ROLE OF METABOLISM IN REGULATING IMMUNE CELL FATE DECISIONS

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ROLE OF METABOLISM IN REGULATING IMMUNE CELL FATE DECISIONS

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EDITORIAL
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Editorial: Role of Metabolism in Regulating Immune Cell Fate Decisions

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Editorial on the Research Topic

Role of Metabolism in Regulating Immune Cell Fate Decisions

Immunometabolism, an interplay between immunological and metabolic processes, describes not only the stepwise adaptation of intracellular metabolic pathways to sustain the bioenergetic demand of an immune response, but also how these metabolic adaptations directly affect immune cell functions and cell fate by controlling transcriptional, post-transcriptional and epigenetic events. The first concepts of immunometabolism date back to the 1930s, when Kempner and Peschel formulated their metabolic concepts of the physiology of inflammation using experiments they performed in a cantharidin-induced skin blister model [as described in Nagy and Haschemi (1)]. Immunometabolism was rediscovered in the twenty-first century, when on one hand, it had emerged that certain chronic, supposedly non-immune, pathologies including obesity contribute to mobilization of the immune system that drives metabolic abnormalities, leading to increased susceptibility to type 2 diabetes, cardiovascular and liver diseases, neurodegeneration and cancer. On the other hand, it was proposed that well-known cellular nutrient sensors, serine/theonine kinases AKT, APMK, LKB1, mTOR, and the transcriptional factor aryl hydrocarbon receptor (AhR) control a fate switch of T cells (2–5). The field has seen a tremendous development since then. This Research Topic contains 16 (Mini)Review, Opinion, and Original Research articles that review and expand our current understanding of the molecular underpinnings of immunological/metabolic cross-talk and metabolism-guided fate decisions of different immune cells during an immune response.

In one of the first articles of the Research Topic, Viola et al. provide a comprehensive overview of the main metabolic pathways in macrophages, and how these pathways are rewired to support the particular functions of the pro-inflammatory (M1) vs. anti-inflammatory (M2) macrophages. Special attention is given to the Krebs cycle metabolites citrate, itaconate and succinate, due to their non-metabolic roles in specific events during macrophage activation, and to disease-associated macrophage metabolic abnormalities. Wilson et al. continue to review the role of macrophage metabolic reprogramming and immune functions in the context of granulomatous diseases. Three examples of granulomatous disease are presented (tuberculosis, schistosomiasis, and sarcoidosis), with important similarities and differences critically discussed, highlighting dysregulated lipid metabolism as a common denominator in granulomatous disease progression. In their Mini Review, Sharif et al. focus on the role of the class I phosphoinositide-3-kinase (PI3K) signaling in sensing nutrients increased in obesity and subsequent rewiring of the metabolism and responses of adipose tissue macrophages, linking metabolically triggered

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inflammation (meta-inflammation) to insulin resistance and diabetes. Meta-inflammation is also studied in the Original Research article by Min et al. who demonstrate that pyruvate dehydrogenase kinase (PDK), which inhibits the pyruvate dehydrogenase-mediated conversion of cytosolic pyruvate to mitochondrial acetyl-CoA, is a metabolic checkpoint for polarization of macrophages to the M1 phenotype. Combined PDK2 and PDK4 deficiency (both global and hematopoietic cell-specific), or alternatively, pharmacological inhibition using a novel PDK inhibitor KPLH1130 prevents M1 macrophage polarization, reduces obesity-associated insulin resistance, and ameliorates adipose tissue inflammation, introducing a viable strategy for the treatment of inflammatory metabolic disorders. In the second Original Research article of this collection, Chapman et al. link proinflammatory stimulation of myeloid cells with ligands of the pattern recognition receptors Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) to metabolic rewiring needed for effector functions by identification of the deubiquitinating enzyme ataxin-3 downstream from TLR2 and NOD2, and demonstrate that ataxin-3 is necessary for optimal mitochondrial respiration and reactive oxygen species production, as well as for intracellular bacterial killing.

Given the particular importance of metabolic remodeling in regulation of T cell development, activation, function, differentiation, and survival, several Review articles within this collection tackle the metabolic control of T cell fate, each from a different angle. Konjar and Veldhoen thoroughly discuss recent insights in metabolic characteristics and phenotypes of CD8 T cell subsets, by side-by-side comparison of naive, circulating memory, effector and tissue resident CD8 T cells. Emphasis is given on tissue resident memory CD8 T cells at the epithelial barriers that show unique metabolic rewiring adapted to their niche in order to fulfill their roles—tissue homeostasis and immediate protection against microbial invasion. The review by Pacella and Piconese addresses the roles and regulation of cellular bioenergetic metabolic pathways in regulatory T cells (Treg) compared to conventional CD4 T cells. By critical analysis of metabolic and functional differences of the two cell types in metabolite-rich (liver, adipose tissue) vs. nutrient-restricted (tumor microenvironment) tissues, they highlight the higher capability of Tregs to adapt to metabolic hurdles, that could be explored for therapeutic purposes. Stark et al. review the metabolic requirements of Th2 cells during their early and late differentiation, focusing on the impact of glucose and lipid metabolism, mTOR activation, the nuclear receptor PPARγ and several extracellular metabolites that directly promote Th2 functions, as well as on metabolic interventions targeting type 2 inflammation. Colamatteo et al. provide a comprehensive review of the role of microRNAs (miRNAs) in regulating T cell metabolism and how the dysregulation of this control can lead to autoimmunity. They also speculate on the possibility that the interplay between miRNAs and metabolism in T cells may help identifying novel miRNA-based therapeutic strategies to treat effector T cell immunometabolic alterations in autoimmune and chronic inflammatory diseases. In their Opinion article, Mondanelli et al. discuss the immunoregulatory interplay between arginine and tryptophan metabolism, connecting deregulated expression of the catabolising enzymes for these amino acids [arginase 1 (ARG1) or indoleamine 2,3-dioxygenase 1 (IDO1), respectively] in neoplasia and autoimmune diseases to functional reprogramming of immune cells, dendritic cells, and T cells in particular. At the cellular level, Audrito et al. give a comprehensive overview on the crosstalk between tumor cells, stromal cells, and infiltrating immune cells (tumor infiltrating lymphocytes, tumor-associated macrophages and neutrophils, myeloid-derived suppressor cells) in tumor microenvironment with specific focus on nicotinamide adenine dinucleotide (NAD) metabolism. The role of the entire "NADome" (NAD metabolites, NAD-biosynthetic and -consuming enzymes) in cancer growth and immune evasion and currently pursuit therapeutic strategies for NADome blockade are critically reviewed.

In a Mini Review, Yerinde et al. discuss a crosstalk between the metabolic and the epigenetic regulation of CD8 T cell differentiation and function and also briefly summarize how metabolic signals from the tumor microenvironment (or virus-infected cells) shape the epigenetic landscape of CD8 T cells. Similarly, Magalhaes et al. draw parallels between mechanisms employed by tumor cells and viruses to influence T cell metabolic regulation and propose an emerging view that metabolic changes in tumors and virally infected cells uniformly create a suppressive microenvironment leading to inhibition of effector CD4 and CD8 T cells. Therefore, these reviews provide an important insight into mechanisms that underlie T cell exhaustion in anti-tumor and anti-viral immunity, which could inspire development of effective therapeutic interventions against it. This view is well-complemented by the Review article of Mayer et al. detailing crucial cellular metabolic pathways that are being utilized by several DNA and RNA viruses for their replication and survival. The dichotomy between the strategy for host cell manipulation—DNA viruses preferentially employ the transcriptional control of key metabolic pathways, while RNA viruses rely on post-transcriptional modifications—is noted and in this view, currently pursuit strategies for metabolism-targeting interventions against different viruses are clearly summarized.

Innate lymphoid cells (ILCs), are relatively recently discovered lymphocytes lacking diversified adaptive antigen receptors. These largely tissue-resident cells play an important role in tissue homeostasis, host defense at mucosal barriers and tissue repair (6). There is increasing evidence linking ILCs with metabolic homeostasis, and immunometabolic regulation of ILCs is an emerging frontier. At a cellular level, Poznanski and Ashkar critically review recent literature to answer the question whether metabolism—or phenotype—can define the functional fate of human natural killer (NK) cells, and provide evidence that indeed differences in metabolism (especially in glucose metabolism) better discriminate between cytotoxic, regulatory and memory NK cells than surface markers. At the whole organ level, Willinger takes a systemic approach, and in his Review thoroughly discusses metabolic signals that regulate ILC homing to the particular tissues and their strategic positioning in healthy and inflamed tissues. Trafficking of both ILC precursors and mature ILCs (NK cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer, LTi cells), including

species-specific differences between humans and mice, are comprehensively covered.

In conclusion, this collection of Review and Original Research articles critically summarizes current understanding of intertwining of metabolic and signaling pathways as ways to determine immune cell fates and ultimately, the ensuing immune response. We anticipate that the articles in the present collection will serve as an inspiration for future research, that will lead to deeper knowledge of cell-intrinsic and -extrinsic metabolic cues regulating cell fate decisions in different immune cell types, and subsequently to a development of novel medicines for immune-mediated diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Pyruvate Dehydrogenase Kinase Is a Metabolic Checkpoint for Polarization of Macrophages to the M1 Phenotype

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Metabolic reprogramming during macrophage polarization supports the effector functions of these cells in health and disease. Here, we demonstrate that pyruvate dehydrogenase kinase (PDK), which inhibits the pyruvate dehydrogenase-mediated conversion of cytosolic pyruvate to mitochondrial acetyl-CoA, functions as a metabolic checkpoint in M1 macrophages. Polarization was not prevented by PDK2 or PDK4 deletion but was fully prevented by the combined deletion of PDK2 and PDK4; this lack of polarization was correlated with improved mitochondrial respiration and rewiring of metabolic breaks that are characterized by increased glycolytic intermediates and reduced metabolites in the TCA cycle. Genetic deletion or pharmacological inhibition of PDK2/4 prevents polarization of macrophages to the M1 phenotype in response to inflammatory stimuli (lipopolysaccharide plus IFN-γ). Transplantation of PDK2/4-deficient bone marrow into irradiated wild-type mice to produce mice with PDK2/4-deficient myeloid cells prevented M1 polarization, reduced obesity-associated insulin resistance, and ameliorated adipose tissue inflammation. A novel, pharmacological PDK inhibitor, KPLH1130, improved high-fat diet-induced insulin resistance; this was correlated with a reduction in the levels of pro-inflammatory markers and improved mitochondrial function. These studies identify PDK2/4 as a metabolic checkpoint for M1 phenotype polarization of macrophages, which could potentially be exploited as a novel therapeutic target for obesity-associated metabolic disorders and other inflammatory conditions.

Keywords: dichloroacetate, high-fat diet, inflammation, insulin resistance, macrophage polarization, metabolic reprogramming, pyruvate dehydrogenase kinase

INTRODUCTION

Macrophage polarization (M1/M2) requires metabolic reprogramming that enhances glycolysis and repurposes mitochondrial function (1–3). Although the importance of these metabolic pathway differences between M1 and M2 macrophages is well-established, our knowledge of the checkpoints in affected metabolic pathways is limited primarily to HIF-1α and pyruvate kinase M2 (PKM2) (4). Obesity-induced insulin resistance is a disease process in which M1 macrophages contribute to adipose tissue (AT) inflammation and insulin resistance. Chronic low-grade inflammation in multiple organs increases the risk of developing obesity, diabetes, cardiovascular diseases, and cancers, indicating a major role for the immune system in the etiology of metabolic disorders (5). Recruitment of M1 macrophages, IFN-γ-secreting Th1 cells, $CD8^+$ T cells, and B cells in the adipose tissue drives the inflammatory response, locally promoting systemic inflammation and impaired insulin action as a result of over-nutrition (6, 7). The phenotypic changes in macrophages that occur in response to over consumption of energy are considered potential therapeutic targets for managing chronic metabolic diseases.

Pyruvate dehydrogenase kinase (PDK) provides a therapeutic target for the Warburg effect in malignant cancers (8) and has been suggested to serve this function during macrophage polarization (9). PDK1 participates in M1 macrophage polarization via HIF-1α-mediated aerobic glycolysis, accounting for the proinflammatory responses (9). In contrast, among the four PDK isozymes, PDK2 and PDK4 are the most strongly associated with metabolic diseases, especially type 2-diabetes (10). Recent work from our laboratory has indicated that dual deficiency of *Pdk2* and *Pdk4* (PDK2/4 DKO) attenuates the lactic acid surge, the proinflammatory markers, and the pain hypersensitivity suggesting a key role for the PDK-PDH-lactic acid axis in the pathogenesis of inflammatory pain mediated by macrophage functional regulation (11). This finding suggests a novel therapeutic approach for many inflammatory conditions but is seemingly at odds with conclusions of others who have addressed the role of the PDKs in macrophage polarization (12–14); we have, therefore, examined this phenomenon in greater depth in the present study.

Here we provide additional evidence for PDK4 induction in macrophages in response to LPS and IFN-γ. We also show that genetic and pharmacological blockage of PDK activity in mice fed a high-fat diet (HFD) represses macrophage M1 polarization, which is correlated with amelioration of adipose tissue inflammation as well as insulin resistance. These findings support the hypothesis that the PDKs are therapeutic targets for inflammatory diseases.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Kyungpook National University and were conducted according to recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old male WT (C57BL/6J) and PDK2/4 DKO mice (15) were either fed a HFD 20% of the calories derived from carbohydrates and 60% from fat (Research Diets; D12492 pellets) for use as a diet-induced obesity (DIO) model or fed an isocaloric control diet (CD) in which 70% of the calories were derived from carbohydrates and 10% from fat (Research Diets; D12450B pellets). The mice were housed and maintained on a 12 h light/dark cycle at 22 ± 2°C. After the mice were sacrificed, the tissues were rapidly collected and freeze-clamped with liquid nitrogen-cooled Wollenberger tongs and stored at −80°C prior to analysis.

Isolation of Peritoneal Macrophages (PMs)

Eight- to ten-week-old WT, PDK2 KO (2KO), PDK4 KO (4KO), and PDK2/4 DKO mice (15–17) were injected with 3% thioglycollate broth, i.p., and then sacrificed 4 days later. Peritoneal lavage was performed twice using 4 mL of 1X PBS, and the harvested cells were then cultured in RPMI1640 (Gibco; 11875-093) supplemented with antibiotics. After 1 h of culture, the suspended cells were discarded, and the adherent cells were used for experiments.

Isolation and Differentiation of Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow cells were collected from the femurs and tibias of 8- to 10-week-old mice. The cells were cultured at 2×10^7 cells/plate in α-MEM medium (WELGENE; LM 008-02) containing 30% L929-conditioned media and 10% FBS for 9 days to allow the differentiation. The established BMDMs were then used for experiments.

Isolation of Peritoneal Macrophages (PMs) by Zymosan a or LPS Treatment

Zymosan-elicited peritoneal macrophages (ZEPMs) and LPS-elicited peritoneal macrophages (LEPMs) were isolated as previously reported (18). Briefly, zymosan A (1 mg/mouse) or LPS (1 mg/kg) were i.p. injected into 8-week-old C57BL/6J mice. One day after the injection, peritoneal fluid was harvested and cells were cultured in RPMI1640 supplemented with antibiotics. After 1 h of culture, the suspended cells were discarded and the adherent cells were used for experiments.

Western Blot Analysis

The tissue cells were lysed using a lysis buffer [20 mM Tris (pH 7.4), 10 mM Na_4P_2OH, 100 mM NaF, 2 mM Na_3VO_4, 5 mM EDTA (pH 8.0), 0.1 mM PMSF, and 1% NP-40] containing protease inhibitors (aprotinin 7 μg/mL and leupeptin 7 μg/mL) and phosphatase inhibitor cocktail. Protein concentrations were measured using BCA protein assay reagent (Thermo Fisher Scientific; 23225). Cell lysates were separated on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Merck Millipore; IPVH00010). The transferred proteins on the membrane were immunoblotted with the following primary antibodies: anti-HIF-1α (1:1,000), anti-iNOS (1:1,000), anti-Arg-1 (1:1,000), anti-PDK2 (1:1,000), anti-PDK4 (1:500), and anti-β-actin (1:5,000). All antibodies were diluted in TBST containing 5% BSA.

Measurement of Oxygen Consumption Rate (OCR)

The OCR was measured using a Seahorse XF-24 Flux Analyzer (Seahorse Biosciences, Billerica, MA, USA). BMDMs were seeded in XF-24 tissue culture plates (24-well) at a density of 1×10^5 cells/well and incubated overnight. The cells were treated with M1 stimulants (LPS 100 ng/mL + IFN-γ 10 ng/mL) with or without dichloroacetate (DCA 10 mM, Sigma; 47795) for 3 h. The assay medium used consisted of XF base medium (Seahorse Biosciences) supplemented with 5.5 mM D-glucose (Sigma-Aldrich; G7528), 1 mM sodium pyruvate (Sigma-Aldrich; S8636), and 1X GlutaMAX™ (Gibco; 35050) and adjusted to pH 7.4. The inhibitors and uncouplers used in this study were as follows: oligomycin A (2 μM, Sigma-Aldrich; 75351), CCCP (carbonyl cyanide 3-chlorophenylhydrazone, 7.5 μM, Sigma-Aldrich; C2759), rotenone (1 μM, Sigma-Aldrich; R8875), and antimycin A (2.5 μM, Sigma-Aldrich; A8674). OCR was normalized to protein concentration.

