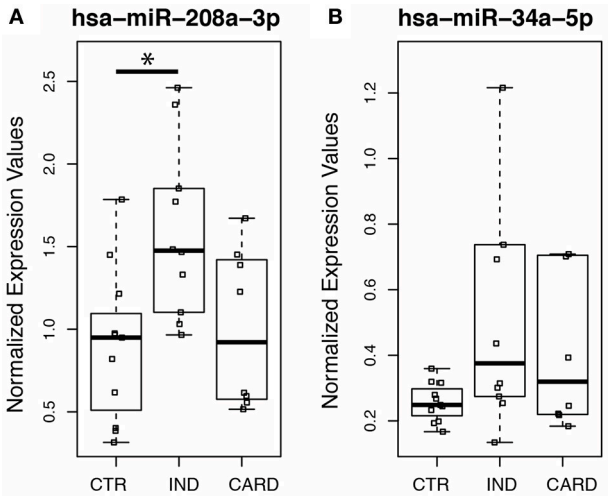

FIGURE 3 | Expression profiling of the heart-specific miR-34a and mir-208a in peripheral blood from chronic chagasic patients using endogenous miRNAs. Quantitative PCR was performed on total miRNA purified from plasma of normal control individuals (CTR), patients at the indeterminate (IND), and cardiac (CARD) clinical forms of Chagas disease. Individual and median values of relative expression of **(A)** hsa-miR-208a-3p or **(B)** hsa-miR-34a-5p normalized by the geometric mean of endogenous non-related hsa-mir-221-3p and hsa-miR-484 expression. Twenty donors, age, and sex matched-individuals were included for each group. Groups were compared by Mann-Whitney non-parametric test and the differences were considered significant at $*p < 0.05$.

prevalent heavy-chain contractile protein in the developed adult heart (Cunha-Neto et al., 2005). Alterations in the miR-208a expression levels are frequently associated with pathological heart dysfunctions, such as hypertrophy, fibrosis, arrhythmias, contractile dysfunction and conduction abnormalities (Cunha-Neto et al., 2005; Wang et al., 2013; Doka et al., 2015; Shyu et al., 2015; Zhang et al., 2017). Indeed, overexpression of miR-208a is associated with coronary heart disease (Zhang et al., 2017), and its therapeutic inhibition leads to recovery of cardiac function during heart disease (Doka et al., 2015). It has been shown that transforming growth factor-β (TGF-β) activates mirR-208a to regulate hypertrophic-related genes and the effect of neutralizating TGF-β antibody attenuates miR-208a induced-expression in cardiomyocyte hypertrophy (Wang et al., 2013).

In fact the TGF-β signaling pathway has been shown to potentiate *T. cruzi* infection and heart damage in both human and experimental murine models (Araújo-Jorge et al., 2002, 2008, 2012; Waghabi et al., 2009). This suppressor cytokine is implicated in several aspects of host-parasite interplay. TGF-β

is responsible for disarming the intracellular defense responses of *T. cruzi*-infected cells, thus potentiating the parasitic burden not only on macrophages but also on cardiac fibroblasts and cardiomyocytes (Araújo-Jorge et al., 2012); regulate the pro-inflammatory immune responses against *T. cruzi* (Gutierrez et al., 2009); and induction of fibrosis and heart injury during the chronicity of the disease (Araújo-Jorge et al., 2002, 2008; Waghabi et al., 2009). Indeed, circulating levels of TGF-β progressively increase during the chronicity of indeterminate stages for the cardiac, presenting a correlation with cardiac dysfunction and progressive fibrosis in Chagas disease (Araújo-Jorge et al., 2002). The regulation of targets by miRNAs is subject to various levels of control, and recent developments have shown that their targets can reciprocally control the level and function of miRNAs (Pasquinelli, 2012). Our data show that during the indeterminate phase of chronic infection, circulating levels of miR-208a are significantly increased. It is possible that there is a mutual regulation of miR-208a and its target genes, preceding the process of progressive fibrosis in Chagas disease during chronic infection.

Our findings therefore suggest a participation of the miR-208a in the early-onset events responsible for activation of the fibrosis and cardiac dysfunction processes in Chagas disease. Furthermore, our findings demonstrate that active myocardial inflammation promoted by *T. cruzi* infection does not yield altered levels of circulating miR-34a. MiR-34a negatively regulates the transcription of the anti-apoptotic factor PNUTS, and its response results in the induction processes of cardiac tissue degeneration (Filipowicz et al., 2008). Recent works have shown that miR-34a is an inducer of cardiomyocyte apoptosis, and its activation is determinant in cardiac senescence processes resulting in the induction of myocardial infarction processes (Filipowicz et al., 2008; Matsumoto et al., 2013). These findings suggest the participation of different mechanisms and signaling pathways of cardiac injury processes in *T. cruzi*-induced cardiomyopathy.

At present, the techniques of risk prediction in patients in the indeterminate phase of Chagas disease are based on the detection of cardiac wall motion abnormalities associated with functional and electrical defects. The presence of echocardiographic abnormalities in association with high interleukin-6 concentrations as a marker of myocardial injury has been highly predictive to screen patients for risk stratification (López et al., 2006). In this line, our findings demonstrating increased expression levels of circulating miR-208a during chronic indeterminate phase of Chagas disease should call attention to its predictive marker to identify patients at risk of developing chagasic heart disease. Subsequent translational studies should determine the levels of miR-208a in the course of chronic infection in order to determine its use as candidate biomarkers to be used in risk-prediction score for patients with Chagas cardiomyopathy.

AUTHOR CONTRIBUTIONS

AM: Conceived and designed the experiments; LL-L, AG, JG-N, and LC: Performed the experiments; LL-L, AG, JG-N, and LC: Analyzed the data; LF, CF, EF, SC, RA, RP, WS, and AM: Contributed reagents, materials, analysis tools; AG, RP, and AM: Wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00269/full#supplementary-material

Supplementary Figure 1 | Intra-cardiac levels of miR-34a expression in *T. cruzi*-induced myocarditis. **(A)** C57BL/6 mice were infected via *i.p.* with 2×10^3 blood-derived trypomastigote forms of Y strain of *T. cruzi* and the parasitemia for each mouse was determined during infection. The values are represented as the mean ± the standard deviation (*SD*) ($n = 5$). **(B)** DNA was extracted from heart tissue at 14 days post-infection and the parasite load was evaluated by quantitative PCR. **(C)** CK-MB levels were analyzed from serum at 14 DPI by enzymatic activity at 37°C. **(D)** MicroRNA-34a levels were measured from serum from non-infected control and *T. cruzi*-infected animals at 14 days post-infection by quantitative RT-PCR. Data are means ± *SD*. and represent the results of three independent experiments performed with 5 mice per treatment. Differences between between the groups were considered significant $^{***}p < 0.001$.