Metabolite Extraction

Isolated cells were cultured overnight in 10% dialyzed FBS media. The medium was replaced with fresh 10% dialyzed FBS medium and cells were then stimulated with LPS (100 ng/mL) and IFN-γ (10 ng/mL) for 12 h. Cells were washed with 3 mL ice-cold 0.9% NaCl twice and then collected in Eppendorf tubes. Cells were resuspended in 200 μL of ice-cold metabolite extraction solution (chloroform:methanol:water 1:3:1, v/v) and then sonicated. After incubation on ice for 1 h, the metabolite samples were collected by centrifugation at 13,000 rpm for 5 min. All the samples were lyophilized and re-suspended in 300 μL of water containing 0.1% formic acid, prior to LC-MS/MS analysis.

Data Presentation and Statistical Analysis

Data were presented using the GraphPad Prism software and statistical analysis was performed using IBM SPSS Statistics (version 21). Statistically significant differences were measured by Student's *t*-test for normally distributed data. Statistical analysis of group comparison was performed by one-way or two-way ANOVA followed by Tukey's HSD (honestly significant difference) *post hoc* test. *p*-values < 0.05 were considered statistically significant. Detailed procedures are included as part of **Supplemental Materials**.

Detailed Procedures

See the **Supplemental Materials**.

RESULTS

PDK2 and PDK4 Are Required for M1 Macrophage Polarization

We explored the role of PDKs in overnutrition-induced AT inflammation. Among the 4 different isoforms of PDK, the mRNA expression of only *Pdk4* was significantly upregulated in the AT of mice fed an HFD compared to those fed a CD (**Figure 1A**). Furthermore, *Pdk4* was more responsive to specific M1 stimulation, which was correlated with the upregulation of *Pdk4*, and M2 stimulation, correlated with *pdk4* downregulation in macrophages (**Figures 1A,B** and **Figures S1A–D**). The induction of HIF-1α and iNOS by M1-only stimulation and M1+M2 stimulation, respectively, to mimic *in vivo* wild-type (WT) conditions, was completely suppressed in PDK2/4 DKO mice but only slightly suppressed in PDK2 or PDK4 KO-peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMDMs) (**Figure 1C**), suggesting that PDK2 and PDK4 can functionally compensate for each other. And we confirmed no change of PDK1 expression in DKO mice (**Figure 1D** and **Figures S1E,F**). Conversely, M2 stimulation with IL-4 caused a greater increase in arginase-1 expression in a time-dependent manner in PMs from PDK2/4 DKO mice compared to those from WT mice (**Figure S1G**). Since HIF-1α is essential for the upregulation of glycolytic genes and, therefore, the activation of inflammatory macrophages, suppression of the increase in HIF-1α by PDK2/4 deficiency in response to LPS + IFN-γ stimulation is especially noteworthy.

RNA-seq analysis revealed increased aerobic glycolysis in response to M1 stimulation; this was indicated by increased expression of glycolysis-related genes and decreased expression of TCA cycle-related genes. This response was practically absent in LPS + IFN-γ-stimulated PMs from PDK2/4 DKO mice (**Figures 1E–G**). Consistently, the decrease in isocitrate dehydrogenase (*Idh*)1 and *Idh*2 mRNA expression levels that normally occur in response to LPS + IFN-γ stimulation was prevented (**Figure 1F**). The expression of *Irg1* required for the production of itaconic acid, an important factor in the antimicrobial response of macrophages was less affected, yet significantly suppressed in the PMs from PDK2/4 DKO mice (**Figure 1F**). To evaluate the mitochondrial function in response to reduced PDK activity, we measured the oxygen consumption rate (OCR) in both PDK2/4-deficient BMDMs and dichloroacetate (DCA; pan PDK inhibitor)-treated BMDMs. M1 polarization significantly reduced basal and maximal OCR in WT-BMDMs; however, the OCR in LPS + IFN-γ treated PDK2/4 DKO-BMDMs was still comparable to that in WT-BMDMs (M0) (**Figure 1H**). Likewise, DCA treatment was found to largely prevent the reduction in basal and maximal OCR in LPS + IFN-γ-treated WT-BMDMs, which are known, otherwise, to have a broken TCA cycle (evidenced by the accumulation of succinate) during M1 polarization (**Figure 1I**) (19). Elevated extracellular lactate and intracellular succinate levels were also significantly attenuated in PDK2/4 DKO-BMDMs compared to that in WT-BMDMs (**Figures S2A,B**). Overall, our data are indicative that genetic as well as pharmacological inhibition of PDK2/4 can block the metabolic reprogramming of aerobic glycolysis and mitochondrial respiration under conditions that normally induce M1 macrophage polarization.

PDK2/4 Deficiency Prevents the Increase in Glycolytic Intermediates and the Decrease in TCA Cycle Intermediates Normally Induced by Treating Macrophages With Inflammatory Stimuli

Based on our finding that mitochondrial respiration is enhanced when M1 polarization is prevented by PDK2/4-deficiency,

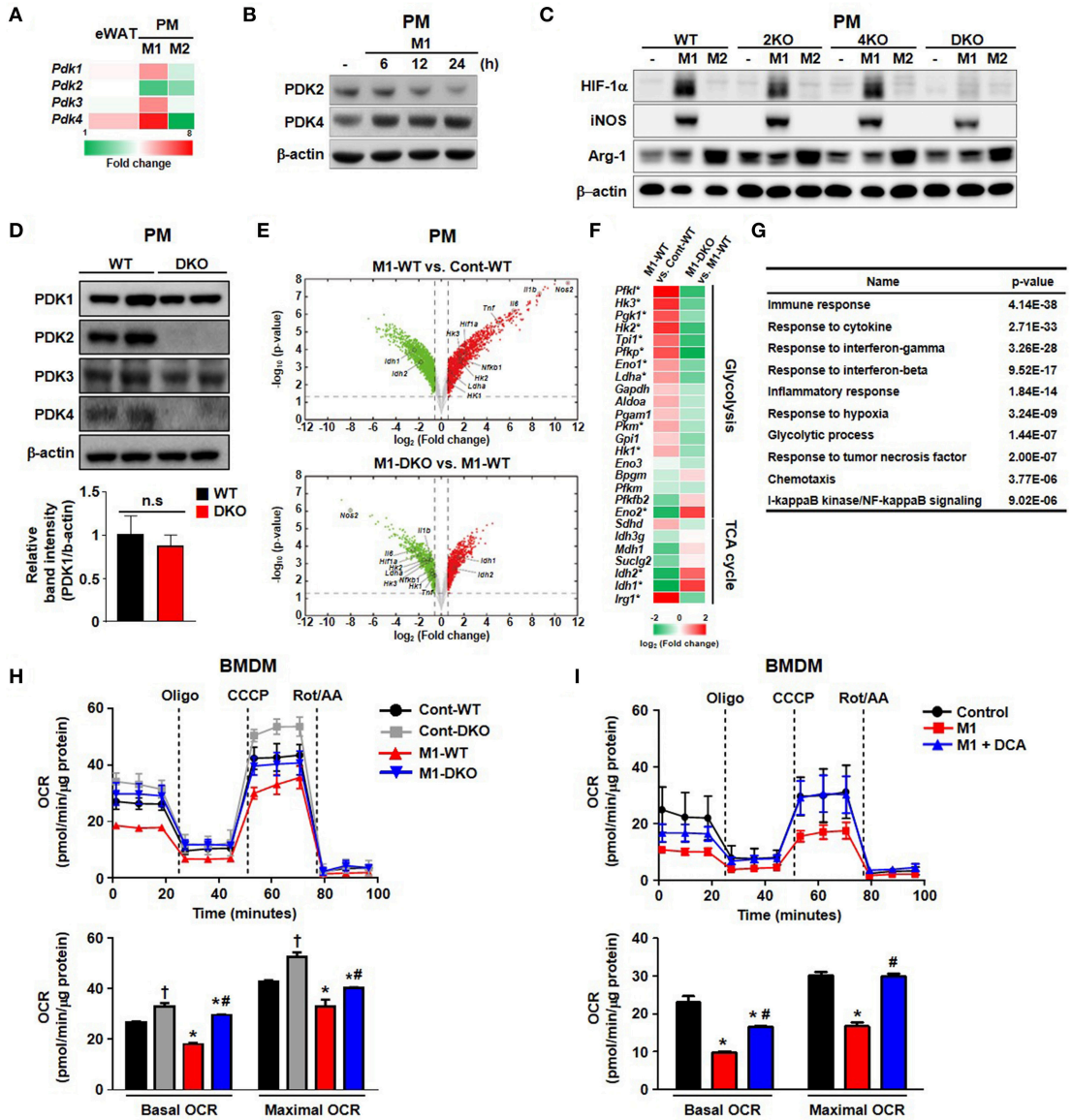

FIGURE 1 | PDK2 and PDK4 are required for M1 macrophage polarization. **(A)** Heat map for relative mRNA levels of PDK isoforms measured by qRT-PCR. eWAT refers to epididymal AT from WT mice fed an HFD in comparison to epididymal AT from WT mice fed a CD for 24 weeks. M1 refers to PM stimulated for 24 h with LPS 100 ng/mL+ IFN-γ 10 ng/mL relative to unstimulated PM. M2 refers to PM stimulated for 24 h with IL-4 10 ng/mL+ IL-10 10 ng/mL relative to unstimulated PMs. $n = 5$–7 per group for AT and $n = 3$ per group for PMs. Values are expressed as mean ± SD for PMs and ± SEM for AT. Statistical analysis was performed by Student's *t*-test. $^{*}p < 0.05$ vs. control. **(B)** PDK expression levels were assessed in PMs after M1 stimulation with LPS 100 ng/mL+ IFN-γ 10 ng/mL for the indicated times. **(C)** M1 and M2 markers in PMs prepared from WT, PDK2 KO, PDK4 KO, and PDK2/4 DKO mice followed by M1 stimulation with LPS 100 ng/mL+ IFN-γ 10 ng/mL or M2 stimulation with IL-4 10 ng/mL+ IL-10 10 ng/mL for 12 h. **(D)** The expressions of PDK isozymes in PMs from WT and DKO mice. **(E)** Volcano plots for the comparisons of M1-WT vs. Cont-WT (upper) and M1-DKO vs. M1-WT (lower). Dotted lines represent the cutoffs for fold change and *p*-value. Red and green dots denote up- and down-regulated genes, respectively. **(F)** Heat map of genes involved in glycolysis and TCA cycle. The color bar represents the gradient of log2 (fold change) of mRNA expression levels in each comparison. The genes that were up- (down-) regulated in M1-WT compared to Cont-WT and down- (up-) regulated in M1-DKO compared to M1-WT at the same time were indicated by an asterisk. **(G)** GOBPs represented by the genes that were up-regulated in M1-WT compared to Cont-WT and down-regulated in M1-DKO compared to M1-WT at the same time. The p-value is the significance of GOBPs being enriched by the genes. **(H,I)** OCRs were measured in WT- or DKO-BMDM in the presence or absence of M1 stimulation by LPS (100 ng/mL) + IFN- γ (10 ng/mL) treatment for 3 h **(H)** and DCA (10 mM) treatment **(I)**; $n = 4$–5 per group. Values are expressed as mean ± SD. Statistical analysis was performed by Student's *t*-test. $^{*}p < 0.05$ vs. (–) M1 stimulation, $^{\dagger}p < 0.05$ vs. control-WT, and $\#p < 0.05$ vs. M1-WT.

we hypothesized that acetyl-CoA (generated by conversion of pyruvate to acetyl-CoA by PDH) might be utilized efficiently by the intermediates of TCA cycle, resulting in increased OXPHOS with less lactate production. To test our hypothesis, we performed $^{13}C_6$-glucose trace analysis (a schematic representation of metabolite labeling with $^{13}C_6$-glucose is given in **Figure 2A**) and assessed the relative mass distribution vector represented by the metabolites derived from $^{13}C_6$-glucose

directly, as well as the total amount of metabolites from glucose metabolism during PDK2/4 deficiency (**Figures 2B,C**) (20). As assessed by two different methods and consistent with the altered expression of glycolytic enzymes (**Figure 1F**), the steady state amounts of metabolites between glucose to lactate were significantly increased in M1-polarized WT-PMs, while PDK2/4 deficiency was found to prevent increases in these intermediates (**Figure 2B**, **Figure S2C**). Although the mass of citrate was not significantly affected, formation of M+2 citrate (blue in the pie chart) from M+6 glucose was reduced in M1-polarized WT-PM but increased by PDK2/4 deficiency, consistent with decreased flux in the former and increased flux in the latter (**Figure 2C**). Although total amounts of succinate and malate were significantly increased, M+2 succinate and M+2 malate levels (blue in the pie chart) were significantly reduced in M1-polarized WT-PM. Interestingly, metabolic flux analysis of these intermediates displayed enhanced enrichments in PDK2/4-deficient conditions (**Figure 2C**; **Figures S2D,E**). These data suggest that increased provision of acetyl-CoA for the maintenance of the TCA cycle by PDK2/4 deficiency prevents the decrease in cellular respiration characteristic of M1-polarized WT macrophages.

Global PDK 2/4 Deficiency in HFD-Fed Mice Reduces Insulin Resistance and AT Inflammation

Greater PDH activity caused by global PDK 2/4 deficiency improves insulin sensitivity (15), suggesting PDKs are involved in the development of whole-body insulin resistance. To determine whether DKO mice show reduced HFD-induced AT inflammation which may contribute to the healthier metabolic phenotype of these mice, WT and DKO mice were fed CD or HFD. As reported previously (15), HFD-fed-PDK2/4 ablated mice displayed lower body weight gain, lower fasting blood glucose levels, improved glucose tolerance, and increased insulin sensitivity along with reduced fat accumulation in the AT and liver (**Figures 3A–E**). Furthermore, the number of infiltrated epididymal AT macrophages was reduced, as indicated by reduction in crown-like structures and the levels of proinflammatory markers Emr1, Cd68, Itgax, and Tnf (**Figures 3F–H**). These data show that global PDK2/4 deficiency attenuates HFD-induced macrophage infiltration and thereby reduces AT inflammation, suggesting that inhibition of PDH activity by the PDKs is involved in the AT inflammation caused by obesity.

Bone Marrow-Specific PDK2/4 Deletion Interferes With HFD-Induced AT Macrophage Infiltration

To investigate the potential effects of innate immune cells on chronic inflammation, a bone-marrow (BM) transplantation mouse model was used to evaluate the direct contribution of BM-derived immune cells from PDK2/4 DKO mice. The method involved irradiation of recipient WT mice to ablate BM cells followed by transplantation via intravenous infusion of donor BM obtained from WT mice or PDK2/4 DKO mice (21); donor as well as recipient mice were maintained on a CD for this study. Four weeks after maintaining all of the mice on a CD, the mice were divided into four groups: 1) WT mice with WT BM that were continued on the CD; 2) WT mice with WT BM that were placed on the HFD; 3) WT mice with DKO BM that were continued on the CD; and 4) WT mice with DKO BM that were placed on the HFD. No differences in any subsequent measurements were observed between the two groups of mice (1 and 3) that were continued on the CD (**Figure 4**). Likewise, no differences in body weight, body size, and food consumption were observed between the two groups of mice (2 and 4) that were maintained on the HFD (**Figures 4A–C**). However, remarkable differences in markers of metabolic dysfunction were observed between the latter two groups of mice. The HFD-fed mice transplanted with WT-BM displayed an increase in fasting blood glucose, AT, and liver fat, while the glucose tolerance and insulin sensitivity was found to be reduced. All of these expected negative consequences of HFD feeding were attenuated in mice transplanted with DKO-BM (**Figures 4D–H**). These findings along with reduced inflammatory responses and no additional anti-inflammatory cytokine production such as IL-10 and TGF-β by PDK2/4 deficiency under sterile inflammatory condition (**Figure 5** and **Figure S3**) are consistent with other reports that have demonstrated improved insulin sensitivity in response to global PDK 2/4 deficiency and, consequently, greater PDH activity (22).

Furthermore, the number of crown-like structures was dramatically increased in the AT of mice with WT BM but not in the mice with DKO-BM (**Figures 6A,B**). To confirm the reduced migrating capacity of PDK2/4 ablated monocyte to fat tissue *in vivo*, we assessed that the PKH26-positive stained macrophage ($F4/80^{+}CD11b^{+}PKH26^{+}$) population derived from DKO monocyte was significantly reduced in the stromal vascular fractions of 4 week HFD mice compared to WT monocyte (**Figure 6C**). Pharmacological inhibition of PDK activity with DCA was found to significantly attenuate MCP-1-induced migration (**Figure 6D**). To mimic the *in vivo* physiological effect of HFD, BMDMs were incubated with palmitate-treated, 3T3-L1-conditioned medium. Interestingly, DCA treatment was found to significantly reduce the migration induced by an unknown chemoattractant in palmitate-treated 3T3-L1 cells (**Figure 6E**). We also confirmed that the increase in M1/M2 ratio as well as levels of proinflammatory markers was significantly blocked in the epididymal AT from WT mice with DKO-BM (**Figures 6F,G** and **Figure S4**). These results are indicative that PDK2/4 deletion and PDK inhibition prevent macrophage polarization and infiltration, thereby preventing inflammation of adipose tissue in response to over consumption of dietary energy.

A Novel PDK Inhibitor Prevents Polarization of Macrophages to the M1 Phenotype and Attenuates Adipose Tissue Inflammation in Obesity

We used a series of previously established, highly specific PDK inhibitors that target structurally conserved ATP-binding pockets in the PDKs (23, 24) to perform an efficacy

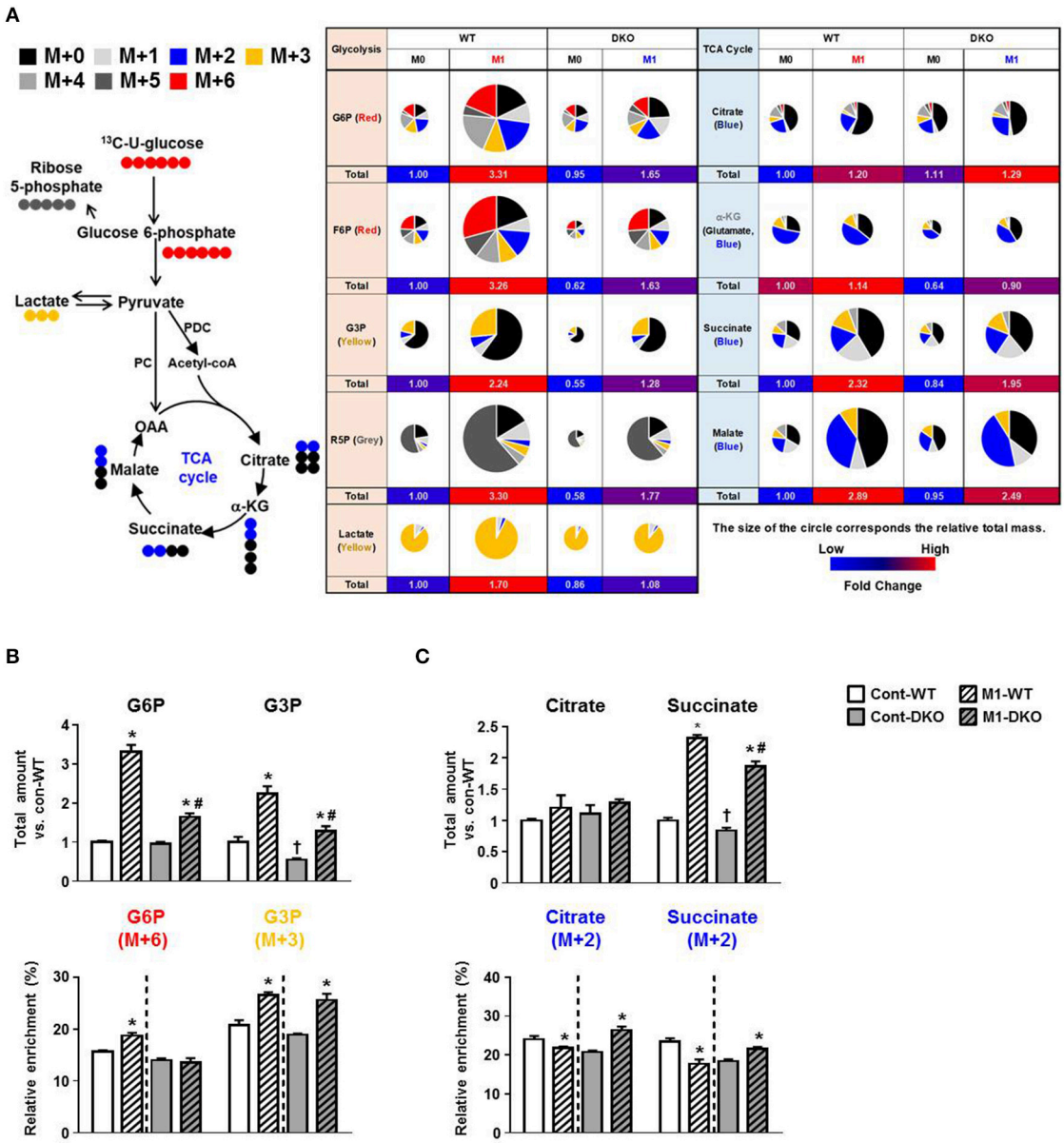

FIGURE 2 | PDK2/4 deletion increases glucose oxidation by macrophages. **(A)** Schematic representation of metabolite labeling by $^{13}C_6$-glucose. The relative amounts of metabolites are represented by the pie charts and the different colors indicate labeling with different mass isotopomers. **(B,C)** The relative amounts and ^{13}C enrichment patterns of the intermediates of the glycolytic **(B)** and TCA cycle **(C)** in WT- and DKO-PMs compared with control-WT; $n = 4$–5 per group. Values are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by Student's *t* test. $^*p < 0.05$ vs. (-) M1 stimulation, $^{\dagger}p < 0.05$ vs. control-WT, and # $p < 0.05$ vs. M1-WT.

test against the inflammatory response (**Figures S5A–C**). In contrast to the high DCA concentrations (0.5∼2 mM) required for the inhibition of PDKs, a novel PDK inhibitor, KPLH1130, was found to significantly inhibit expression of proinflammatory cytokines including TNFα, IL-6, and IL-1β in various types of macrophages at much lower (5–10 μM) concentrations (**Figures 7A–D, 8A–E**; **Figures S5D–F**). In addition to reduced migration capacity by KPLH1130 treatment, iNOS, nitric oxide, and HIF-1α levels were significantly reduced by pharmacological PDK inhibition in various types of macrophages (**Figures 7E–G, 8F–H**; **Figures S5G–M**). KPLH1130 also prevented the decrease in basal and maximal OCR caused by M1 polarizing conditions in BMDMs (**Figure 7H**). We also found that KPLH1130 administration improved the glucose tolerance of HFD-fed mice (**Figure 7I**). Similarly, these effects were not mediated by induced anti-inflammtory cytokines (**Figures 8I–K**). Taken together, our results are indicative that KPLH1130 can effectively

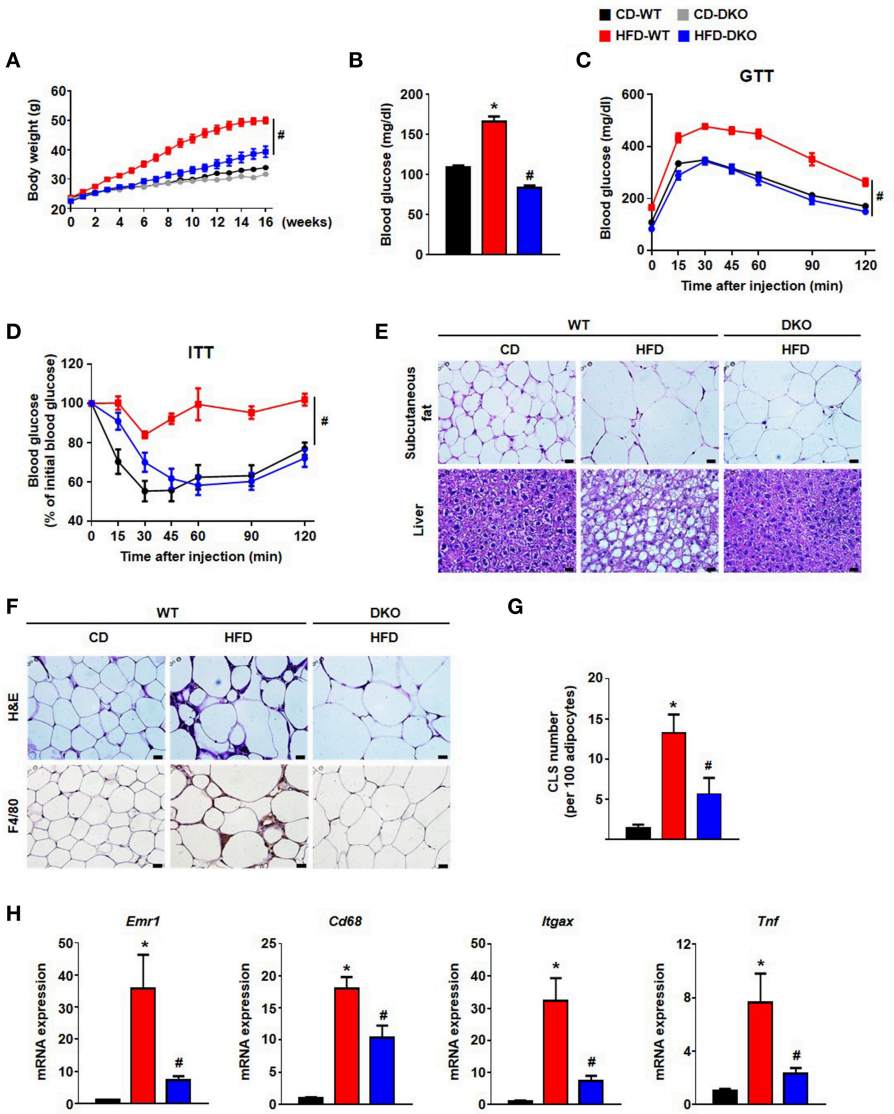

FIGURE 3 | PDK2/4 deficiency ameliorates adipose tissue inflammation and insulin resistance in mice fed a high fat diet. **(A–D)** Measurements of body weights **(A)**, fasting blood glucose levels **(B)**, glucose tolerance test (1.5 g/kg, i.p.) after 16 h fasting **(C)** and insulin tolerance test (0.75 U/kg, i.p.) after 6 h fasting **(D)** were performed in CD or HFD-fed mice; $n = 8$–14 per group. **(E)** Representative morphology of the subcutaneous AT and liver from mice fed CD or HFD for 24 weeks, as shown by H&E staining; magnification: 400X; scale bar: 20 μm. **(F,G)** Representative morphology of the epididymal AT, as shown by H&E staining or IHC staining for F4/80; magnification: 400X; scale bar: 20 μm **(F)**. Quantification of crown-like structures in the epididymal AT; $n = 6$–9 per group **(G)**. **(H)** mRNA expression levels of inflammatory genes in the epididymal AT; $n = 7$–12 per group. Values are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test. $^*p < 0.05$ vs. CD-WT, and $\#p < 0.05$ vs. HFD-WT.

attenuate inflammatory responses induced by obesity-associated metabolic dysfunction.

DISCUSSION

This study shows that PDK2/4 deficiency blocks polarization of resting macrophages to the M1 phenotype in response to LPS and INF-γ. Our findings establish that the regulation of PDH activity by the PDKs plays an important role in M1 macrophage polarization. The evidence for this includes a) dramatically induced PDK4 expression by LPS + INF-γ; b) reduced HIF-1α levels and suppression of the Warburg effect (phenomenon characterized by upregulation of glycolytic enzymes and down regulation of TCA cycle enzymes) in response to PDK2/4 deficiency; c) PDK2/4 deficiency-mediated check on the pool size of glycolytic intermediates that would, otherwise, feed into anabolic pathways required for proliferation of macrophages; and d) significantly reduced levels of NO and proinflammatory cytokines including TNFα, IL-6, and IL-1β in response to PDK2/4 deficiency. These findings suggest that sterile

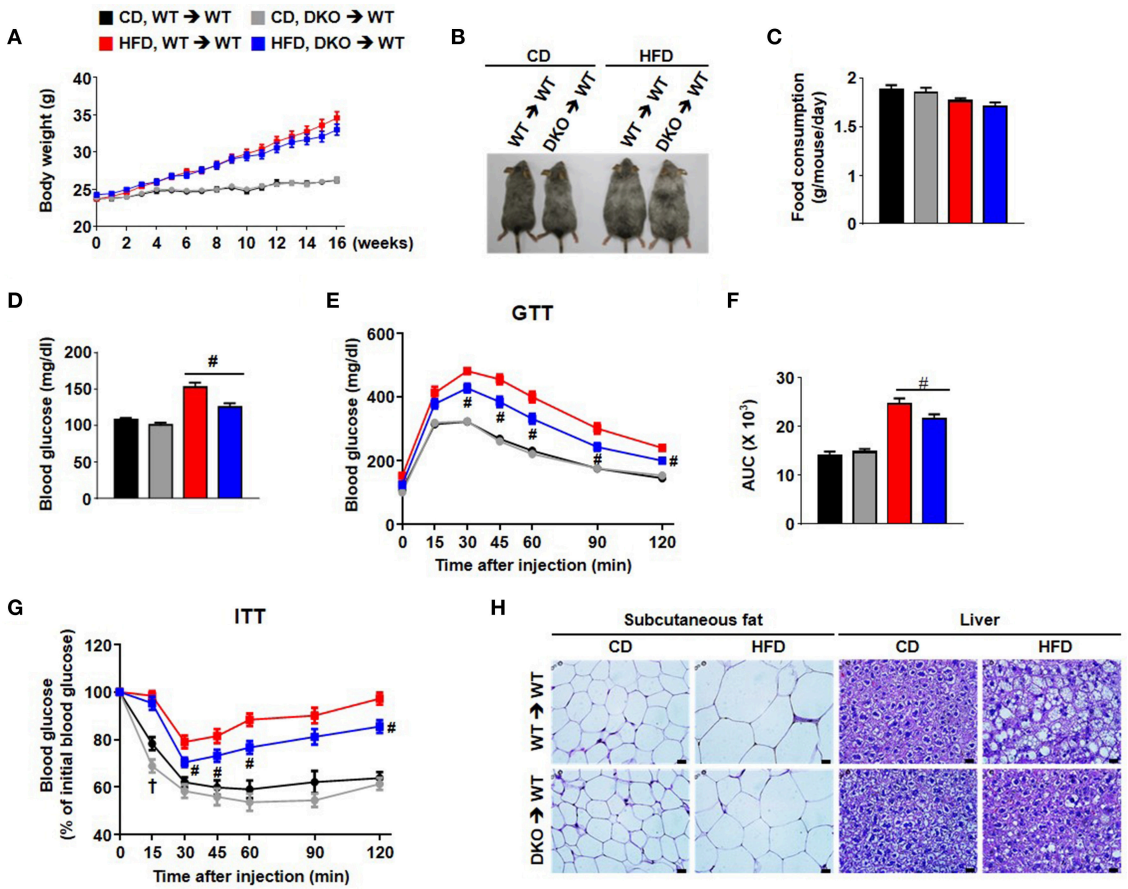

FIGURE 4 | Hematopoietic cell-specific PDK2/4 deficiency attenuates insulin resistance in mice fed a high-fat diet. **(A)** Body weights were measured during feeding of the different diets; $n = 12$–26 per group. **(B)** Representative mice of each group **(C)** Food consumption was measured per day in each group; $n = 7$ per group. **(D–G)** Measurement of fasting blood glucose levels **(D)**, glucose tolerance test (1.5 g/kg, i.p.) after 16 h fasting **(E,F)**, and insulin tolerance test (0.75 U/kg, i.p.) after 6 h fasting **(G)** was performed in both CD and HFD-fed mice; $n = 11$–26 per group. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way or two-way ANOVA followed by Tukey's HSD. # $p < 0.05$ vs. HFD-WT-BMT. **(H)** Representative morphology of the subcutaneous AT and liver from WT- and DKO-BMT mice as shown by H&E staining; magnification: 400X; scale bar: 20 μm.

inflammatory conditions, such as neuroinflammatory diseases, obesity, and steatohepatitis may be amendable to therapeutic intervention with PDK inhibitors.

Markers of AT inflammation including the number of infiltrated AT macrophages in response to over-nutrition, M1 macrophage population, presence of crown-like structures, and the levels of proinflammatory markers were found to be significantly reduced in the global PDK2/4 DKO mice compared to that in WT mice. These indicators of AT inflammation are associated with the metabolic dysfunctions characteristic of obesity including hyperglycemia, glucose intolerance, and insulin resistance. These findings clearly establish an important role for PDKs and, therefore, the regulation of the activity of the PDH complex in obesity-induced AT inflammation and metabolic dysfunction. To directly determine if these effects could be due to prevention of M1 macrophage polarization by PDK deficiency, macrophage-specific PDK deficiency was induced by transplanting PDK2/4-deficient myeloid cells into irradiated WT mice that were subsequently fed an HFD to induce obesity. Whereas, the AT of obese mice transplanted with normal myeloid cells became inflamed as evidenced by the accumulation of crown-like structures and protein markers for M1 macrophages, the AT of mice transplanted with PDK2/4-deficient cells remained free of inflammation; additionally, the negative effects on blood glucose levels, glucose tolerance, and insulin sensitivity were partially ablated. The observation that glucose tolerance and insulin sensitivity in HFD-fed DKO-BMT mice is only partially improved in contrast to the almost complete protection from HFD-induced glucose intolerance, insulin insensitivity, obesity, and macrophage infiltration observed in the organs of PDK2/4 DKO mice is suggestive that the beneficial effects of PDK deficiency are not totally due to inhibition of macrophage polarization. In other words, increased PDH activity in tissue other than myeloid cells plays a role in the beneficial effects of global PDK2/4 deficiency in HFD-fed mice.