REFERENCES

Adams, J. E., Abendschein, D. R., and Jaffe, A. S. (1993). Biochemical markers of myocardial injury. Is MB creatine kinase the choice for the 1990s? *Circulation* 88, 750–763. doi: 10.1161/01.CIR.88.2.750

Andrade, L. O., and Andrews, N. W. (2005). The Trypanosoma cruzi-host-cell interplay: location, invasion, retention. *Nat. Rev. Microbiol.* 3, 819–823. doi: 10.1038/nrmicro1249

Araújo-Jorge, T. C., Waghabi, M. C., Bailly, S., and Feige, J. J. (2012). The TGF-β pathway as an emerging target for Chagas disease therapy. *Clin. Pharmacol. Ther.* 92, 613–621. doi: 10.1038/clpt.2012.102

Araújo-Jorge, T. C., Waghabi, M. C., Hasslocher-Moreno, A. M., Xavier, S. S., Higuchi Mde, L., Keramidas, M., et al. (2002). Implication of transforming growth factor-beta1 in Chagas disease myocardiopathy. *J. Infect. Dis.* 186, 1823–1828. doi: 10.1086/345882

Araújo-Jorge, T. C., Waghabi, M. C., Soeiro Mde, N., Keramidas, M., Bailly, S., and Feige, J. J. (2008). Pivotal role for TGF-β in infectious heart disease: the case of *Trypanosoma cruzi* infection and consequent Chagasic myocardiopathy. *Cytokine Growth Factor Rev.* 19, 405–413. doi: 10.1016/j.cytogfr.2008.08.002

Basso, D., Pesarin, F., Salmaso, L., and Solari, A. (2009). "Nonparametric one-way ANOVA," in *Permutation Tests for Stochastic Ordering and ANOVA. Theory and Applications with R* (New York, NY: Springer), 133–172. doi: 10.1007/978-0-387-85956-9

Boon, R. A., Iekushi, K., Lechner, S., Seeger, T., Fischer, A., Heydt, S., et al. (2013). MicroRNA-34a regulates cardiac ageing and function. *Nature* 495, 107–110. doi: 10.1038/nature11919

Burleigh, B. A., and Andrews, N. W. (1995). The mechanisms of *Trypanosoma cruzi* invasion of mammalian cells. *Annu. Rev. Microbiol.* 49, 175–200. doi: 10.1146/annurev.mi.49.100195.001135

Callis, T. E., Pandya, K., Seok, H. Y., Tang, R. H., Tatsuguchi, M., Huang, Z. P., et al. (2009). MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *J. Clin. Invest.* 119, 2772–2786. doi: 10.1172/JCI36154

Campos, C. F., Cangussú, S. D., Duz, A. L., Cartelle, C. T., Noviello, Mde L., Veloso, V. M., et al. (2016). Enteric neuronal damage, intramuscular denervation and smooth muscle phenotype changes as mechanisms of chagasic megacolon: long-term murine model of *Tripanosoma cruzi* infection. *PLoS ONE* 11:e0153038. doi: 10.1371/journal.pone.0153038

Cunha-Neto, E., Dzau, V. J., Allen, P. D., Stamatiou, D., Benvenutti, L., Higuchi, M. L., et al. (2005). Cardiac gene expression profiling provides evidence for cytokinopathy as a molecular mechanism in Chagas' disease cardiomyopathy. *Am. J. Pathol.* 167, 305–313. doi: 10.1016/S0002-9440(10)62976-8

de Souza, M. M., Franco, M., Almeida, D. R., Diniz, R. V., Mortara, R. A., da Silva, S., et al. (2001). Comparative histopathology of endomyocardial biopsies in chagasic and non-chagasic heart transplant recipients. *J. Heart Lung Transp.* 20, 534–543. doi: 10.1016/S1053-2498(00)00320-X

Dóka, G., Radik, M., Krenek, P., Kyselovic, J., and Klimas, J. (2015). 1a.02: microrna-208a and its host gene cardiac myosin heavy chain Myh6 are involved in hypertrophic heart dysfunction. *J. Hypertens* 33(Suppl. 1), e1. doi: 10.1097/01.hjh.0000467352.59037.c9

Elizari, M. V., and Chiale, P. A. (1993). Cardiac arrhythmias in Chagas' heart disease. *J. Cardiovasc. Electrophysiol.* 4, 596–608. doi: 10.1111/j.1540-8167.1993.tb01247.x

Eulalio, A., Mano, M., Dal Ferro, M., Zentilin, L., Sinagra, G., Zacchigna, S., et al. (2012). Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* 492, 376–381. doi: 10.1038/nature11739

Faragó, N., Zvara, A., Varga, Z., Ferdinandy, P., and Puskás, L. G. (2011). Purification of high-quality micro RNA from the heart tissue. *Acta Biol. Hung.* 62, 413–425. doi: 10.1556/ABiol.62.2011.4.7

Ferreira, L. R., Frade, A. F., Santos, R. H., Teixeira, P. C., Baron, M. A., Navarro, I. C., et al. (2014). MicroRNAs miR-1, miR-133a, miR-13 miR-208a and miR-208b are dysregulated in chronic Chagas disease cardiomyopathy. *Int. J. Cardiol.* 175, 409–417. doi: 10.1016/j.ijcard.2014.05.019

Filipowicz, W., Bhattacharyya, S. N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* 9, 102–114. doi: 10.1038/nrg2290

Gutierrez, F. R., Mineo, T. W., Pavanelli, W. R., Guedes, P. M., and Silva, J. S. (2009). The effects of nitric oxide on the immune system during *Trypanosoma cruzi* infection. *Mem. Inst. Osw. Cruz* 104(Suppl. 1), 236–245. doi: 10.1590/S0074-02762009000900030

Higuchi Mde, L., Lopes, E. A., Saldanha, L. B., Barretto, A. C., Stolf, N. A., Bellotti, G., et al. (1986). Immunopathologic studies in myocardial biopsies of patients with Chagas' disease and idiopathic cardiomyopathy. *Rev. Inst. Med. Trop. Sao Paulo* 28, 87–90. doi: 10.1590/S0036-46651986000200004

Hu, Z., Dong, J., Wang, L. E., Ma, H., Liu, J., Zhao, Y., et al. (2012). Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. *Carcinogenesis* 33, 828–834. doi: 10.1093/carcin/bgs030

Kollien, A. H., and Schaub, G. A. (2000). The development of Trypanosoma cruzi in triatominae. *Parasitol. Today* 16, 381–387. doi: 10.1016/S0169-4758(00)01724-5

Kolosova, I., Nethery, D., and Kern, J. A. (2011). Role of Smad2/3 and p38 MAP kinase in TGF-β1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. *J. Cell. Physiol.* 226, 1248–1254. doi: 10.1002/jcp.22448

Lepletier, A., de Almeida, L., Santos, L., da Silva Sampaio, L., Paredes, B., González, F. B., et al. (2014). Early double-negative thymocyte export in *Trypanosoma cruzi* infection is restricted by sphingosine receptors and associated with human Chagas disease. *PLoS Negl. Trop. Dis.* 8:e3203. doi: 10.1371/journal.pntd.0003203