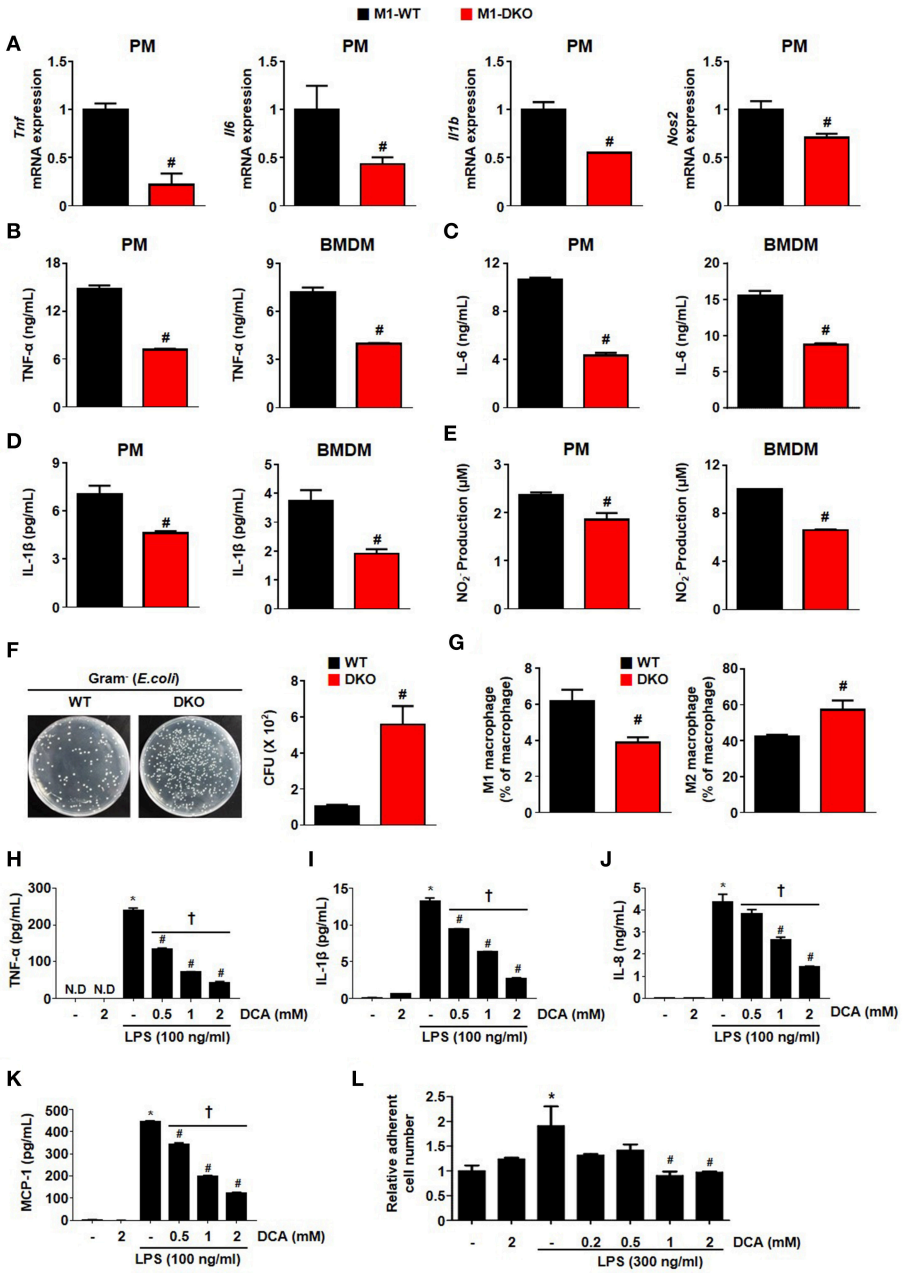

FIGURE 5 | Suppression of PDK2/4 in macrophages attenuates the secretion of proinflammatory effectors in response to inflammatory stimuli. **(A–E)** The levels of mRNA expression **(A)** and secreted proinflammatory effectors **(B–E)** were measured after treatment of WT- and DKO-PMs or -BMDMs with LPS 100 ng/mL+ IFN- γ 10 ng/mL for 12 h; $n = 3$–4 per group. **(F)** Bactericidal effect was assessed by counting the colony numbers in WT- and DKO-PMs; $n = 5$ per group. **(G)** Effect on PDK2/4 deficiency on the *in vivo* macrophage population in LPS (1 mg/kg)-injected mice; $n = 5$ per group. Values are expressed as mean ± SEM. Statistical analysis was performed using Student's *t*-test. #$p < 0.05$ vs. WT. **(H–K)** Secreted proinflammatory cytokine levels were determined at different DCA concentrations in THP1 cells treated with LPS 100 ng/mL for 24 h; $n = 3$ per group. **(L)** Adhesion assay was performed in collagen (10 μg/mL)-coated wells with DCA-treated THP-1 cells incubated with LPS (300 ng/mL) for 24 h; $n = 3$ per group. Values are expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test. *$p < 0.05$ vs. (–) LPS, #$p < 0.05$ vs. only LPS, and $^{\dagger}p < 0.05$ vs. 2 mM DCA. N.D, not detected.

The present study also shows that KPLH1130, a novel PDK-specific inhibitor, blocks M1 polarization and attenuates proinflammatory responses. Additionally, we have observed that M2-polarized PDK2/4 DKO macrophages express elevated levels of arginase-1—a superior competitor to iNOS for the substrate arginine—which has anti-inflammatory functions. The metabolic activation of macrophages triggered by glucose, insulin, and palmitate plays a key role in adipose-driven inflammation

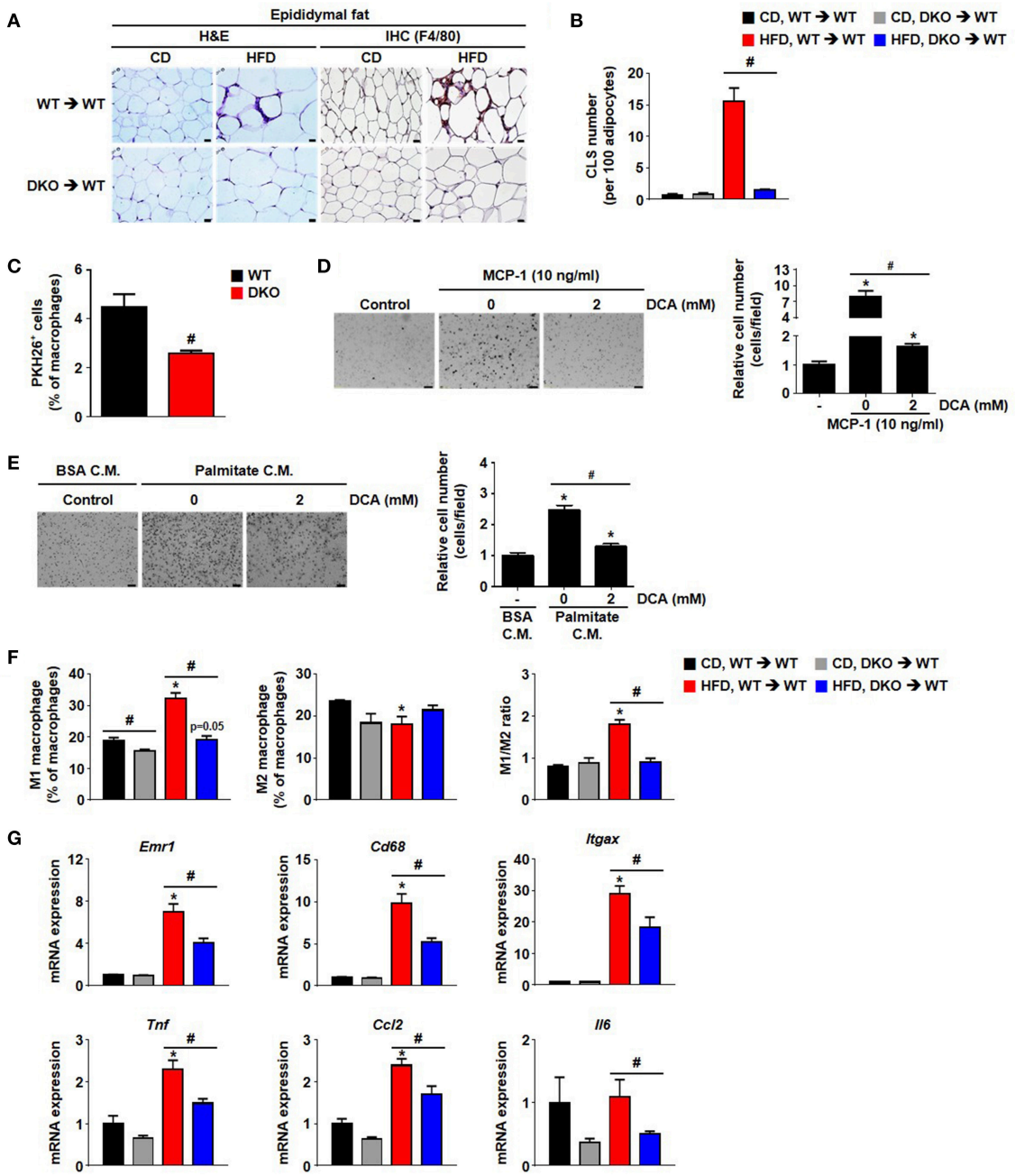

FIGURE 6 | Hematopoietic cell-specific PDK2/4 deficiency attenuates adipose tissue inflammation by preventing the recruitment of M1 macrophages. **(A,B)** Representative morphology of the epididymal AT by H&E staining or IHC staining for F4/80; magnification: 400X; scale bar: 20 μm **(A)**. The numbers of crown-like structures in the epididymal AT were determined; $n = 8$ per group **(B)**. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's HSD. #$p < 0.05$ vs. HFD-WT-BMT. **(C)** PKH26^{+} cell population was measured by FACS analysis; $n = 4$ per group. Values are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test. #$p < 0.05$ vs. WT monocyte donor group. **(D)** MCP-1-induced migration was evaluated in DCA-treated BMDMs using a trans-well migration assay; $n = 3$ per group; magnification: 200X; scale bar: 50 μm. The relative number of migrated cells per field was counted. Values are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test. *$p < 0.05$ vs. control, #$p < 0.05$ vs. MCP-1 induction. **(E)** 3T3-L1-conditioned medium (C.M.)-induced migration was evaluated in DCA-treated BMDMs using a trans-well migration assay; $n = 3$ per group; magnification: 200X; scale bar: 50 μm. The relative number of migrated cells per field was counted. Values are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test. *$p < 0.05$ vs. control, #$p < 0.05$ vs. palmitate C.M. **(F)** M1 and M2 macrophage populations and their ratios were determined in the stromal vascular fraction of WT- and DKO-BMT; $n = 3$ per group. Values are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test. *$p < 0.05$ vs. corresponding CD-fed group, # $p < 0.05$ compared with WT- and DKO-BM donor group. **(G)** mRNA expression levels of inflammatory genes in the epididymal AT; $n = 7$–12 per group. Values are expressed as mean ± SEM. Statistical analysis was performed by Student's *t*-test. *$p < 0.05$ vs. CD, #$p < 0.05$ vs. WT-BMT.

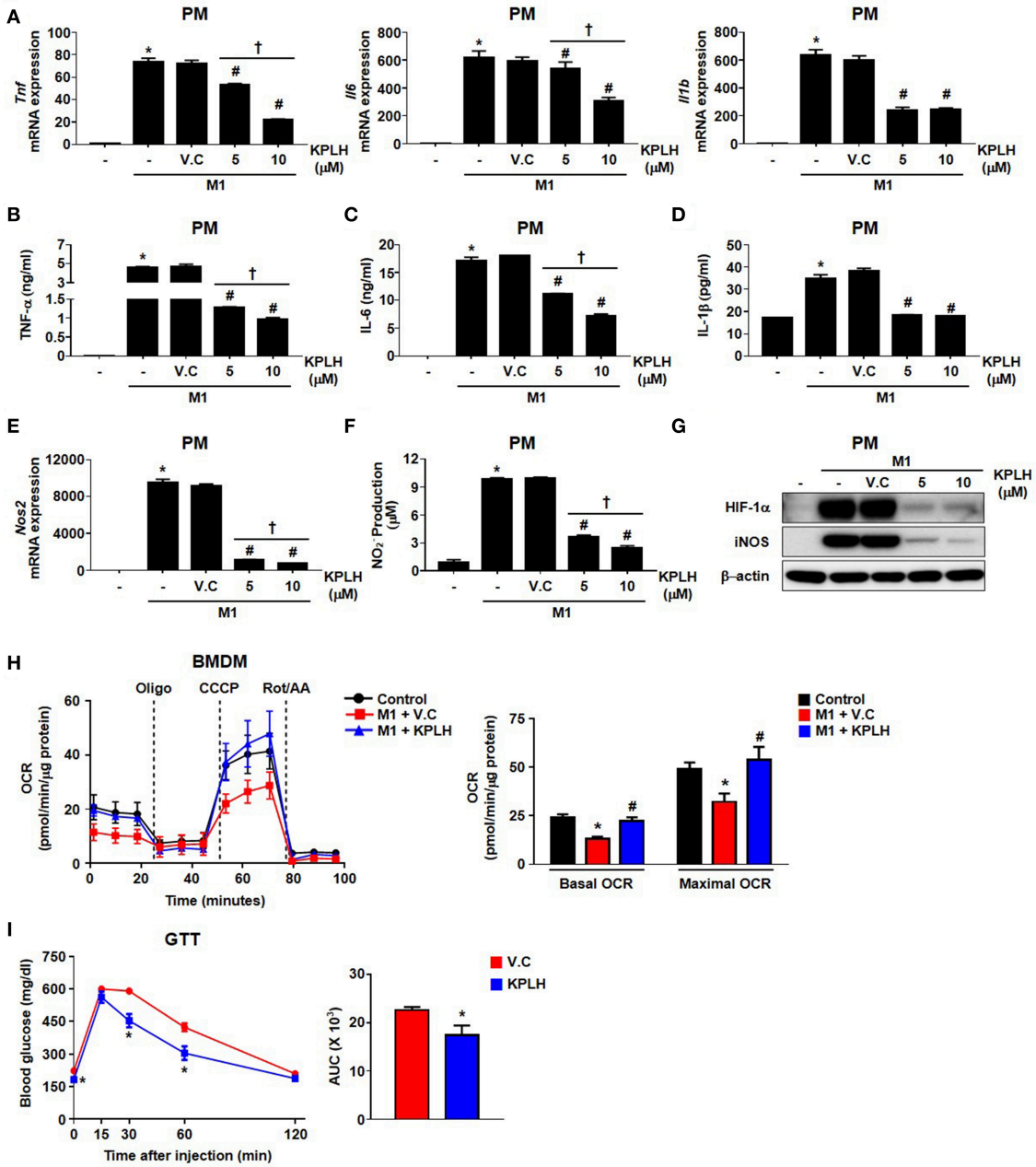

FIGURE 7 | A new PDK inhibitor, KPLH1130, attenuates the secretion of pro-inflammatory effectors by stimulated macrophages and improves glucose tolerance in HFD-fed mice. **(A–F)** The levels of mRNA expression **(A,E)** and secreted proinflammatory effectors **(B–D,F)** were measured with different KPLH1130 concentrations in PMs treated for 12 h with LPS (100 ng/mL) + IFN-γ (10 ng/mL); $n = 3$–6 per group. **(G)** M1 markers were assessed with different KPLH1130 concentrations in PMs treated with LPS (100 ng/mL) + IFN-γ (10 ng/mL) for 12 h. **(H)** OCRs were measured in KPLH1130 (10 μM) ± M1 stimulation by LPS (100 ng/mL) + IFN-γ (10 ng/mL) for 3 h; $n = 4$–5 per group. Values are expressed as mean ± SD. Statistical analysis was performed by Student's *t*-test. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. M1 only, and $^{\dagger}p < 0.05$ vs. KPLH1130 (10 μM). **(I)** Glucose tolerance test (1.5 g/kg, i.p.) after 6 h fasting was measured after HFD for 14 weeks with KPLH1130 (70 mg/kg, 4 weeks); $n = 6$ per group. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way followed by Tukey's HSD. $^{*}p < 0.05$ vs. vehicle control.

which results in insulin resistance—a process distinct from classical activation (25). Both genetic and pharmacological inhibition of PDK in macrophages significantly suppressed proinflammatory responses including M1 polarization markers, bactericidal activity, adherence, and migration. These findings support the notion that PDKs are potential targets for the treatment of inflammation as well as the negative metabolic repercussions of inflammatory conditions.

Gene set enrichment analysis identified the signaling pathways associated with inflammatory responses. Interestingly, the

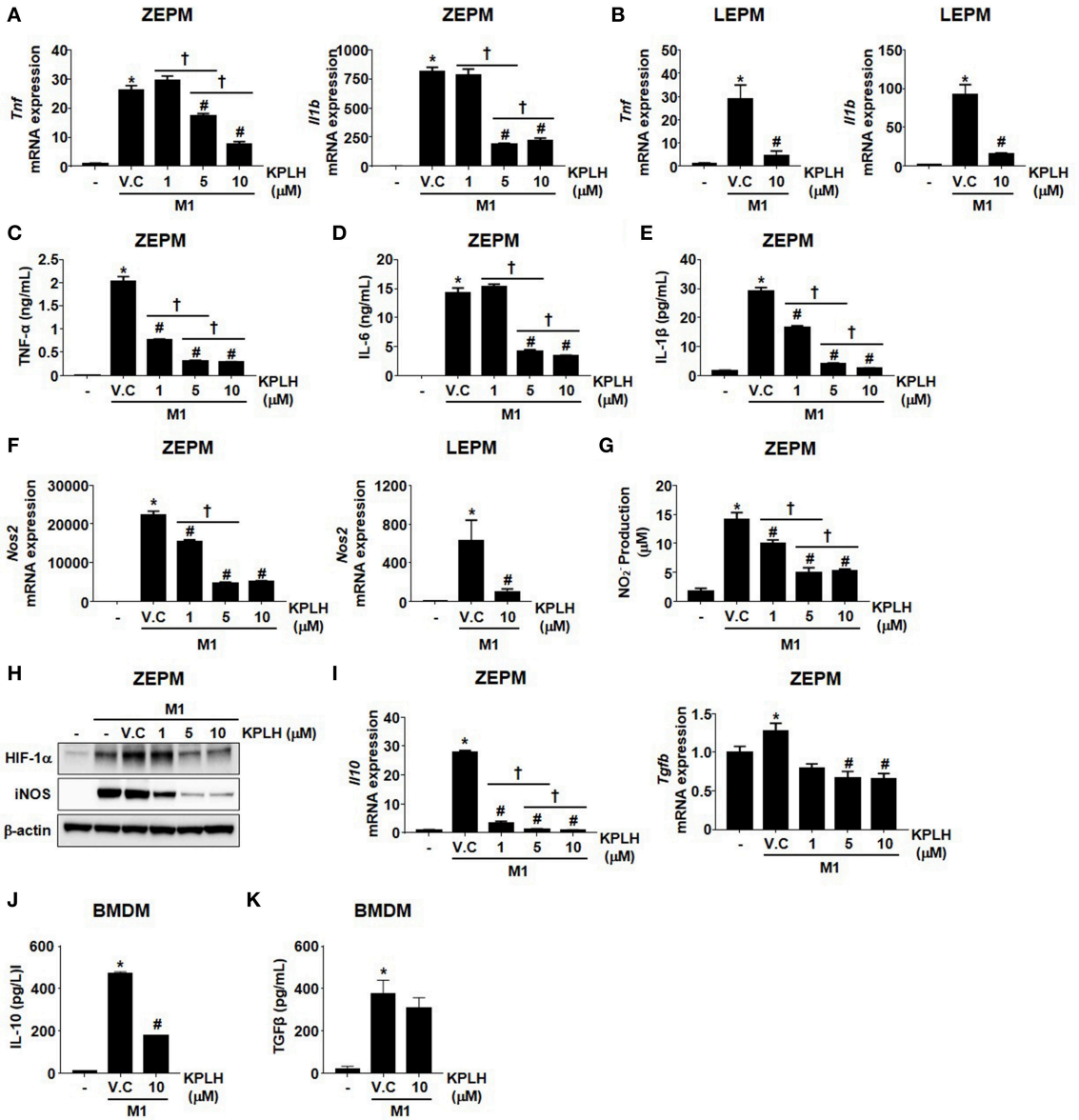

FIGURE 8 | Macrophage activation is suppressed by the novel PDK inhibitor, KPLH1130 using Zymosan-elicited PMs and LPS-elicited PMs. **(A–G)** mRNA expression **(A,B,F)** and secreted proinflammatory effectors **(C–E,G)** were measured following KPLH1130 (10 μM) treatment of ZEPMs or LEPMs incubated with LPS (100 ng/mL) + IFN-γ (10 ng/mL) for 12 h; $n = 3$ per group. **(H)** M1 markers were assessed following KPLH1130 (10 μM) treatment of ZEPMs incubated with LPS (100 ng/mL) + IFN-γ (10 ng/mL) for 12 h. **(I–J)** mRNA expression of IL-10 and TGFβ **(I)** and secreted IL-10 **(J)** and TGFβ **(K)** were measured following KPLH1130 (10 μM) treatment of ZEPMs and BMDMs, respectively incubated with LPS (100 ng/mL) + IFN-γ (10 ng/mL) for 12 h; $n = 3$ per group. Values are expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test. $^{*}p < 0.05$ vs. control, $\#p < 0.05$ vs. M1+V.C and $^{\dagger}p < 0.05$ vs. different doses of KPLH1130.

decrease in *Idh* expression, indicating a metabolic break in the TCA cycle of M1-polarized macrophages (26), was significantly attenuated after PDK2/4 deletion. PDK2/4 deletion prevented the reduction in mitochondrial OXPHOS that normally occurs in response to M1 stimulation. A metabolic switch from OXPHOS to aerobic glycolysis is followed by increased production of lactate and the accumulation of citrate and succinate by TLR4 activation (27, 28). According to our stable isotope flux study, PDK 2/4 ablation prevented the metabolic breaks associated with citrate and succinate accumulation as well as the increased lactate production in M1-polarized macrophages. In the case of PDK2 deficiency in hepatocytes, ^{13}C-glucose tracer analysis has suggested that PDC flux increases but TCA cycle intermediate flux decreases due to shunted ketogenesis (29).

Although the same effects induced by PDK2/4 deficiency on macrophage polarization are also induced by PDK1 deficiency

(9), PDK1 is not sensitive to induction by LPS stimulation (13), and PDK1 deficiency is less effective in preventing the expression of the glycolytic enzymes (9). Nevertheless, the finding that deficiencies of PDK1 and PDK2/4 block macrophage polarization to the proinflammatory phenotype indicates that reducing PDH activity by phosphorylation is required for LPS-induced macrophage polarization. Surprisingly, it has been reported that LPS-induced macrophage polarization is also prevented by a number of manipulations that strongly inhibit PDH flux. These include knockdown of pyruvate dehydrogenase phosphatase 1 (12), pharmacological inhibition of pyruvate import into the mitochondria (13), and upregulation of PDK2 by VSIG4 (14). These findings suggest that complete inhibition of PDH is not compatible with proinflammatory macrophage polarization. Indeed, pyruvate oxidation through PDH is necessary for the synthesis of the antimicrobial metabolite itaconate, an important product of mature proinflammatory macrophages (13). The finding that macrophage polarization is prevented by both inhibition of PDH and PDK deficiency suggests that LPS-induced polarization of macrophages is extremely sensitive to PDH flux. This is consistent with the evidence that reprogramming of the mitochondrial processes, particularly glutaminolysis, is also necessary for the inflammatory responses of M1 macrophages (30). If flux through PDH is too high or too low, changes in gene expression, reprogramming of glycolysis and the TCA cycle, and polarization to the M1 phenotype are prevented. Perhaps changes in the levels of metabolites transmit information to the nucleus when conditions are right for the reprogramming of gene expression necessary for polarization. The location of PDH allows it to precisely control the relationship between glycolysis and the TCA cycle and therefore serves as a sensitive checkpoint of bioenergetic reprogramming. In spite of the complexity, this makes PDH a sensitive target for therapeutic intervention in inflammatory conditions, suggesting that PDK2 and PDK4 in immune cells are potential targets for the treatment of inflammatory metabolic disorders.

ETHICS STATEMENT

All experiments were approved by the Institutional Animal Care and Use Committee of Kyungpook National University and were conducted according to recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

B-KM, SP, DK, RAH, and I-KL conceived and designed research. B-KM, H-JK, HH, C-MH, B-JC, JYL, CO, EY, HK, and DYH performed experiments. B-KM, SP, B-GK, DH, Y-HK, TL, and C-HL analyzed data. B-KM, SP, J-HJ, RAH, and I-KL interpreted results of experiments. B-KM and SP prepared figures. B-KM, SP, RAH, and I-KL drafted manuscript. B-KM, SP, JK, Y-KC, K-GP, AC, JL, RAH, and I-KL edited and revised the manuscript. B-KM, SP, H-JK, RAH, and I-KL approved final version of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00944/full#supplementary-material

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REVIEW
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What Defines NK Cell Functional Fate: Phenotype or Metabolism?

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NK cells are capable of an array of functions that range widely from their classic anti-tumor and anti-viral cytotoxic effector functions, to their critical regulatory roles in controlling inflammatory immune responses and promoting tissue growth. However, the mechanisms that polarize NK cells to these distinct and opposing functions are incompletely understood. NK cell functional subsets are primarily identified and studied based on phenotype, which has served as an accessible means for profiling NK cells and does offer information on NK cell activation state. However, inconsistencies have emerged in using classic phenotypes to inform function, which raise the questions: Can phenotype in fact define NK cell functional fate? What factors do profile and drive NK cell fate? In other immune cells, cell metabolism has been shown to critically determine subset polarization. There is a growing body of evidence that cell metabolism is integral to NK cell effector functions. Glucose-driven glycolysis and oxidative metabolism have been shown to drive classic NK cell anti-tumor and anti-viral effector functions. Recent studies have uncovered a critical role for metabolism in NK cell development, education, and memory generation. In this review, we will draw on the evidence to date to investigate the relationship between NK cell phenotype, metabolism, and functional fate. We explore a paradigm in which the differential activity of metabolic pathways within NK cells produce distinct metabolic fingerprints that comprehensively distinguish and drive the range of NK cell functional abilities. We will discuss future areas of study that are needed to develop and test this paradigm and suggest strategies to efficiently profile NK cells based on metabolism. Given the emerging role of metabolism in driving NK cell fates, profiling and modulating NK cell metabolism holds profound therapeutic potential to tune inflammatory and regulatory NK cell responses to treat disease.

Keywords: NK cell, cell metabolism, phenotype, NK cell subsets, glycolysis, mitochondria, CD56, innate immunity

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INTRODUCTION

Natural Killer (NK) immune cells are capable of an array of functions that range widely from their classic innate anti-tumor and anti-viral effector functions, to their regulatory roles in modulating other immune cells and promoting tissue growth (1). These distinct functions play critical yet paradoxical roles in a host of diseases. Cytotoxic NK cells are known to have an imperative role in the clearance of virally-infected and malignant cells (2); however, signals in the tumor environment polarize NK cells to regulatory subsets that support tumor progression and suppress other cytotoxic immune cells (3–5). While pathogenic in the context of cancer, regulatory NK cells have critical homeostatic roles in tissue growth and immune tolerance in contexts such as placental development

and fetal tolerance in pregnancy (6–8), and regulating fibrosis and immune cells in the liver (9–11). Despite the critical importance of mounting the appropriate NK cell response in different disease contexts, the mechanisms that polarize NK cells to these distinct and opposing functional fates are incompletely understood.

To date, NK cell functional subsets have been primarily identified and studied based on phenotypic markers. In their most classic definition, human NK cells are broadly dichotomized into $CD56^{bright}CD16^{-}$ regulatory NK cells with greater cytokine-producing capabilities, and $CD56^{dim}CD16^{+}$ anti-tumor/anti-viral NK cells with greater cytotoxic functions (12, 13). Additional receptor families function to tune NK cell activation and effector responses; these include activating Natural Cytotoxicity Receptors (NKp30, NKp44, and NKp46), activating and inhibitory CD94/NKG2 receptors that recognize non-classical MHC, and inhibitory KIR receptors that recognize classical MHC. The differential expression of these receptors is used to further specify NK cell developmental stages, effector subsets, and memory populations (14). Although phenotype is an accessible means by which to profile NK cells and provides information on NK cell activation state, reports are increasingly emerging that highlight discrepancies between NK cell phenotypic classification and their effector functions. These discrepancies present the questions: Can phenotype in fact define NK cell functional fate? What factors do define and drive NK cell fate?

A cell's ability to generate energy through metabolism enables its functional capacities. Indeed, there is a growing body of evidence that cell metabolism is integral to NK cell effector functions. It has been established that glucose-driven glycolysis and oxidative metabolism are required for classic NK cell anti-tumor and anti-viral effector functions (15–18). Recent studies have also emerged that point to critical roles of metabolism in NK cell development, education, and memory responses (19–23). In other immune cell subsets including T cells and macrophages, cell metabolism critically determines subset polarization. Up-regulation of glycolytic metabolism drives the polarization of pro-inflammatory T cells and macrophages (24–27). In contrast, a shift to respiration-derived ATP drives the polarization and functions of regulatory macrophages and T cells (24–26, 28). The generation of memory in T cells is marked by an increase in mitochondrial respiratory capacity, which enables a more rapid and robust secondary immune response (29, 30). Even within a terminally polarized subset, the activation of different metabolic pathways leads to distinct functional outcomes (31). While metabolism has been shown to regulate certain NK cell effector functions, the role of metabolism in broadly determining and defining different NK cell functional fates remains to be fully characterized.

Herein, we explore the relationship between NK cell phenotype, metabolism, and functional fate. Drawing on the evidence to date, we investigate the utility and roles of NK cell phenotype and metabolic activity in identifying and determining NK cell effector fate. We propose that differential activity in NK cell metabolic pathways, but not phenotype, produces distinct and subset-defining fingerprints that comprehensively distinguish and drive the range of possible NK cell functional abilities.

NK CELL FATE BASED ON PHENOTYPE: CAN WE JUDGE A BOOK BY ITS COVER?

For decades, NK cell phenotype has been integrally conflated with effector function and used as the principle means of classifying NK cells into distinct subsets. For instance, the stages of NK cell maturation are demarcated by the expression of specific phenotypic markers. Less mature human peripheral blood (pb)NK cells are defined as $CD56^{bright}CD16^{-}$ and express CD94 and the NKG2A inhibitory receptor (32, 33). As NK cells mature, they down-regulate CD56 expression and up-regulate CD16, becoming $CD56^{dim}CD16^{+}$ (34). These lose expression of NKG2A and acquire expression of KIR inhibitory receptors. A final step of NK cell maturation is marked by the acquisition of CD57. $CD56^{dim}CD57^{+}$ NK cells are considered to be the most mature and terminally differentiated subset (32).

Contrary to this classic maturation paradigm, it is now understood that $CD56^{dim}$ NK cells can in fact up-regulate CD56 expression upon cytokine activation and become $CD56^{bright}$ (35). Thus, high CD56 expression does not necessarily differentiate less mature from mature NK cells, as it can also indicate mature, activated NK cells. Other recent studies provided evidence that $CD57^{+}$ NK cells are not terminally differentiated. It was found that upon single cell expansion with K562 feeder cells, some clones derived from originally $CD56^{dim}CD57^{+}$ NK cells had lost CD57 expression. Moreover, clones from $NKG2A^{-}$ populations were capable of reacquiring NKG2A expression (36). Following autologous stem cell transplant in patients with lymphoma or myeloma, a unique $CD56^{bright}$ NK cell population was found to be the predominant NK cell subset following leukocyte regeneration. These young $CD56^{bright}$ NK cells had high expression of CD57 and KIRs and potent degranulation (37). These studies demonstrate that unless NK cells are capable of regressing through maturation, classic NK cell development markers cannot definitively specify the stage of NK cell maturation.

Recent studies have also introduced discrepancies in the $CD56^{bright}/CD56^{dim}$ phenotypic dichotomization of regulatory and cytotoxic NK cells. Wagner et al. demonstrated that following priming with IL-15, $CD56^{bright}$ pbNK cells not only had greater cytokine production compared to $CD56^{dim}$ pbNK cells, but also displayed greater degranulation and killing in response to tumor cell targets. Importantly, they negated the possibility that this was due to up-regulation of CD56 on $CD56^{dim}$ NK cells, as they observed this effect even with pre-sorted $CD56^{bright}$ NK cells (38). In addition, evidence has emerged to suggest that $CD56^{bright}$ does not unanimously define NK cells with the strongest cytokine-producing capabilities. The hepatic NK cell population is highly enriched in $CD56^{bright}$ cells compared to pbNK cells; yet, these have recently been shown to have reduced IFN-γ and TNFα production in response to stimulation compared to pbNK cells, despite the majority of pbNK cells being $CD56^{dim}$ (39). Building further on this phenomenon, $CD56^{superbright}$

NK cells are considered highly immunoregulatory. Notably, $CD56^{superbright}CD16^{-}$ uterine NK cells are instrumental in promoting angiogenesis and tissue remodeling required for healthy placental development in pregnancy. These regulatory functions lend themselves as well to enhancing, rather than inhibiting, tumor progression (7). However, in stark contrast to their characteristic regulatory functions, highly cytotoxic $CD56^{superbright}$ NK cells produced following NK cell expansion with K562-based feeder cells were recently described. These $CD56^{superbright}$ expanded NK cells eliminated large ovarian tumors in xenograft models. What's more, within the expanded NK cell population, degranulation, cytotoxicity, and IFN-γ, increased with increasing CD56 brightness (40). Together, these studies indicate that CD56 expression cannot distinguish regulatory from cytotoxic NK cells. Indeed, $CD56^{bright}$ NK cells can be any combination of mature or less mature, and cytotoxic or regulatory.

Since $CD56^{bright}$ NK cells can be either cytotoxic or regulatory, the question that pursuantly arises is whether another phenotypic marker may better define these functional subsets. CD16 expression is classically used in conjunction with CD56, with $CD16^{+}$ NK cells considered cytotoxic and $CD16^{-}$ NK cells categorized as regulatory (13). It has been demonstrated that upon activation, $CD56^{dim}CD16^{+}$ NK cells up-regulate CD56, culminating in a $CD56^{bright}CD16^{+}$ cytotoxic NK cell population (35). Thus, it could be postulated that CD16 expression distinguishes $CD56^{bright}$ cytotoxic and regulatory NK cells. However, highly cytotoxic IL-15-primed $CD56^{bright}$ NK cells remained predominantly $CD16^{-}$ (38). Moreover, Siewiera et al. have demonstrated regulatory capacities in $CD16^{+}$ NK cells. They reported that following culture in TGF-β/IL15/IL18, pbNK cells acquired regulatory functions, as they produced high levels of VEGF, and had reduced cytotoxicity and production of IFN-γ and TNFα, but retained high expression of CD16 (41). These studies call into question the relevance of CD16 for determining cytotoxic and regulatory NK cell subsets.

Turning to other NK cell markers does not seem to address the above discrepancies. Both regulatory and cytotoxic NK cells can express high levels of activating receptors including NKG2D, NKp30, NKp44, and NKp46, but stimulation through these receptors instigates distinct functions in regulatory and cytotoxic NK cells (7, 41, 42). While it has been reported that expression of different splice variants in these receptors partly contributes to determining downstream function (41), the fundamental mechanisms that determine the receptor isoform expressed remain unknown. Inhibitory receptor expression is equally uninformative, as both regulatory and cytotoxic NK cells can express high or low levels of NKG2A and/or KIRs (32, 38, 40, 43, 44). In all, a specific combination of NK cell phenotypic markers that consistently distinguishes regulatory from cytotoxic NK cells remains elusive (**Figure 1**).