Li, Y., Zhang, L., Liu, F., Xiang, G., Jiang, D., and Pu, X. (2015). Identification of endogenous controls for analyzing serum exosomal miRNA in patients with hepatitis B or hepatocellular carcinoma. *Dis. Mark.* 2015:893594. doi: 10.1155/2015/893594

López, L., Arai, K., Giménez, E., Jiménez, M., Pascuzo, C., Rodríguez-Bonfante, C., et al. (2006). C-reactive protein and interleukin-6 serum levels increase as Chagas disease progresses towards cardiac failure. *Rev. Esp. Cardiol.* 59, 50–56. doi: 10.1157/13083649

Machado, F. S., Jelicks, L. A., Kirchhoff, L. V., Shirani, J., Nagajyothi, F., Mukherjee, S., et al. (2012). Chagas heart disease: report on recent developments. *Cardiol. Rev.* 20, 53–65. doi: 10.1097/CRD.0b013e31823efde2

Machado, F. S., Martins, G. A., Aliberti, J. C., Mestriner, F. L., Cunha, F. Q., and Silva, J. S. (2000). *Trypanosoma cruzi*-infected cardiomyocytes produce chemokines and cytokines that trigger potent nitric oxide-dependent trypanocidal activity. *Circulation* 102, 3003–3008. doi: 10.1161/01.CIR.102.24.3003

Marin-Neto, J. A., Cunha-Neto, E., Maciel, B. C., and Simões, M. V. (2007). Pathogenesis of chronic Chagas heart disease. *Circulation* 115, 1109–1123. doi: 10.1161/CIRCULATIONAHA.106.624296

Matsumoto, S., Sakata, Y., Suna, S., Nakatani, D., Usami, M., Hara, M., et al. (2013). Circulating p53-responsive microRNAs are predictive indicators of heart failure after acute myocardial infarction. *Circ. Res.* 113, 322–326. doi: 10.1161/CIRCRESAHA.113.301209

Noireau, F., Diosque, P., and Jansen, A. M. (2009). *Trypanosoma cruzi*: adaptation to its vectors and its hosts. *Vet. Res.* 40:26. doi: 10.1051/vetres/2009009

Pasquinelli, A. E. (2012). MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat. Rev. Genet.* 13, 271–282. doi: 10.1038/nrg3162

Pereira, I. R., Vilar-Pereira, G., Silva, A. A., and Lannes-Vieira, J. (2014). Severity of chronic experimental Chagas' heart disease parallels tumour necrosis factor and nitric oxide levels in the serum: models of mild and severe disease. *Mem. Inst. Oswaldo Cruz* 109, 289–298. doi: 10.1590/0074-0276140033

Rassi, A. Jr., Rassi, A., and Marin-Neto, J. A. (2009). Chagas heart disease: pathophysiologic mechanisms, stratification. *Mem. Inst. Oswaldo Cruz* 104(Suppl. 1), 152–158. doi: 10.1590/S0074-02762009000900021

Rassi, A. Jr., Rassi, A., and Marin-Neto, J. A. (2010). Chagas disease. *Lancet* 375, 1388–1402. doi: 10.1016/S0140-6736(10)60061-X

Ritz, C., and Spiess, A. N. (2008). qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. *Bioinformatics* 24, 1549–1551. doi: 10.1093/bioinformatics/btn227

Rossi, M. A., Tanowitz, H. B., Malvestio, L. M., Celes, M. R., Campos, E. C., Blefari, V., et al. (2010). Coronary microvascular disease in chronic Chagas cardiomyopathy including an overview on history, pathology, and other proposed pathogenic mechanisms. *PLoS Negl. Trop. Dis.* 4:e674. doi: 10.1371/journal.pntd.0000674

Shyu, K. G., Wang, B. W., Cheng, W. P., and Lo, H. M. (2015). MicroRNA-208a increases myocardial endoglin expression and myocardial fibrosis in acute myocardial infarction. *Can. J. Cardiol.* 31, 679–690. doi: 10.1016/j.cjca.2014.12.026

Tarleton, R. L. (2015). CD8+ T cells in *Trypanosoma cruzi* infection. *Semin. Immunopathol.* 37, 233–238. doi: 10.1007/s00281-015-0481-9

Teixeira, A. R., Hecht, M. M., Guimaro, M. C., Sousa, A. O., and Nitz, N. (2011). Pathogenesis of chagas' disease: parasite persistence and autoimmunity. *Clin. Microbiol. Rev.* 24, 592–630. doi: 10.1128/CMR.00063-10

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.1. doi: 10.1186/gb-2002-3-7-research0034

Waghabi, M. C., de Souza, E. M., de Oliveira, G. M., Keramidas, M., Feige, J. J., Araújo-Jorge, T. C., et al. (2009). Pharmacological inhibition of transforming growth factor beta signaling decreases infection and prevents heart damage in acute Chagas' disease. *Antim. Agents Chemother.* 53, 4694–4701. doi: 10.1128/AAC.00580-09

Wang, B. W., Wu, G. J., Cheng, W. P., and Shyu, K. G. (2013). Mechanical stretch via transforming growth factor-beta1 activates microRNA-208a to regulate hypertrophy in cultured rat cardiac myocytes. *J. Formosan Med. Assoc.* 112, 635–643. doi: 10.1016/j.jfma.2013.01.002

World Health Organization (2017). *Chagas Disease (American Trypanosomiasis). Fact Sheet*. Geneva.

Zhang, L., and Tarleton, R. L. (1999). Parasite persistence correlates with disease severity and localization in chronic Chagas' disease. *J. Infect. Dis.* 180, 480–486. doi: 10.1086/314889

Zhang, M., Fraser, D., and Phillips, A. (2006). ERK, p38, and Smad signaling pathways differentially regulate transforming growth factor-beta1 autoinduction in proximal tubular epithelial cells. *Am. J. Pathol.* 169, 1282–1293. doi: 10.2353/ajpath.2006.050921

Zhang, Y., Li, H. H., Yang, R., Yang, B. J., and Gao, Z. Y. (2017). Association between circulating microRNA-208a and severity of coronary heart disease. *Scand. J. Clin. Lab. Invest.* 77, 379–384. doi: 10.1080/00365513.2017.1328740

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REVIEW
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Human Kinetoplastid Protozoan Infections: Where Are We Going Next?

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Kinetoplastida trypanosomatidae microorganisms are protozoan parasites exhibiting a developmental stage in the gut of insect vectors and tissues of vertebrate hosts. During the vertebrate infective stages, these parasites alter the differential expression of virulence genes, modifying their biological and antigenic properties in order to subvert the host protective immune responses and establish a persistent infection. One of the hallmarks of kinetoplastid parasites is their evasion mechanisms from host immunity, leading to disease chronification. The diseases caused by kinetoplastid parasites are neglected by the global expenditures in research and development, affecting millions of individuals in the low and middle-income countries located mainly in the tropical and subtropical regions. However, investments made by public and private initiatives have over the past decade leveraged important lines of intervention that if well-integrated to health care programs will likely accelerate disease control initiatives. This review summarizes recent advances in public health care principles, including new drug discoveries and their rational use with chemotherapeutic vaccines, and the implementation of control efforts to spatially mapping the kinetoplastid infections through monitoring of infected individuals in epidemic areas. These approaches should bring us the means to track genetic variation of parasites and drug resistance, integrating this knowledge into effective stewardship programs to prevent vector-borne kinetoplastid infections in areas at risk of disease spreading.

Keywords: parasitic infection, kinetoplastid protozoans, *Trypanosoma cruzi*, *Leishmania*, *Trypanosoma brucei*

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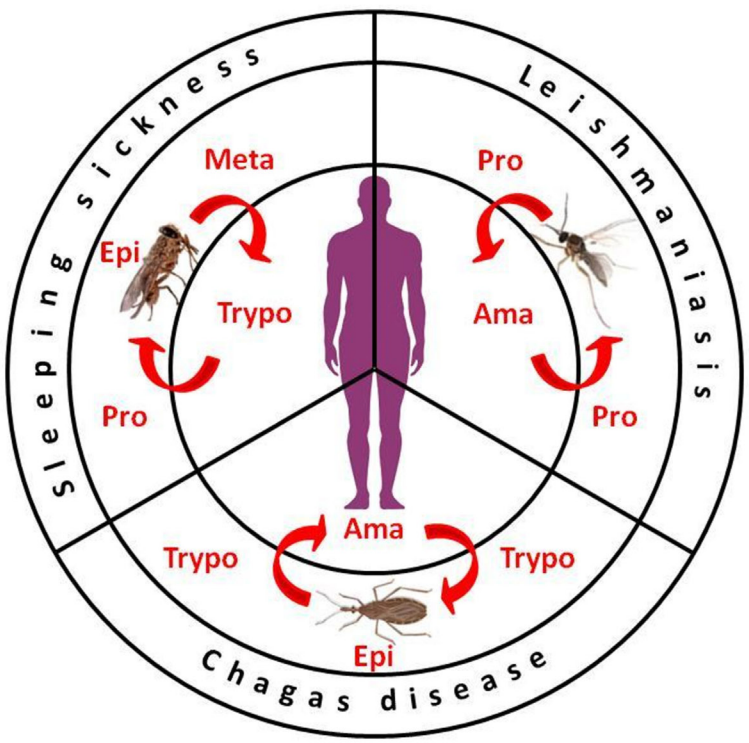
INTRODUCTION

Protozoan infections are one of the most devastating causes of human death worldwide. These infections are caused by protozoan parasites, microorganisms originally classified in the Kingdom Protozoa, which comprises a diverse group of unicellular eukaryotes (1). Although the majority of the protozoan exists as free-living microorganisms in different aquatic and humid environments, there are many species living in association with host organisms, causing severe human diseases (1, 2). This is the case of Kinetoplastid parasites, a group of flagellated protozoans that parasitize most plant and animal species; and cause human diseases with public health threats and social-economic effects (3).

The kinetoplastids are a monophyletic group related to the euglenids. These microorganisms are distinguished from other protozoan groups mainly by the presence of kinetoplasts, a granule that contains "kDNA," a DNA located in the mitochondria, associated with the base of the flagella (3). Three distinct kinetoplastids cause human disease: *Trypanosoma brucei* [human African trypanosomiasis (HAT) or sleeping sickness], *Trypanosoma cruzi* (Chagas disease), and *Leishmania* spp. (leishmaniasis) (3, 4), which are still recognized as neglected tropical diseases (NTDs) by the World Health Organization. These two genera of parasites are found in the blood and/or tissues of infected humans and are transmitted by arthropod vectors (**Figure 1**) (5).

Although the advances in the development of drug therapies and vector control agents against kinetoplastid diseases (6), new strategies are required for global elimination of epidemies. The main limiting efforts for this accomplishment obviously relies on the global investments in R&D for these NTDs when compared to other diseases with higher levels of financial support, such as malaria, tuberculosis, and HIV, known as "big three" (7). The analysis of the global sums of expenditures for each of these diseases, and their correlations with the social impact indexes on public health indicates a correlation that goes far beyond the political question (8). Such correlation is proportional to the severity of each disease (**Figure 2**), as to the metric of the disability-adjusted



Kinetoplastid	Vector	Invertebrate Stages(IS)/Vertebrate stages(VS)	Disease
Trypanosoma cruzi	Triatomine bugs	Epimastigotes (IS), metacyclic tripomastigotes (IS/IV), trypomastigotes (VS), amastigotes (VS)	Chagas
Trypanosoma brucei	Tsetse flies	Procyclic trypomastigotes (IS), epimastigotes (IS), metacyclic trypomastigotes (IS/VS), trypomastigotes (VS)	African Trypanosomiasis
Leishmania spp	Phlebotomine sandflies	Promastigotes (IV), amastigotes (VS)	Leishmaniasis

FIGURE 1 | Kinetoplastid transmission cycles. The life cycles of kinetoplastid protozoan parasites show development stages in both invertebrate and vertebrate hosts. In leishmaniasis, promastigotes (flagellate and mobile forms) are inoculated into the skin along with the saliva of phlebotomine vectors (Diptera: Psychodidae). In the vertebrate hosts, they are maintained inside phagocytic cells under the proliferative form, amastigotes. When ingested by insect vectors, the amastigote forms are transformed into promastigotes which multiply in the intestinal tissue, then migrate as metacyclic promastigotes into the stomodal valve to be later injected into the skin during blood meal. In Chagas' disease, the etiologic agent *Trypanosoma cruzi* is transmitted to vertebrate hosts as a metacyclic trypomastigote forms by infected triatomine bugs (Triatominae: Reduviidae) during blood feeding. Once in the vertebrate, trypomastigotes differentiate into intracellular amastigote forms. These proliferative stages multiply by binary division, and then differentiate into trypomastigotes, which are released into the bloodstream. When the triatomine bug takes a blood meal from an infected vertebrate host containing circulating parasites, the ingested trypomastigotes forms to differentiate into epimastigotes in the medium intestine of the vector, multiplying by binary division, after which they differentiate into infective metacyclic trypomastigote forms. In sleep sickness or human African trypanosomiasis, the parasite *Trypanosoma brucei* is transmitted by the bite of the tsetse fly (Glossinidae: Glossina). The parasite exists in the saliva of the invertebrate vector and is injected when the insect feeds on human blood. Unlike *Trypanosoma cruzi*, trypomastigotes of *T. brucei* do not invade host cells and, therefore, does not differentiate into intracellular amastigote forms. Instead, *T. brucei* parasites multiply as trypomastigotes in the blood of infected vertebrate host. The parasite cycle continues when a new vector feeds on a contaminated individual. In the invertebrate vector, the parasites differentiate into proliferative epimastigotes forms, invading the insect salivary glands to continue the cycle.