A similar challenge arises in phenotypically defining memory NK cells. Memory NK cells are generated in response to a number of stimuli, including hapten-induced contact hypersensitivity, infections, cytokine activation, and pregnancy (6, 45–47). Secondary responses of memory NK cells are variegated and depend on the sensitizing stimulus. Some memory NK cells have adaptive-like antigen-specific secondary responses, while others have an innate-like non-antigen-specific recall response (46, 47). What's more, memory responses have now been identified in both cytotoxic and regulatory NK cells (6, 45). However, what does remain consistent and broadly defines memory subsets is their functional capacity to have a rapid and enhanced response upon re-stimulation.

Studies have largely used phenotypic markers to define memory NK cells. Extensively studied memory NK cells include those generated in response to cytomegalovirus (CMV) infection. In humans, these have been predominantly defined as $CD56^{dim}NKG2C^{+}$ NK cells, which preferentially expand in response to acute infection. These $NKG2C^{+}$ NK cells undergo contraction following acute infection, but persist long-term and specifically increase in response to human CMV (HCMV) re-activation and produce high levels of IFN-γ (48). Certainly, NKG2C plays a direct role in shaping HCMV adaptive NK cell responses. NKG2C on HCMV-adaptive NK cells has been shown to display fine peptide specificity through differential recognition of polymorphic UL40-encoded peptides. These UL40 polymorphisms enabled selective recognition of distinct strains of HCMV and selective activation, proliferation, and differentiation of $NKG2C^{+}$ adaptive NK cells (49). HCMV memory NK cells are considered to have a mature phenotype, as they lack NKG2A, have lower levels of NKp30 and NKp46, and have high KIR and CD57 expression (22, 48, 50). Although NKG2C is principally used to identify HCMV-memory NK cells, recent studies have challenged the requirement for NKG2C in generating HCMV-memory NK cells. Memory NK cell responses to HCMV have been reported in NKG2C-deficient humans and upon HCMV re-activation in patients transplanted with NKG2C-deficient cord blood (51, 52). Therefore, expression of NKG2C is not necessary to define HCMV-memory NK cells.

In addition, memory NK cells do not unanimously express a classically mature phenotype. Cytokine-induced memory-like (CIML) NK cells generated by IL-12/IL-15/IL-18 pre-activation are primarily $CD56^{dim}$, but express CD25, NKG2A, CD94, and CD69, and lack expression of KIRs and CD57 (53, 54). Memory NK cells are also not limited to the $CD56^{dim}$ compartment. Frequencies of long-lived BCG-reactive $CD56^{bright}$ and $CD56^{dim}$ NK cells were shown to be elevated for up to 1 year after BCG re-vaccination in adults and were predominantly $KIR2DL2/DL3^{-}$ and $CD57^{-}$. BCG re-vaccination induced greater IFN-γ expression in both $CD56^{bright}$ and $CD56^{dim}$ NK cells for up to 1 year compared to NK cells prior to BCG re-vaccination. Furthermore, perforin expression was also enhanced upon BCG stimulation in $CD56^{bright}$ NK cells 1 year following BCG-revaccination, compared to $CD56^{bright}$ NK cells pre-BCG-revaccination (55). Recently, memory NK cells have been described in the context of pregnancy. Pregnancy-trained memory uterine NK cells (PTuNKs) were reported in the deciduae of multigravid women. PTuNK cells have enhanced regulatory function including VEGF production, relative to uNK cells from primigravid deciduae, which supported enhanced vascularization and tissue growth. Given their tissue-residency, it is perhaps not surprising that PTuNK cells have a unique phenotype relative to other memory subsets, as they

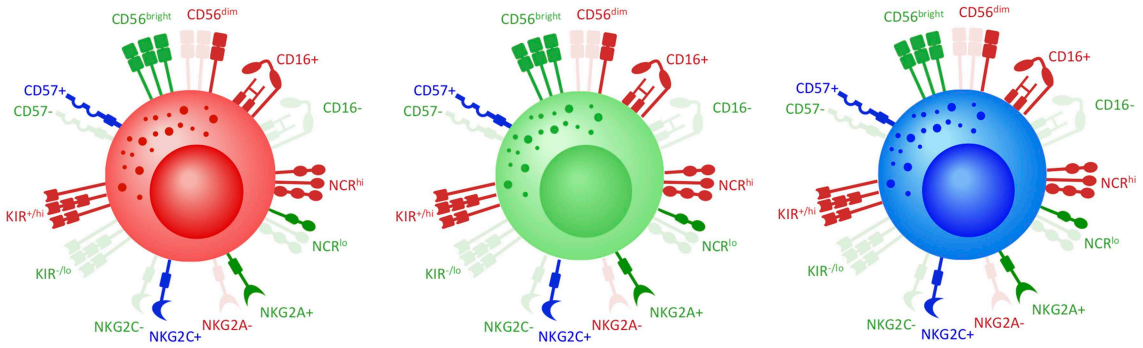

FIGURE 1 | Same covers, different stories: phenotype does not distinguish NK cell functional fates. To date, phenotype has been used as a principal means of studying and classifying NK cell functional subsets. However, there is mounting evidence that demonstrates significant discrepancies in the classic phenotype definition of NK cell subsets. For instance, while cytotoxic NK cells are classically defined as $CD56^{dim}CD16^{+}$, recent studies have demonstrated that $CD56^{bright}$ NK cells in fact hold the greatest cytotoxic potential. It has now been shown that regulatory NK cells, classically defined as $CD56^{bright}CD16^{-}$, can also in fact express CD16. Memory NK cells also present as either $CD56^{bright}$ or $CD56^{dim}$ and $CD16^{+}$ or $CD16^{-}$. Other phenotypic markers do not further distinguish NK cell subsets: cytotoxic, regulatory, and memory NK cells can all express either high, low, or absent levels of Natural Cytotoxicity Receptors (NCRs), NKG2A, NKG2C, KIRs, and CD57. Thus, not only can NK cells with the same functional fate express a range of possible phenotypes, but NK cells with opposing functions can express the same phenotype. Indeed, the range of classic NK cell phenotypic profiles are expressed across NK cell fates and are thus insufficient to distinguish NK cell functional subsets. Red denotes receptors classically associated with cytotoxic NK cells; green denotes receptors classically associated with regulatory NK cells; blue denotes receptors classically associated with memory NK cells.

were predominantly $CD56^{bright}CD16^{-}$, expressed high levels of NKG2C, but unchanged levels of NKp30 and NKp46 (6). It is possible that certain phenotypic traits may distinguish antigen-specific from non-specific memory NK cells. Nevertheless, there remains no unifying phenotype to identify NK cells with memory capacity (**Figure 1**). Furthermore, the mechanisms within NK cells that ultimately drive and identify the capacity for an enhanced secondary response are not fully elucidated.

If phenotype is unable to consistently identify NK cell subsets and functional fate (**Figures 1**, **2A**), what other factors might achievably distinguish NK cell fate? Such identifying features likely lie at the heart of mechanisms that drive NK cell function.

IF AN NK CELL CANNOT BE DEFINED BY HOW IT LOOKS, COULD IT BE DEFINED BY HOW IT IS FUELED?

As highly plastic innate responders, NK cell effector fate is molded by its environment. NK cells integrate a number of environmental cues which determine downstream function. In the absence of activating signals, naïve NK cells remain in a resting quiescent state. In response to inflammatory signals, cytotoxic effector NK cell responses are marked by robust proliferation and synthesis of proinflammatory cytokines and cytotoxic machinery. Upon resolution of inflammation, NK cells curb their response, returning to a quiescent state. It is noteworthy that this shifting functional profile represents a profound shift in energy dynamics from a low-energy quiescent state to one with substantial energetic and biosynthetic requirements. Further, NK cells exert distinct functions across different tissue sites that vary greatly in nutrient types and availability. From these factors arises a parallel between NK cell energetics and functional fates.

Indeed, the capacity of a cell to generate energy through metabolism has emerged as an important factor in enabling immune cell effector functions. The burgeoning field of NK cell metabolism has uncovered the involvement of cell metabolism throughout different steps of NK cell fate determination, including development, cytotoxic effector responses, and generation of long-lived memory populations. Thus, could distinct metabolic profiles comprehensively identify and drive NK cell functional fates?

At Their Inception, NK Cell Development Is Enabled by Metabolic Signals

NK cell development is an energy-intensive process as it requires high levels of proliferation of NK cells in the bone marrow. As NK cells progress through development, their proliferation slows and they progress to state of quiescence in the periphery (56, 57). In line with this, gene expression analysis has demonstrated that metabolic pathways associated with cell growth are up-regulated in developing NK cells whereas gene signatures associated with metabolic quiescence, such as fatty acid catabolism and aerobic metabolism, are predominant in resting NK cells in the periphery (56). While studies have yet to comprehensively measure the metabolic activity of NK cells at different stages of development, key metabolic regulators have emerged as pivotal drivers of NK cell maturation.

The mechanistic target of rapamycin (mTOR) is a major energy sensor in the cell that integrates signals for nutrient availability, growth, and activation. In response to such signals,

mTOR up-regulates glycolytic metabolism and biosynthetic processes (58). A seminal study by Marçais et al. revealed the critical role for mTOR in murine NK cell development. They found that mice with an NK cell-specific deletion of mTOR had normal levels of developing NK cells in the bone marrow, but only trace levels of NK cells in the periphery (56). Other studies have further demonstrated that mTOR signaling is required for the early stages of NK cell development (19, 20). The protein E4BP4 plays an indispensible role in the commitment to the NK cell lineage by promoting the transcription of Eomes (59). Downstream of IL-15 signaling, mTOR activation by the kinase PDK1 was found to be required for E4BP4 expression in bone marrow NK cells. Indeed, knock-out of PDK1 in NK cells arrested NK cell development in early stages by inhibiting mTOR activation and reducing expression of the transferrin receptor CD71 and the amino acid transporter CD98 (20). Tight regulation of mTOR activity is critical for NK cell development, as the expression of *Tsc1*, a repressor of mTOR, is required to prevent deregulated proliferation and resulting exhaustion in response to IL-15 in developing NK cells (19). Together, these studies present mTOR-mediated metabolic signaling as a central node in NK cell development.

The evidence to date has demonstrated the requirement for metabolic regulators in NK cell development and that a shifting metabolic expression profile parallels development. These studies portray a baseline energy-intensive, biosynthetic metabolic fingerprint for developing NK cells in the bone marrow, which shifts to a more energy-conservative fingerprint as NK cells progress to the periphery. Further developing and characterizing the metabolic fingerprints of NK cells throughout development may more broadly define the maturation stages of NK cells, particularly for later stages in which phenotype fails to do so and for NK cells at different tissue sites. Assessing the baseline metabolic fingerprint in such instances holds potential to more comprehensively define the degree of NK cell maturity.

Greater Glucose-Driven Metabolic Fitness Identifies NK Cells With the Greatest Cytotoxic Capacity

Although metabolically quiescent at baseline, mature cytotoxic NK cells up-regulate the rate of glucose-driven glycolysis and oxidative phosphorylation (OxPhos) upon stimulation (15–17, 60, 61). Increases in these metabolic pathways are accompanied by increased expression of the nutrient receptors Glut1, CD71, and CD98 (16, 56, 60). Activated cytotoxic NK cells primarily rely on glucose to fuel mitochondrial ATP production, as inhibition of glycolysis via the competitive inhibitor 2-deoxyglucose (2DG) impaired ATP production comparably to direct inhibition of OxPhos by the ATP synthase inhibitor oligomycin (61). The activation-induced increase in NK cell glucose metabolism has been shown to be regulated by mTOR, glutamine, and transcription factors cMyc and Srebp. mTOR activity is highly up-regulated in NK cells in response to stimulation (16, 56, 60, 62). In murine NK cells, knock-out or inhibition of mTOR has been shown to prevent the increases in glycolysis, glycolytic enzymes, and nutrient receptor expression upon activation (16, 56, 63). Similarly in human NK cells, the up-regulation of glycolysis in response to IL-15 and IL-2 is dependent upon mTOR, although independent from mTOR in response to IL-15/IL-12 stimulation (60). cMyc is another key driver of glucose metabolism in immune cells (64). In response to IL-2/IL-12 stimulation, glutamine uptake by NK cells was found to drive the expression of cMyc which in turn was required for the activation-induced increase in glycolysis and OxPhos by up-regulating glycolytic machinery and mitochondrial biogenesis (17). Srebp transcription factors promote the catabolism of glucose to cytosolic citrate in NK cells by supporting the activity of the citrate-malate shuttle which shuttles glucose-derived citrate from the mitochondria to the cytosol. Activity of the shuttle in turn produces mitochondrial NADH which fuels elevated OxPhos in NK cells (15).

This up-regulation in glucose-driven glycolysis and OxPhos is critical for NK cell cytotoxicity and IFN-γ production. Direct inhibition of glycolysis in murine NK cells, by 2DG or by substituting glucose with galactose in culture, inhibited IFN-γ and granzyme B expression in response various modes of stimulation including TLR-, cytokine-, or activation receptor-mediated stimulation (16, 61). In line with the evidence that glucose is the primary fuel driving OxPhos in cytotoxic NK cells, inhibition of OxPhos by glucose-depleted media or the ATP synthase inhibitor oligomycin also potently inhibited receptor-mediated IFN-γ production (61). Mah et al. demonstrated the pivotal role for NK cell glucose metabolism in the defense against infection: inhibition of glycolysis with 2DG impaired NK cell clearance of MCMV-infected cells in mice and compromised control of viremia and resulting survival of the mice (18). Arrest of NK cell glucose metabolism has also been shown to play a critical role in obesity-induced NK cell dysfunction. The increased presence of fatty acids in obesity led to lipid accumulation in NK cells, resulting in a PPAR-mediated increase in NK cell lipid metabolism and abrogation of glycolysis and OxPhos. These metabolic changes impaired polarization of cytotoxic machinery and resulting anti-tumor NK cell functions (65). Consistent with its effects on glucose metabolism, mTOR inhibition broadly impaired cytotoxic NK cell functions, including IFN-γ, perforin, and granzyme B expression and degranulation and cytotoxicity in response to tumor cell targets (56, 63). Moreover, inhibition of either cMyc expression, Srebp activity, or the citrate-malate shuttle all critically impaired NK cell IFN-γ production and cytotoxicity (15, 17). In human NK cells, elevated OxPhos is essential for NK cell cytotoxicity and IFN-γ production in response to IL-2 and IL-15/IL-12 stimulation. Heightened glycolysis was also shown to be required for NK cell cytotoxicity following 24 h IL-15 stimulation and for a robust IFN-γ response to IL-15/IL-12, particularly in $CD56^{bright}$ NK cells (18, 60). Keating et al. demonstrated metabolic differences between $CD56^{bright}$ and $CD56^{dim}$ human pbNK cells (60). In line with their greater cytotoxic functions following cytokine priming, $CD56^{bright}$ pbNK cells had enhanced mTOR-mediated expression of nutrient receptors and glucose uptake in response to cytokine stimulation as compared to $CD56^{dim}$ pbNK cells. Thus, up-regulation of glucose-driven glycolysis and OxPhos

characterizes and enables the activation of cytotoxic NK cell functions.

Accumulating evidence is demonstrating that the most highly cytotoxic NK cells are endowed with greater glucose-driven metabolic fitness. Extended pre-activation of NK cells for 3–5 days with IL-15 induces greater IFN-γ production in response to receptor-mediated activation as compared to short-term (4–24 h) IL-15 pre-activation (56, 61). During extended IL-15 stimulation, NK cells undergo profound metabolic reprogramming as compared to short-term stimulation, as they greatly increase both their rate and overall capacity for glycolysis and OxPhos (56, 61). Following such metabolic reprogramming, NK cell IFN-γ production was no longer susceptible to the inhibition of OxPhos alone; rather, aggressive inhibition of both glycolysis and OxPhos by treating NK cells with oligomycin in glucose-free media was required to suppress IFN-γ (61). Such metabolic reprogramming has demonstrated improved outcomes in the context of infection. Pre-treatment of mice with the IL-15 super-agonist complex ALT-803 for 3 days prior to MCMV infection increased basal and maximal glycolytic and oxidative metabolism and rescued impaired viral clearance in response to 2-DG treatment (18). Similar metabolic reprogramming also underpins the enhanced cytotoxic functions of licensed NK cells. The process of licensing during NK cell development requires that inhibitory KIRs expressed by the NK cell recognize self-MHC, the result of which lowers the threshold required for activation and enables greater effector potential in licensed NK cells compared to their unlicensed counterparts (66). Schafer et al. demonstrated that glycolytic metabolism is a critical regulator of NK cell licensing (21). In NK cells expanded with IL-21-expressing K562 feeder cells, highly functional licensed NK cells had greater glycolysis and glycolytic reserve compared to less functional unlicensed NK cells. Despite this increased conversion of glucose to lactate, expanded licensed NK cells sustained comparable levels of mitochondrial respiration as unlicensed NK cells, indicating a greater net level of glucose-derived energy production. While unlicensed NK cell cytotoxicity was highly sensitive to inhibition of OxPhos with oligomycin, the cytotoxicity of licensed NK cells was more resistant to metabolic inhibition. Similar to following extended IL-15 stimulation, the cytotoxicity of licensed NK cells was only inhibited upon aggressive abrogation of glucose metabolism by overnight incubation in glucose-free media, 2DG and oligomycin (21). Together, these findings demonstrate that a greater capacity for glucose-fueled energy production identifies NK cells with enhanced cytotoxic functions (**Figure 2B**). Moreover, greater metabolic fitness in these highly functional NK cells imparts greater flexibility in using either glycolysis or OxPhos to fuel their cytotoxic functions, rendering them more resistant to metabolic stressors.