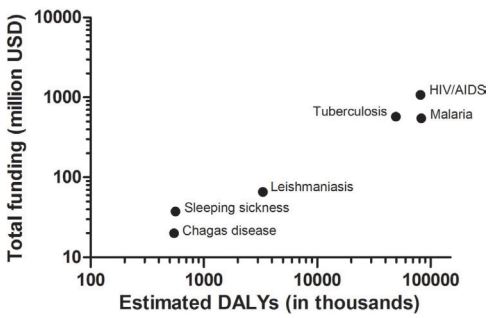


FIGURE 2 | Overall funding for research and development investment correlated with disability-adjusted life years, estimated from the Global Burden of Disease Study.

life year (DALY), an index calculated as the sum of the years of life lost due to premature mortality of patients and the years lost due to disability for people living with the illness (9).

In spite of the low priority of the investments received, studies in the past decades have pointed out the paths for developing effective actions to control human kinetoplastid protozoan infections through a better understanding of the pathogen–vertebrate host interactions during their life cycles, disease pathogenesis in the hosts, and methods that allow the diagnosis, even in the acute phase of the infection (3, 4). This has provided a better opportunity to prospect new therapeutic targets, more effective drug development approaches, and promising vaccines that, together, may combat these diseases more robustly in the near future (5).

TRENDS IN DRUG DEVELOPMENT FOR KINETOPLASTID INFECTIONS

Treatment approaches based on drug therapies in kinetoplastid infections are scarce and have been considered to be highly toxic (10). However, interest in research and exploration of new approaches has given a sign of change. Since kinetoplastid infections lead to chronic persistent diseases, it is critical that drug therapy interventions would be focused on the acute phase, with hopes of achieving complete eradication of disease transmission. To that end, it is necessary to establish technical procedures that allow early diagnosis of acute kinetoplastid infection (5). This intervention approach aims to act at a stage of infection in which pathogen has not yet established itself and is present with a low parasitism burden and few down modulatory mechanisms in place over the host immune responses (1, 11, 12).

In addition, a rapid and efficient intervention during acute phase of the infection, when the host is still under low tissue parasitism, decreases the chances of acquiring drug resistance (13, 14). In Chagas disease, benznidazole and nifurtimox have long been the only clinical treatment options for infection (15). However, progress has been made in the research of new promising drugs against the disease. E1224, a pro-drug of ravuconazole, has shown some efficiency in combating the disease (16). The results of the first phase 2 clinical trial in Bolivia, conducted by drugs for neglected diseases initiative, have shown satisfactory protective results for E1224. This drug was effective in controlling *T. cruzi* parasitism in infected patients. Furthermore, its use in combination with benzanidazole is more effective as compared to monotheraphy-based protocols (16).

To treat HAT, five drugs have been approved: pentamidine, melarsoprol, eflornithine, suramin, and nifurtimox (17). Pentamidine and suramin are used in monotherapies in the early stage of *T.b. gambiense* and *T.b. rhodesiense* infections, whereas melarsoprol is used for the second stage of the disease (18, 19). Nifurtimox has been used since 2009 in combination with eflornithine, mostly in the second stage of *T.b. gambiense* infection. The combination therapy protocols for these two drugs have been improved, although there are still practical restrictions to their potential use in large-scale applications (20). Currently, new drugs designed to improve patient care are being considered to meet current elimination targets. Most of them have been optimized to undergo clinical trials (17).

Fexinidazole and oxaborole SCYX-7158, are already being studied in clinical evaluation as oral therapies in phase IIb/III and phase I trials, respectively (21, 22). Other important R&D studies have focused on the virulence responses of the parasite. The cell surface of African trypanosomes (*T. brucei* spp.) is covered by a dense coat of glycoconjugates that play important roles in the evasion of host immune responses (23). New strategies in drug discovery against HAT aim the development of specific carbohydrate-binding agents capable of inhibiting the action of glycosyltransferases and glycosidases of the parasite, thus altering the nature of the parasite's cell-surface glycans as a treatment target for sleeping sickness (24).

In leishmaniasis, a notable progress in the disease treatment has been made in the preparation of new formulations of amphotericin B, using liposome carriers (25). In addition, new drugs have been extensively studied in clinical trials, although the effects of HIV co-infection in endemic areas contribute to drug unresponsiveness during therapies (26). The use of paromomycin, an aminoglycoside class antibiotic, has also been shown to be efficient at low cost as a first-line drug (27). Alternatively, studies have suggested the effective action of drugs that act on a broad spectrum against *Leishmania* parasites. This is the case of miltefosine, a drug that is also used in the treatment of dogs with leishmaniasis in Brazil (28). The miltefosine has an inhibitory action on several biological pathways of the parasite, such as cytochrome C oxidase, synthesis of phosphatidylcholine, and disruption of parasite Ca^{2+} homeostasis (29–31).

In addition, drug discovery researches for kinetoplastids have benefited from investments made in the field of protozoan genomics. This is clearly seen in the use of data mining, annotation, and analysis of *Leishmania* parasite genome that has lead to the creation of LeishCyc database (32). This systematic approach will allow a complete mapping of *Leishmania* transcriptomics, proteomics, and metabolomics enabling the development of new compounds that can be used in high-throughput screening approaches to search selective drugs against *Leishmania*

parasites (32). In fact omics-based analyses have facilitated the broadening of R&D researches in the field of drug development. Lipidomics analyses have yielded the characterization of lipid structures, protein lipidation pathways in post-translational modifications, and their putative functions for kinetoplastid parasites (33, 34). This knowledge will allow the search for new classes of anti-parasitic pharmaceuticals.

RESEARCH AND DEVELOPMENT OF NEW VACCINES AGAINST KINETOPLASTID PARASITES

The major challenge in the preventive control of kinetoplastid infections and other neglected tropic infectious diseases is undoubtedly the implementation of low-cost vaccines for public health programs in affected countries, most of them were low- and middle-income countries (5, 35). This accomplishment would enable to structure intervention actions in health care at the level of mass treatment programs, thus avoiding disease re-emergence. To reach this ideal scenario, there are serious social-political obstacles to overcome in order to ensure the development of clinical tests. The most incisive limitation is unquestionably economic, resulting from a scarce financial incentives and market failures in view of the geopolitical areas where epidemics occur (35). Such constraints are likely to arouse less interest from financial and pharmaceutical institutions in the market development strategies for vaccines against NTDs in general.

There are no licensed vaccines for kinetoplastid infections yet, reinforcing the need for their development, particularly in countries where they are epidemic. Most of the ongoing vaccine studies have been conducted at the level of basic research (5). Particularly, recent advances in the construction of manipulative parasites through genome engineering using CRISPR/Cas9 and Cre recombinase have been dedicated to reprogramming their genome, allowing the identification of regulatory genes associated with the cell fates in the host–parasite interplay (36). These critical virulence genes represent good candidates to be studied as vaccine targets.

Interesting, some kinetoplastid vaccines undergoing clinical trials are promising, since they are cost-effective and show long-term protection against both cutaneous and visceral leishmaniasis. This is the case of vaccines developed by the Infectious Diseases Research Institute, which has included protective *Leishmania* antigen epitopes in its vaccine formulations used in clinical trials (37, 38). Another prominent proposal comes from initiative studies in cooperation with National Institutes of Health. They present a more elaborate vaccine formulation, including protective recombinant *Leishmania* antigens in combination with sand fly salivary gland antigens, capable of inducing a more robust host immune response, considering vector–host interactions in the transmission of *Leishmania* parasites (39).

In the case of Chagas disease, initial studies have pointed out potential antigens in experimental mice models capable of inducing host protective immune responses. They were also effective in reducing cardiac parasite loads and disease pathology, increasing host survival indices. Those studies have proposed the use of Tc24 (calcium binding protein associated with flagellar pocket of *T. cruzi*) and TSA-1 (Trypomastigote surface antigen-1) by a global consortium (40–42). These are the first candidates to be used in vaccine formulations to prevent Chagas disease. Furthermore, the use of vaccines as a therapeutic approach to treat chagasic patients has also been proposed (42).

KEY CHALLENGES TO KINETOPLASTID PARASITE CONTROL: INTEGRATING NEW APPROACHES INTO ERADICATION STRATEGIES

To eradicate kinetoplastid infections in humans, we must gain a better understanding of variants of pathogens and their vectors, the transmission models among their hosts, and efficacious preventive vaccine approaches, to understand and design strategies to intervene in the real dynamics of disease spreading in epidemic areas (43). To achieve that, diagnostic tests capable of detecting infections in the acute phase, and accurate analysis of drug resistance in the parasite spreading are essential to trace a better correlation between variants of pathogens and the clinical spectrum of the disease. Another important step of intervention in the transmission cycles of vector diseases is regarding the application of health care models based on geographic information system (GIS) projects and technologies (44). By using this system, we will be able to follow the infection cycle in terms of spatial data, geographical and geodatabases, which allows us to create mapping models to predict potential areas of risk for transmission of vector-borne diseases (45).

The GIS technology is able to compile multi-analysis related to public health data collected from endemic urban and wild areas, including analyses of disease dispersal, genetic variation of parasites, vector and host habitats, and their relationship with microclimates in affected areas. These metadata analyses can be correlated with geographic positioning system to establish interventions of potential infections, thus allowing for a rational use of medications to prevent the emergence of drug resistance in the context of disease surveillance (44, 45). Nowadays, diverse stochastic models are used as tools for estimating probability distributions of potential outcomes from the understanding of environmental and ecological networks involved in the cycles of vector-borne diseases. These geographic models have allowed disseminating critical information concerning public health surveillance for endemic areas (**Figure 3**).

In kinetoplastid infections, spatial clustering using GIS technology when applied with accurate diagnostic test, analysis of the phylogenetic distribution of *Leishmania* spp. together with surveillance has been demonstrated to be of great value in predicting areas of risk. Epidemiological analysis of the transmission cycles in Europe, and in recent kala-azar outbreaks in Nepal, Kenya, and Brazil, have helped to precisely define that the priority of intervention is in the vector control with the use of insecticides, in order to block the transmission cycle of disease in potential risk areas, identified to be priority to reduce the spread of infection (46–49).

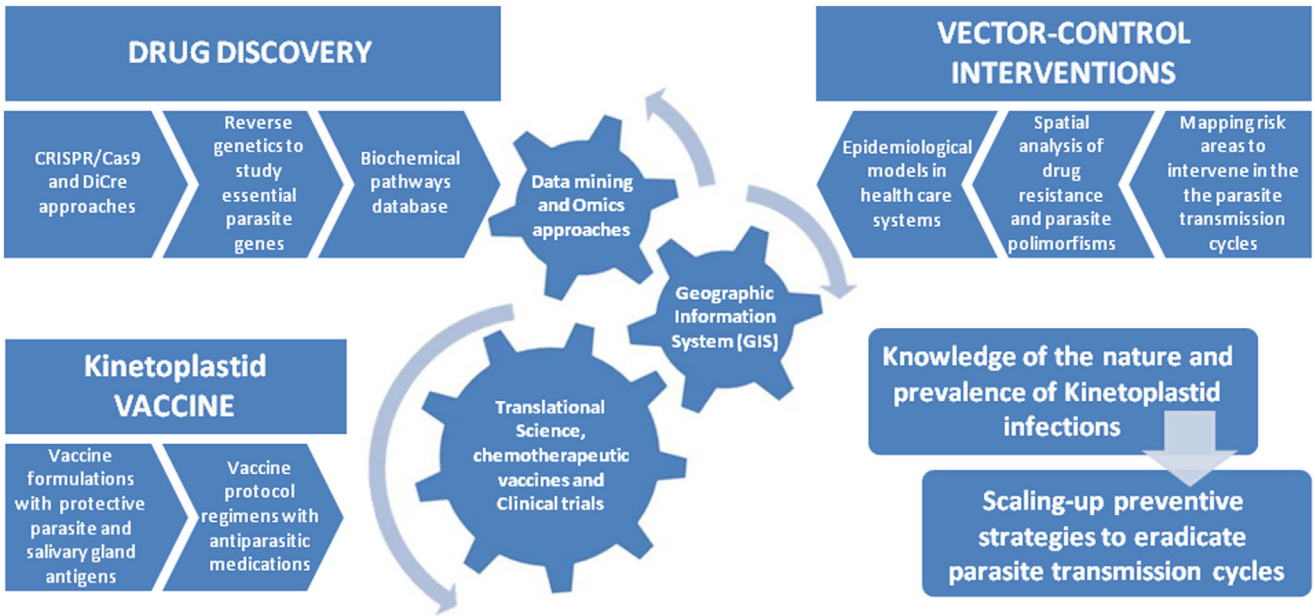


FIGURE 3 | Integrating new approaches into control strategies for kinetoplastid infections. In the past decade, genetic sequencing efforts and manipulation of trypanosomatid genomes have elucidated the characterization of virulence factors and parasite biochemical pathways involved in the pathogenesis disease. These advances allowed the design of new drug targets and therapeutic vaccines capable of reducing the parasitic burden thus controlling the infection and its clinical symptoms. The integration of these tools with epidemiological interventions into public health programs should yield substantial gains in controlling the transmission cycles of these vector-borne diseases. In this line, important studies in the field of vector control interventions have defined a multi-parametric analysis using geographic information system technologies to monitor spatial analysis of drug resistance and parasite polymorphisms to delineate epidemic-prone areas. Those studies have offered a powerful platform for prospecting and development of tools and technologies capable of effectively defining elimination programs in the control and eradication of kinetoplastid diseases.