A parallel emerges when considering the phenotype and metabolism of highly cytotoxic NK cells. It is known that upon extended cytokine stimulation including IL-15, in addition to increasing their capacity for glucose metabolism, NK cells also up-regulate CD56 expression and become predominantly $CD56^{bright}$ (35). Indeed, evidence has separately demonstrated that 1) $CD56^{bright}$ pbNK cells have a greater propensity for glucose metabolism compared to $CD56^{dim}$ NK cells (67), and 2) $CD56^{bright}$ pbNK cells have superior cytotoxicity and IFN-γ production compared to $CD56^{dim}$ NK cells following priming with IL-15 (38). Moreover, a recent report suggests that highly functional licensed expanded NK cells have greater CD56 expression than their less functional unlicensed counterparts. Following the same feeder cell expansion with IL-21-expressing K562 cells, expanded NK cell degranulation and IFN-γ production increased with CD56 expression: the most cytotoxic and thus licensed NK cells were $CD56^{superbright}$ whereas the least cytotoxic were $CD56^{dim}$ (40). Together these studies expose an inextricable tie between heightened cytotoxic potential, capacity for glucose metabolism, and degree of CD56 expression. Thus, in the context of cytotoxic NK cells, both CD56 expression and heightened glucose metabolism are indicative of subsets with the greatest functional capacity. However, highly functional regulatory NK cells are also $CD56^{bright}$, which raises the question: can distinct metabolic programs differentiate highly cytotoxic $CD56^{bright}$ NK cells from highly regulatory $CD56^{bright}$ NK cells?

Glucose Metabolism—The Weight That Tips the Balance Between Cytotoxic and Regulatory NK Cells?

Research on NK cell metabolism has largely focused on the metabolic pathways that generate anti-viral and anti-tumor effector NK cells and it is clear that enhanced glucose metabolism is critical for such functions. On the other hand, while regulatory NK cells have critical homeostatic anti-inflammatory roles in the body, there is a dearth of knowledge about the role of metabolism in driving regulatory NK cell polarization and functions. In order to more fully understand the role of metabolism in the polarization of NK cell functions, it will be critical for future studies to investigate the metabolic profile of regulatory NK cells. For instance, is the metabolism of highly functional NK cells similar irrespective of whether the downstream functions are cytotoxic or regulatory? Or do regulatory NK cells predominantly utilize distinct, non-glucose-driven metabolic pathways which determine their regulatory functional fate?

The metabolism of a cell is largely dictated by its microenvironment, including factors such as nutrient and oxygen availability. Thus, in considering the above questions, it is worthwhile to examine microenvironments that favor regulatory NK cell polarization, such as the uterus, tumors, and the liver, and the metabolic profile these may shape:

Following implantation in the uterine endometrium, the early growth of the embryo occurs in hypoxic conditions in the uterus (68). Hypoxia in turn stimulates the secretion of VEGF and other angiogenic factors from uNK cells to support the demands for increased vascularization (69, 70). In addition, TGFβ plays an important role in inducing regulatory uNK cell functions (41, 69–71). Many parallels are evident between regulatory uNK cells and tumor-associated (TA-)NK cells. As a result of rapid tumor cell proliferation, tumors have aberrant vascularization resulting in large pockets of hypoxia (72, 73). In addition, high levels of anti-inflammatory cytokines such as TGFβ are produced by other tumor-associated immune cells and are known to impair

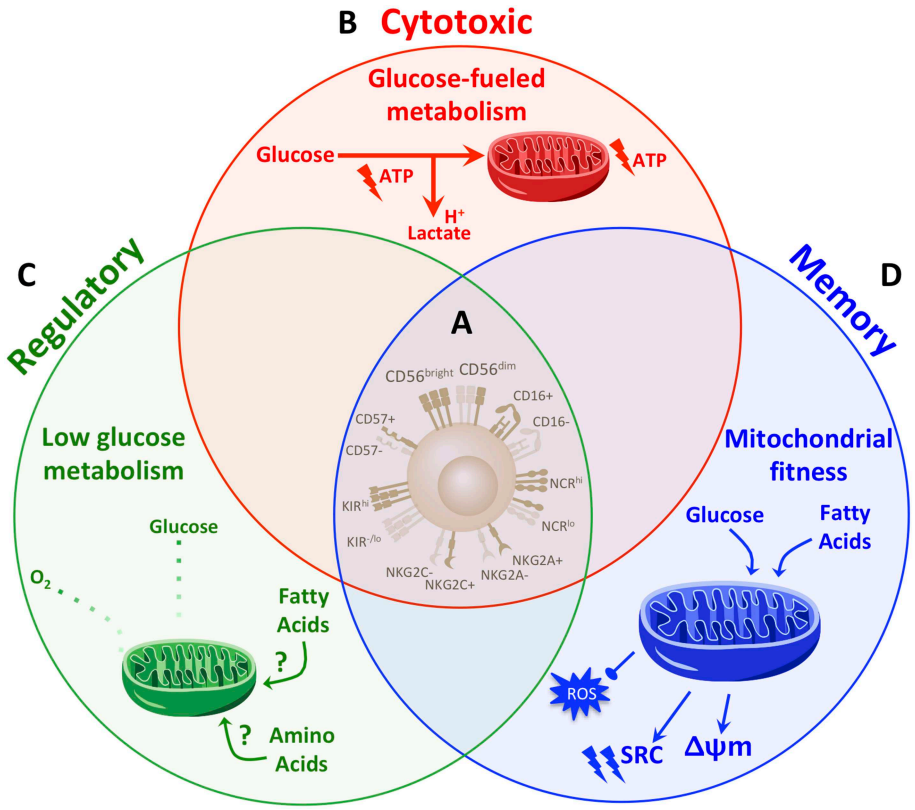

FIGURE 2 | Distinct metabolic fingerprints, but not phenotype, underpin NK cell functional fates. **(A)** Though widely used to define NK cell subsets, classic NK cell phenotypic markers are proving insufficient to comprehensively identify NK cell fates. The range of archetypal NK cell phenotypes are in fact expressed across NK cells with different functional fates. In the absence of distinguishable phenotypes to reliably determine NK cell fate and functional potential, determining what drives and identifies NK cell fate will be instrumental. **(B–D)** Studies so far have demonstrated that distinct metabolic profiles drive NK cell functions. **(B)** Cytotoxic NK cells are fueled primarily by glucose. Upon activation, cytotoxic NK cells increase rates of glucose-driven glycolysis and OxPhos which in turn drive cytotoxic functions. A greater capacity for glucose metabolism through glycolysis and OxPhos identifies NK cells with the greatest cytotoxic abilities. **(C)** NK cells are polarized to a regulatory fate under hypoxic and glycolysis-limiting conditions. Thus, regulatory functions are promoted under low levels of glycolysis and OxPhos and may rely on fuels other than glucose, such as fatty acids or amino acids. **(D)** Memory NK cells exhibit enhanced mitochondrial fitness. During the contraction phase of an immune response, NK cells undergo autophagy to clear dysfunctional mitochondria, which is required for the generation of a memory NK cell pool. Memory NK cells exhibit an increased spare respiratory capacity (SRC) and membrane potential ($\Delta\psi m$) and reduced levels of reactive oxygen species (ROS). While memory NK cells maintain an enhanced capacity for glucose metabolism, they also up-regulate genes involved in lipid metabolism. Such a diversification in fuels in memory NK cells may provide metabolic adaptability to support longevity and the greater energy demands for enhanced function upon re-activation. Taken together, a paradigm in which distinct metabolic fingerprints comprehensively distinguish and drive the range of NK cell functional fates warrants further exploration.

NK cell anti-tumor functions (67). Similar to uterine NK cells, TA-NK cells in a number of cancers have been shown to have poor cytotoxicity, but secrete the angiogenic factors VEGF and PlGF and, through a mechanism involving TGFβ, acquire a uNK-like $CD56^{bright}CD16^{-}$ phenotype (4, 5, 74). The liver is another site enriched in regulatory NK cells and anti-inflammatory cytokines including TGFβ and IL-10 (75, 76). Liver-resident NK cells have been shown to suppress the proliferation of T cells and B cells through their secretion of IL-10, and to inhibit the anti-viral activity of T cells through PD1/PDL1 engagement (77, 78). Moreover, the liver is predominantly hypoxic, with oxygen levels dipping as low as 1.3% in the healthy liver, which can be further exacerbated by infection and fibrosis (79–82). Hypoxia has been shown to be an important factor in limiting the anti-viral activity of NK cells in HCV+ patients, but does not affect their regulatory activity against liver fibrosis (82).

Hypoxia and anti-inflammatory cytokines stand out as hallmarks across environments that foster regulatory NK cell functions. The critical role of hypoxia and TGFβ in directly polarizing NK cells to a regulatory state was highlighted by a study that demonstrated that *in vitro* culture of cytotoxic pbNK cells in TGFβ+IL-15 under hypoxic conditions was sufficient to convert pbNK cells to regulatory NK cells that secreted high levels of VEGF and had poor cytotoxicity (69). The induction of regulatory NK cells by hypoxia and TGFβ suggests that there are vastly different metabolic requirements for regulatory NK cell functions compared to the glucose-driven glycolytic and respiratory requirements for cytotoxic functions. In contrast to the ability of regulatory NK cells to thrive in hypoxic

conditions and in line with the reliance of cytotoxic NK cells on glucose-driven OxPhos, hypoxia suppresses NK cell anti-tumor and anti-viral effector functions (82, 83). Studies have also demonstrated that TGFβ inhibits NK cell cytotoxic functions by suppressing glucose-driven glycolysis and OxPhos (63, 84). It follows then, that regulatory NK cell functions induced by TGFβ are not reliant on, and perhaps even inhibited by, elevated glucose metabolism. Further supporting this notion is a recent study which demonstrated that the tumor environment directly limits glycolysis in NK cells. The lung tumor microenvironment increased NK cell expression of fructose-1,6-bisphosphatase (FBP1), a rate-limiting enzyme in gluconeogenesis, which reduced glucose flux through glycolysis (85); thus, regulatory functions such as VEGF and PlGF production by TA-NK cells are unlikely to rely on elevated glucose metabolism. Another recent study demonstrated that in response to cytokine stimulation, regulatory liver-resident $CD56^{bright}$ NK cells had lower expression of the glucose transporter Glut1, but higher expression of the amino acid transporter CD98 and the CD71 transferrin receptor compared to pbNK cells (86). These findings support a model in which regulatory NK cells are less reliant on glucose metabolism compared to cytotoxic NK cells, and may utilize other fuels, such as amino acids and/or fatty acids, to support their functions (**Figure 2C**).

Indeed, the evidence to date indicates that dependence on glucose-driven glycolysis and OxPhos may be a central node in tipping the balance between cytotoxic and regulatory NK cell fate. If this proves true, these distinct metabolic fingerprints would consistently distinguish cytotoxic from regulatory NK cells, addressing the drawbacks encountered in phenotypically defining these subsets. Future studies investigating the metabolic parameters that govern regulatory NK cell functions will be imperative to establishing this paradigm.

Deep Breaths Bring Longevity—Mitochondrial Respiration at the Core of NK Cell Memory

The generation of memory NK cells requires a shift from the energy-intensive effector phase of the immune response to a contraction phase, characterized by a curtailment in proliferation and effector functions, resulting in the generation of resting long-lived memory NK cells. The ability of memory NK cells to mount a more robust and rapid effector response upon re-stimulation compared to naïve activated NK cells suggests an enhanced ability to draw upon energy reserves upon re-activation. Given the fundamental shifts between energy expenditure and quiescence between effector, contraction, and recall phases of an immune response, it is perhaps not surprising that metabolic changes have been found to be both necessary and sufficient to drive the formation and enhanced recall responses of other memory immune cells (29, 30, 87, 88).

In NK cells, restoring mitochondrial function following an effector response has been shown to be critical in the generation of memory NK cells during murine CMV infection. O'Sullivan et al. demonstrated that during the peak of the effector phase in response to MCMV, virus-specific NK cells developed reduced mitochondrial fitness, marked by a decrease in mitochondrial membrane potential and increase in ROS production. In contrast, the contraction phase was marked by the clearance of dysfunctional mitochondria in NK cells through autophagy, resulting in the restoration of mitochondrial membrane potential and reduced ROS. The clearance of dysfunctional mitochondria through autophagy was required for the generation and survival of MCMV-memory NK cells, as the inhibition of autophagy impaired the formation of the memory NK cell pool, while treatment with an ROS scavenger restored memory formation. Further, enhancement of autophagic activity through the inhibition of mTOR or activation of the metabolic regulator AMPK enriched the memory-NK cell population (23).

Enhanced mitochondrial fitness has emerged as a defining characteristic of HCMV-adaptive NK cells in humans (**Figure 2D**). NK cells from HCMV-seropositive donors had greater levels of maximal respiration, but not glycolysis, compared to NK cells from seronegative donors. Further, sorted $CD56^{dim}NKG2C^{+}$ HCMV-adaptive NK cells had comparable basal levels of glycolysis and OxPhos, but an increased capacity for these pathways, compared to donor-matched $CD56^{dim}NKG2C^{-}$ canonical NK cells. These findings indicate that adaptive NK cells have a greater ability to up-regulate glycolysis and OxPhos upon activation. The enhanced respiratory capacity of adaptive compared to canonical NK cells was supported by greater mitochondrial membrane potential and expression of genes involved in the electron-transport chain. The increased mitochondrial fitness of adaptive NK cells was found to be regulated by the chromatin-modifying protein ARID5B, and necessary for enhanced NK cell survival and effector function (22).

A question that remains to be addressed is which metabolic fuel(s) is/are primarily used by adaptive NK cells. The greater glycolytic capacity in HCMV-adaptive NK cells points to greater glucose metabolism. However, gene expression analysis of HCMV-adaptive NK cells also revealed an increase in genes involved in lipid catabolism (22). Interestingly, autophagy is a key regulator of lipid catabolism (89); thus, it is plausible that a diversification and flexibility in fuel sources contributes to the resilient longevity and recall effector response in HCMV-adaptive NK cells (**Figure 2D**).

Although the above studies have demonstrated a metabolic basis for the generation and function of memory NK cells, these have been limited to assessing adaptive NK cells in the context of CMV infection. While phenotypic variations span different memory NK cell subsets, the memory-traits of extended survival and enhanced recall effector responses, for which the metabolic adaptations are necessary in the context of CMV, are traits that remain consistent across memory NK cells. Thus it is likely that across memory NK cell subsets, increased mitochondrial fitness and fuel flexibility are broadly required to support longevity and that an enhanced respiratory capacity is necessary to derive the energy required for enhanced function upon re-activation. An important area of future study will be to verify that similar metabolic reprogramming

broadly drives the formation and function of other memory-NK cell subsets, including cytokine-induced memory-like NK cells, BCG-memory NK cells, and pregnancy-trained uterine NK cells.

CONCLUDING REMARKS

Research on NK cell biology is continuing to evolve and uncover a wider scope of NK cell functional fates. However, as knowledge on the complexity and plasticity of NK cells has grown, so too have incongruities in the classic phenotypic definitions of NK cell subsets. Classic NK cell phenotypes have undoubtedly served as an accessible measure for profiling NK cells and provide indications on NK cell activation state. Nevertheless, in many instances phenotype in fact confounds functional fates, as NK cells that express the same phenotype can have divergent functions, and NK cells with similar functional potential can express a range of phenotypic possibilities. Indeed, identifiable phenotypes that are sufficient to profile NK cell functional fates are lacking (**Figure 2A**). The shortcomings of phenotype in informing function expose a need to further search for what identifies NK cell fate and develop strategies to efficiently profile NK cells by these factors. Turning to a central hub that governs NK cell fate holds promise to address these drawbacks.

Metabolism has emerged as an intriguing factor to distinguish NK cell functional fates. The field of NK cell metabolism is still in early stages, but studies so far point to distinct metabolic profiles as drivers of NK cell functional potential which may thus serve as reliable fingerprints to identify functional fates (**Figures 2B–D**). A heightened capacity for glucose metabolism through glycolysis and OxPhos identifies NK cells with the greatest cytotoxic capacity. In contrast, regulatory NK cells thrive in hypoxic and glycolysis-limiting conditions, suggesting that regulatory functions can be effected with minimal levels of OxPhos and glycolysis. Longevity in its essence requires adaptability and endurance—memory NK cells are marked by enhanced mitochondrial fitness, achieved by the clearance of dysfunctional mitochondria, increased mitochondrial respiratory capacity and membrane potential, and reduced levels of damaging reactive oxygen species. Collectively, the evidence to date suggests a paradigm in which distinct metabolic fingerprints, but not phenotype, comprehensively distinguish and drive the range of NK cell functional fates (**Figure 2**).

Certainly, significant questions remain to fully delineate and validate this paradigm. For instance, what metabolic fuels and pathways are principally used by regulatory NK cells? Are low levels of glucose metabolism sufficient to support regulatory functions or do regulatory NK cells use an alternate fuel, such as fatty acids, as a more efficient means of energy generation in low oxygen conditions? Does enhanced mitochondrial fitness span all memory NK cell types? Are there metabolic pathways that further distinguish memory cytotoxic from memory regulatory NK cells? Are other factors, such as transcription markers and epigenetic modifications, required in conjunction with metabolism to determine NK cell fate? Such questions present exciting avenues for future research and will provide a more comprehensive understanding of the mechanisms and role of metabolism in tuning the spectrum of NK cell functions.