CONCLUDING REMARKS

Neglected tropical diseases caused by kinetoplastid protozoan parasites are considered endemic in lower-middle-income economies, although in many countries they are in process of epidemiological containment and elimination. However, the eradication of these diseases is a complex issue that must involve health management policies, coordinated among the affected countries, such as the development of public policies to combat parasitic diseases and improvement of social conditions. This will only be possible through the cooperation of local governments in a participatory search for bilateral relations between public and private interests capable of fostering translational studies that guarantee the access of the population to appropriate treatments. The development of accurate diagnosis capable of identifying these diseases in the acute phase of infection, as well as the identification of parasite polymorphisms and variants of drug resistance, together with application of health care models based on GIS technologies will enable both preventive and therapeutic actions based on new generations of drugs and vaccines currently developed for these neglected diseases.

AUTHOR CONTRIBUTIONS

AM conceived the manuscript. AAF and AM wrote the manuscript. AAF, KG-P, MPN, KZ, LF, LP, DON, LC, and AM participated in the preparation of the manuscript.

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REFERENCES

1. Sibley LD. Invasion and intracellular survival by protozoan parasites. *Immunol Rev* (2011) 240(1):72–91. doi:10.1111/j.1600-065X.2010.00990.x
2. Kling JC, Körner H. Different regulatory mechanisms in protozoan parasitic infections. *Int J Parasitol* (2013) 43(6):417–25. doi:10.1016/j.ijpara.2013.02.001
3. Jackson AP, Otto TD, Aslett M, Armstrong SD, Bringaud F, Schlacht A, et al. Kinetoplastid phylogenomics reveals the evolutionary innovations associated with the origins of parasitism. *Curr Biol* (2016) 26(2):161–72. doi:10.1016/j.cub.2015.11.055
4. Portman N, Gull K. The paraflagellar rod of kinetoplastid parasites: from structure to components and function. *Int J Parasitol* (2010) 40(2):135–48. doi:10.1016/j.ijpara.2009.10.005
5. Hotez PJ, Pecoul B, Rijal S, Boehme C, Aksoy S, Malecela M, et al. Eliminating the neglected tropical diseases: translational science and new technologies. *PLoS Negl Trop Dis* (2016) 10(3):e0003895. doi:10.1371/journal.pntd.0003895

6. Majumder HK, de Souza W, Chang KP. Target identification and intervention strategies against kinetoplastid protozoan parasites. *Mol Biol Int* (2011) 2011:185413. doi:10.4061/2011/185413
7. Hotez PJ. Blue marble health and "the big three diseases": HIV/AIDS, tuberculosis, and malaria. *Microbes Infect* (2015) 17(8):539–41. doi:10.1016/j.micinf.2015.05.004
8. Pedrique B, Strub-Wourgaft N, Some C, Olliaro P, Trouiller P, Ford N, et al. The drug and vaccine landscape for neglected diseases (2000-11): a systematic assessment. *Lancet Glob Health* (2013) 1(6):e371–9. doi:10.1016/S2214-109X(13)70078-0
9. Horstick O, Tozan Y, Wilder-Smith A. Reviewing dengue: still a neglected tropical disease? *PLoS Negl Trop Dis* (2015) 9(4):e0003632. doi:10.1371/journal.pntd.0003632
10. Gilbert IH. Drug discovery for neglected diseases: molecular target-based and phenotypic approaches. *J Med Chem* (2013) 56(20):7719–26. doi:10.1021/jm400362b
11. Morgan GW, Hall BS, Denny PW, Carrington M, Field MC. The kinetoplastida endocytic apparatus. Part I: a dynamic system for nutrition and evasion of host defences. *Trends Parasitol* (2002) 18(11):491–6. doi:10.1016/S1471-4922(02)02391-7
12. Ouaissi A, Ouaissi M. Molecular basis of *Trypanosoma cruzi* and *Leishmania* interaction with their host(s): exploitation of immune and defense mechanisms by the parasite leading to persistence and chronicity, features reminiscent of immune system evasion strategies in cancer diseases. *Arch Immunol Ther Exp (Warsz)* (2005) 53(2):102–14.
13. Fairlamb AH, Gow NA, Matthews KR, Waters AP. Drug resistance in eukaryotic microorganisms. *Nat Microbiol* (2016) 1(7):16092. doi:10.1038/nmicrobiol.2016.92
14. Alsford S, Kelly JM, Baker N, Horn D. Genetic dissection of drug resistance in trypanosomes. *Parasitology* (2013) 140(12):1478–91. doi:10.1017/S003118201300022X
15. Paucar R, Moreno-Viguri E, Pérez-Silanes S. Challenges in Chagas disease drug discovery: a review. *Curr Med Chem* (2016) 23(28):3154–70. doi:10.2174/0929867323999160625124424
16. Chatelain E. Chagas disease drug discovery: toward a new era. *J Biomol Screen* (2015) 20(1):22–35. doi:10.1177/1087057114550585
17. Cullen DR, Mocerino M. A brief review of drug discovery research for human African trypanosomiasis. *Curr Med Chem* (2017) 24(7):701–17. doi:10.2174/0929867324666170120160034
18. Babokhov P, Sanyaolu AO, Oyibo WA, Fagbenro-Beyioku AF, Iriemenam NC. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathog Glob Health* (2013) 107(5):242–52. doi:10.1179/2047773213Y.0000000105
19. Kuepfer I, Schmid C, Allan M, Edielu A, Haary EP, Kakembo A, et al. Safety and efficacy of the 10-day melarsoprol schedule for the treatment of second stage Rhodesiense sleeping sickness. *PLoS Negl Trop Dis* (2012) 6(8):e1695. doi:10.1371/journal.pntd.0001695
20. Schmid C, Kuemmerle A, Blum J, Ghabri S, Kande V, Mutombo W, et al. In-hospital safety in field conditions of nifurtimox eflornithine combination therapy (NECT) for *T. b. gambiense* sleeping sickness. *PLoS Negl Trop Dis* (2012) 6(11):e1920. doi:10.1371/journal.pntd.0001920
21. Mesu VKBK, Kalonji WM, Bardonneau C, Mordt OV, Blesson S, Simon F, et al. Oral fexinidazole for late-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a pivotal multicentre, randomised, non-inferiority trial. *Lancet* (2018) 391(10116):144–54. doi:10.1016/S0140-6736(17)32758-7
22. Jacobs RT, Nare B, Wring SA, Orr MD, Chen D, Sligar JM, et al. SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human African trypanosomiasis. *PLoS Negl Trop Dis* (2011) 5(6):e1151. doi:10.1371/journal.pntd.0001151
23. Mugnier MR, Stebbins CE, Papavasiliou FN. Masters of disguise: antigenic variation and the VSG coat in *Trypanosoma brucei*. *PLoS Pathog* (2016) 12(9):e1005784. doi:10.1371/journal.ppat.1005784
24. Castillo-Acosta VM, Balzarini J, González-Pacanowska D. Surface: a therapeutic opportunity for diseases. *Trends Parasitol* (2017) 33(10):775–87. doi:10.1016/j.pt.2017.06.009
25. Zaioncz S, Khalil NM, Mainardes RM. Exploring the role of nanoparticles in amphotericin B delivery. *Curr Pharm Des* (2017) 23(3):509–21. doi:10.2174/1381612822666161027103640
26. Jha TK. Drug unresponsiveness & combination therapy for kala-azar. *Indian J Med Res* (2006) 123(3):389–98.
27. Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. *Expert Opin Investig Drugs* (2008) 17(5):787–94. doi:10.1517/13543784.17.5.787
28. Andrade HM, Toledo VP, Pinheiro MB, Guimarães TM, Oliveira NC, Castro JA, et al. Evaluation of miltefosine naturally infected with *L. infantum* (=L. chagasi) in Brazil. *Vet Parasitol* (2011) 181(2–4):83–90. doi:10.1016/j.vetpar.2011.05.009
29. Luque-Ortega JR, Rivas L. Miltefosine (hexadecylphosphocholine) inhibits cytochrome C oxidase in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* (2007) 51(4):1327–32. doi:10.1128/AAC.01415-06
30. Rakotomanga M, Blanc S, Gaudin K, Chaminade P, Loiseau PM. Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. *Antimicrob Agents Chemother* (2007) 51(4):1425–30. doi:10.1128/AAC.01123-06
31. Benaim G, García-Marchán Y, Reyes C, Uzcanga G, Figarella K. Identification of a sphingosine-sensitive Ca2+ channel in the plasma membrane of *Leishmania mexicana*. *Biochem Biophys Res Commun* (2013) 430(3):1091–6. doi:10.1016/j.bbrc.2012.12.033
32. Doyle MA, MacRae JI, De Souza DP, Saunders EC, McConville MJ, Likić VA. LeishCyc: a biochemical pathways database for *Leishmania major*. *BMC Syst Biol* (2009) 3:57. doi:10.1186/1752-0509-3-57
33. Biagiotti M, Dominguez S, Yamout N, Zufferey R. Lipidomics and anti-trypanosomatid chemotherapy. *Clin Transl Med* (2017) 6(1):27. doi:10.1186/s40169-017-0160-7
34. Ritzefeld M, Wright MH, Tate EW. New developments in probing and targeting protein acylation in malaria, leishmaniasis and African sleeping sickness. *Parasitology* (2017) 8:1–18.
35. Beaumier CM, Gillespie PM, Hotez PJ, Bottazzi ME. New vaccines for neglected parasitic diseases and dengue. *Transl Res* (2013) 162(3):144–55. doi:10.1016/j.trsl.2013.03.006
36. Duncan SM, Jones NG, Mottram JC. Recent advances in *Leishmania* reverse genetics: manipulating a manipulative parasite. *Mol Biochem Parasitol* (2017) 216:30–8. doi:10.1016/j.molbiopara.2017.06.005
37. Raman VS, Duthie MS, Fox CB, Matlashewski G, Reed SG. Adjuvants for *Leishmania* vaccines: from models to clinical application. *Front Immunol* (2012) 3:144. doi:10.3389/fimmu.2012.00144
38. Alvar J, Croft SL, Kaye P, Khamesipour A, Sundar S, Reed SG. Case study for a vaccine against leishmaniasis. *Vaccine* (2013) 31(Suppl 2):B244–9. doi:10.1016/j.vaccine.2012.11.080
39. Cecílio P, Pérez-Cabezas B, Fernández L, Moreno J, Carrillo E, Requena JM, et al. Pre-clinical antigenicity studies of an innovative multivalent vaccine for human visceral leishmaniasis. *PLoS Negl Trop Dis* (2017) 11(11):e0005951. doi:10.1371/journal.pntd.0005951
40. Dumonteil E, Bottazzi ME, Zhan B, Heffernan MJ, Jones K, Valenzuela JG, et al. Accelerating the development of a therapeutic vaccine for human Chagas disease: rationale and prospects. *Expert Rev Vaccines* (2012) 11(9):1043–55. doi:10.1586/erv.12.85
41. Seid CA, Jones KM, Pollet J, Keegan B, Hudspeth E, Hammond M, et al. Cysteine mutagenesis improves the production without abrogating antigenicity of a recombinant protein vaccine candidate for human Chagas disease. *Hum Vaccin Immunother* (2017) 13(3):621–33. doi:10.1080/21645515.2016.1242540
42. Konduri V, Halpert MM, Liang D, Levitt JM, Cruz-Chan JV, Zhan B, et al. Genetic adjuvantation of a cell-based therapeutic vaccine for amelioration of chagasic cardiomyopathy. *Infect Immun* (2017) 85(9):e127–117. doi:10.1128/IAI.00127-17
43. DebRoy S, Prosper O, Mishoe A, Mubayi A. Challenges in modeling complexity of neglected tropical diseases: a review of dynamics of visceral leishmaniasis in resource limited settings. *Emerg Themes Epidemiol* (2017) 14:10. doi:10.1186/s12982-017-0065-3
44. Bergquist NR. Vector-borne parasitic diseases: new trends in data collection and risk assessment. *Acta Trop* (2001) 79(1):13–20. doi:10.1016/S0001-706X(01)00099-7
45. Palaniyandi M. The role of remote sensing and GIS for spatial prediction of vector-borne diseases transmission: a systematic review. *J Vector Borne Dis* (2012) 49(4):197–204.

46. Ryan JR, Mbui J, Rashid JR, Wasunna MK, Kirigi G, Magiri C, et al. Spatial clustering and epidemiological aspects of visceral leishmaniasis in two endemic villages, Baringo District, Kenya. *Am J Trop Med Hyg* (2006) 74(2): 308–17.
47. Khanal B, Picado A, Bhattarai NR, Van Der Auwera G, Das ML, Ostyn B, et al. Spatial analysis of *Leishmania donovani* exposure in humans and domestic animals in a recent kala azar focus in Nepal. *Parasitology* (2010) 137(11):1597–603. doi:10.1017/S0031182010000521
48. Tsokana CN, Sokos C, Giannakopoulos A, Mamuris Z, Birtsas P, Papaspyropoulos K, et al. First evidence of *Leishmania* infection in European brown hare (*Lepus europaeus*) in Greece: GIS analysis and phylogenetic position within the *Leishmania* spp. *Parasitol Res* (2016) 115(1):313–21. doi:10.1007/s00436-015-4749-8
49. Sevá AD, Mao L, Galvis-Ovallos F, Tucker Lima JM, Valle D. Risk analysis and prediction of visceral leishmaniasis dispersion in São Paulo State, Brazil. *PLoS Negl Trop Dis* (2017) 11(2):e0005353. doi:10.1371/journal.pntd.0005353

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