While metabolism is a promising means by which to identify NK cell fates, another critical question is whether it is achievable to efficiently profile NK cells based on metabolism. In this regard, similar to assessing classic phenotypic markers, a number of metabolic measures are frequently assessed via cytometry or microscopy, allowing for multiplex analyses that comprehensively measure a number of metabolic features. These include functional mitochondrial measures, such as mitochondrial mass, structure, membrane potential, ROS production, and oxidative stress (22, 29). Further, the relative activity of major metabolic regulators such as mTOR and AMPK are routinely measured by the phosphorylation status of these and their downstream targets (16, 56, 60, 90). Although not a direct measure of metabolic activity, the expression of cell surface nutrient receptors such as Glut1, CD71, and CD98 have been shown to reflect certain metabolic states in NK cells (16, 60). Finally, measuring the activities of key metabolic enzymes through cytometry has shown to be a valid and reliable means of assessing the metabolic configurations of immune cells and can additionally be accomplished *in situ* (91). With these applicable and efficient methods at hand, it will be imperative that future work characterize and validate combinations of metabolic measures that comprehensively indicate and discern the metabolic fingerprints of NK cell fates.

Given the critical and distinct roles that NK cells play in a host of diseases, therapeutically modulating NK cell function is garnering increasing attention. With distinct metabolic fingerprints at the heart of NK cell functional fates, profiling and modulating metabolism hold promise as powerful therapeutic strategies to predict and control NK cell fate.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Metabolic Signature of Macrophage Responses

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Macrophages are a heterogeneous population of immune cells playing several and diverse functions in homeostatic and immune responses. The broad spectrum of macrophage functions depends on both heterogeneity and plasticity of these cells, which are highly specialized in sensing the microenvironment and modify their properties accordingly. Although it is clear that macrophage phenotypes are difficult to categorize and should be seen as plastic and adaptable, they can be simplified into two extremes: a pro-inflammatory (M1) and an anti-inflammatory/pro-resolving (M2) profile. Based on this definition, M1 macrophages are able to start and sustain inflammatory responses, secreting pro-inflammatory cytokines, activating endothelial cells, and inducing the recruitment of other immune cells into the inflamed tissue; on the other hand, M2 macrophages promote the resolution of inflammation, phagocytose apoptotic cells, drive collagen deposition, coordinate tissue integrity, and release anti-inflammatory mediators. Dramatic switches in cell metabolism accompany these phenotypic and functional changes of macrophages. In particular, M1 macrophages rely mainly on glycolysis and present two breaks on the TCA cycle that result in accumulation of itaconate (a microbicide compound) and succinate. Excess of succinate leads to Hypoxia Inducible Factor 1α (HIF1α) stabilization that, in turn, activates the transcription of glycolytic genes, thus sustaining the glycolytic metabolism of M1 macrophages. On the contrary, M2 cells are more dependent on oxidative phosphorylation (OXPHOS), their TCA cycle is intact and provides the substrates for the complexes of the electron transport chain (ETC). Moreover, pro- and anti-inflammatory macrophages are characterized by specific pathways that regulate the metabolism of lipids and amino acids and affect their responses. All these metabolic adaptations are functional to support macrophage activities as well as to sustain their polarization in specific contexts. The aim of this review is to discuss recent findings linking macrophage functions and metabolism.

Keywords: macrophage, metabolism, inflammation, metabolic rewiring, immune cross-talk

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INTRODUCTION

From a historical perspective, macrophages ("*makros*" = big, "*phagein*" = to eat) were discovered in the 19th century by the Russian zoologist Metchnikoff, in a seminal study on starfish larvae. Metchnikoff observed that few hours after pinning them with small thorns of a tangerine tree, the thorns were surrounded by cells that he supposed to have origin from blood in response

to injury (1). Macrophages were found in tissues as resident cells patrolling their surroundings and removing invading pathogens, apoptotic cells, and debris, thus maintaining tissue integrity. The first hypothesis was that tissue macrophages may differentiate from monocytes that exit the bloodstream during inflammation. However, it is now established that while monocyte-derived macrophages have origin in the bone marrow by definitive haematopoiesis, tissue macrophage progenitors derive from yolk sac and fetal liver, during primitive and definitive haematopoiesis (2). Interestingly, embryo-derived macrophages retain self-renewal potential, whereas monocyte-derived cells are terminally differentiated (3).

Despite of these differences, it is clear that both monocyte-derived and tissue-resident macrophages play a pivotal role in the maintenance of tissue homeostasis and in tissue regeneration after injury. In humans for example, the tissue cellular turnover rate has been estimated to be more or less 1 million cells per second each day (4): the removal of apoptotic cells is constantly provided mainly by macrophages that reside in tissues, through an immunologically silent process known as efferocytosis. One of the hallmarks of this process is represented by the release of anti-inflammatory cytokines that prevent the development of inflammation, such as interleukin (IL) 10 and transforming growth factor beta (TGF-β) (5, 6). Indeed, defects in the clearance of apoptotic cells are directly linked to the development of inflammation and autoimmune diseases (4).

On the other hand, when an inflammatory process is triggered by the perturbation of tissue homeostasis, bone-marrow derived monocytes that circulate in the blood-stream are attracted to the site of inflammation, through a specific milieu of pro-inflammatory chemokines secreted by resident macrophages, stromal and endothelial cells. At the site of inflammation, monocytes differentiate into macrophages, which cooperate with resident cells for sustaining immunity or promoting resolution of inflammation and tissue regeneration (7).

THE TWO POLES OF MACROPHAGES ACTIVATION

Macrophages are extremely plastic cells being able to change rapidly their functional profile through a process defined as polarization. Macrophage polarization is indeed the process by which macrophages respond to stimuli coming from the local microenvironment and acquire a specific functional phenotype.

Based on specific programs of gene expression leading to the acquisition of different markers on the cellular surface, the secretion of certain cytokines as well as to metabolic adaptations, macrophages are usually classified into classically activated, pro-inflammatory or M1 macrophages (8, 9), and alternatively activated, anti-inflammatory, or M2 macrophages (10, 11). A classification of the different phenotypes is reported in **Table 1**.

Pro-inflammatory macrophages are induced by microbial products, such as the lipopolysaccharide (LPS) and other Toll-like receptors (TLRs) ligands, or by cytokines secreted by T_H-1 lymphocytes, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). From the functional point of view, M1 macrophages are characterized by their ability to kill pathogens and present their antigens to T lymphocytes for initiation of adaptive responses. Thus, they express CD80, CD86, CIITA, major histocompatibility complex class II receptor (MHC-II), cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) and they produce high levels of pro-inflammatory cytokines, such as TNF-α, IL1-β, IL-6, IL-12, and IL-23, and promote T_H-1 responses [extensively reviewed in (12, 13)]. The expression of these cytokines is mainly controlled by the activation and nuclear translocation of the transcription factor NF-κB (nuclear factor kappa-light-chain enhancer of B-cell) (14, 15), together with STAT1 (Signal transducer and activator of transcription) (16, 17), STAT3 (18), IRF4 (IFN-γ regulatory factor) (19), HIF1α (Hypoxia induced factor 1 alpha), and AP1 (activator protein 1) (20).

M2 or anti-inflammatory macrophages are induced by IL-4 or IL-13 secreted by innate and adaptive immune cells, such as mast cells, basophils, and T_H-2 lymphocytes (10, 11). Alternatively-activated macrophages are characterized by an anti-inflammatory profile, which permits resolution of inflammation and tissue repair. They express high levels of mannose receptor (CD206), the decoy receptor IL-1R as well as the IL-1R antagonist, and produce pro-fibrotic factors such as the transforming growth factor beta (TGF-β) and insulin-like growth factor 1 (IGF-1), thus actively suppressing inflammation and promoting repair (21). In addition, markers and effectors associated with M2 polarization include STAT6, GATA3 (GATA binding protein 3), SOCS1 (suppressor of cytokine signaling 1), PPARγ (peroxisome proliferator-activated receptor gamma), CD163, CD36, FIZZ1 (found in inflammatory zone 1), matrix metalloproteases (MMPs), and arginase 1 (ARG1) (22). The increased arginase activity results in production of polyamines and collagen and favors tissue remodeling and wound healing (21). Finally, M2 macrophages induce angiogenesis and lymphangiogenesis by producing vascular endothelial growth factor A (VEGF-A), endothelial growth factor (EGF), platelet-derived growth factor (PDGF), and IL-8 (23).

In addition to this phenotype induced by IL-4/Il-13 (also known as M2a), specific profiles of M2 macrophages may be induced by different stimuli, including TGFβ, IL-10, immune complexes, or glucocorticoids (24). Thus, M2b or regulatory macrophages–induced by stimulation with immune complexes and TLR ligands or by IL-1R agonists–produce both pro- and anti-inflammatory cytokines, such as IL-10, IL-1β, and TNF-α, and regulate both immune and inflammatory reactions; on the other hand, the M2c subset is activated by glucocorticoids or IL-10 and exhibits a strong anti-inflammatory profile by releasing IL-10 and TGF-β. Finally, M2d macrophages, also known as tumor-associated macrophages (TAMs), are induced by TLR ligands and A2 adenosine receptor (A2R) agonists, or by IL-6; they secrete high levels of IL-10, TGF-β, and VEGF and low IL-12, TNF-α, and IL-1β, and contribute to tumor angiogenesis, growth and metastasis (25).

Considering the complexity of the tissue microenvironment and the plasticity of macrophages, it is clear that a static vision of M1–M2 polarization adopted from *in vitro* experiments may not

TABLE 1 | A schematic summary of macrophage polarization.

Polarization	Stimuli	Released cytokines	Surface markers	Metabolic enzymes	Transcription factors	Functions
M1	LPS + IFN-γ	TNF-α, IL-1β, IL-6, IL-12, IL-23	CD80, CD86, CIITA, MHC-II	iNOS, PFKFB3, PKM2, ACOD1	NF-κB (p65), STAT1, STAT3, IRF-4, HIF1α, AP1	Bacterial killing, tumor resistance, Th1 response
M2a	IL-4/IL-13	IL-10, TGF-β	CD206, CD36, IL1Ra, CD163	ARG1, CARKL	STAT6, GATA3, SOCS1, PPARγ	Anti-inflammatory response, tissue remodeling, wound healing
M2b	IC, TLR ligands/IL-1Ra	IL-10, IL-1β, IL-6, TNF- α	CD86, MHC II	ARG1, CARKL	STAT3, IRF4, NF-κB (p50)	Tumor progression, immunoregulation, Th2 response
M2c	Glucocorticoids/IL-10	IL-10, TGF- β	CD163, TLR1, TLR8	ARG1, GS	STAT3, STAT6, IRF4, NF-κB (p50)	Phagocytosis of apoptotic bodies, tissue remodeling, immunosuppresion
M2d (TAM)	TLR ligands + A2R/IL-6	IL-10, VEGF	CD206, CD204, CD163	ARG1, IDO	STAT1, IRF3, NF-κB (p50)	Angiogenesis, tumor progression

A2R, adenosine receptor 2; ACOD1, aconitate decarboxylase 1; AP-1, Activator proten 1; ARG1, Arginase 1; CARKL, carbohydrate kinase- like; IC, immunocomplexes; IDO, indoleamine dioxygenase; iNOS, inducible Nitric Oxide Synthase; GATA3, GATA binding protein 3; GS, glutamine synthetase; HIF1α, Hypoxia-inducible factor 1-alpha; IFN-γ, Interferon gamma; IL-, interleukin; IRF, interferon regulatory factor; MHC-II, major histocompatibility complex class 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PPARγ, Peroxisome proliferator-activated receptor gamma; SOCS1, Suppressor of cytokine signaling 1; STAT, Signal transducer and activator of transcription; TNF-α, Tumor necrosis factor alpha; TGF-β, transforming growth factor beta; TLR, toll like receptor; VEGF, Vascular endothelial growth factor.

fully describe macrophage polarization *in vivo*, which has to be considered as an extremely dynamic and tissue-specific process.

MACROPHAGE METABOLISM

In addition to the functional properties mentioned above, macrophage polarization involves also metabolic reprogramming (**Figure 1**). Thus, depending on the stimuli received by the microenvironment, macrophages can switch from an aerobic profile, based on oxidative phosphorylation, to an anaerobic one, based on glycolysis, and vice versa.

The first studies in the field of immune cell metabolism appeared in the 1950s, with the discovery that neutrophils depend on aerobic glycolysis, a process defined as "Warburg effect" (26). Indeed, this metabolic pathway was first recognized by Otto Warburg, during his research on tumor cells, which are characterized by increased glucose uptake, high rate of glycolysis, followed by lactic acid fermentation in conjunction with a reduced level of oxidative phosphorylation (OXPHOS), even in the presence of abundant oxygen. In this setting, aerobic glycolysis occurs to produce energy and to generate biosynthetic intermediates (26, 27). In 1970, Hard et al. observed that M1 macrophages display enhanced glycolysis accompanied by decreased oxygen consumption (28, 29). Almost 20 years later, Newsholme et al. demonstrated that the rate of glycolysis increased dramatically during phagocytosis or upon macrophage activation by inflammatory stimuli. Indeed, now we know that pro-inflammatory macrophages utilize glycolysis (29, 30) and the pentose phosphate pathway (PPP) (31, 32) to meet their ATP requirements, whereas the Krebs cycle is broken at two points (32, 33), and OXPHOS as well as the fatty acid oxidation (FAO) are downregulated (32, 34, 35). In contrast, in M2 macrophages the Krebs cycle is intact and their metabolic activity is characterized by enhanced FAO and OXPHOS (32).

Starting from these and other observations, the concept of "immunometabolism" has been introduced to indicate that, in addition to provide energy supporting immune activity in specific contexts, these metabolic adaptations directly affect immune cell functions by controlling transcriptional and post-transcriptional events. In the next paragraph we will describe the main metabolic blocks and the modulation of their fluxes for sustaining the different functional states of macrophages.

GLYCOLYSIS AND THE PENTOSE PHOSPHATE PATHWAY (PPP)

Glycolysis is one of the simplest ways to generate energy within the cell (**Figure 2**). The glycolytic metabolic pathway takes place in the cytosol and it converts glucose to pyruvate, thus generating two molecules of ATP per unit of glucose. Although glycolysis is relatively inefficient in ATP production, it provides metabolic intermediates for biosynthetic pathways to support the synthesis of ribose, amino acids, and fatty acids that are crucial for metabolic adaptation of the cell. Furthermore, glycolysis supplies the PPP, allowing the production of NADPH and ribose-5-phosphate. In parallel to glycolysis, PPP occurs in the cytosol and consists of two distinct phases. In the oxidative phase, the energy from metabolic conversion of glucose-6-phosphate into ribulose-5-phosphate is used for the reduction of $NADP^+$ into NADPH. NADPH is then used by several enzymes, including the NADPH oxidase, which generates reactive oxygen species (ROS) to kill pathogens and plays a crucial role in macrophage responses (36, 37). Moreover, high levels of NADPH offer protection against oxidative stress, by providing reducing power for generation of

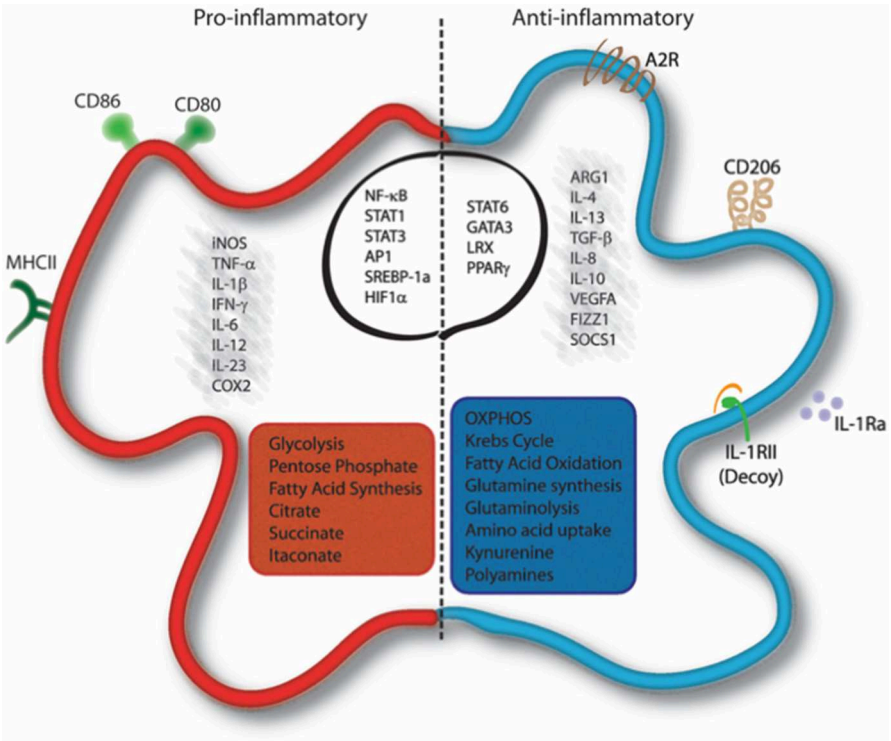

FIGURE 1 | Molecular and metabolic signatures of macrophage activation. Pro-inflammatory stimuli induce the activation of specific pathways through the activation of transcription factors such as NF-κB, STAT1, STAT3, AP-1, SREBP-1, and HIF1α, which trigger the expression of markers like iNOS, COX-2, CD80, CD86, and MHC-II and the release of IL-1β, TNF-α, IFN-γ, IL-6, IL-12, and IL-23. Cells undergo a metabolic reprogramming toward glycolysis, the pentose-phosphate pathway, and fatty acid synthesis. This associates to interruption of the Krebs cycle, ROS formation and efflux of citrate, which supports NADPH and PGE2 synthesis, and succinate, which stabilizes HIF-1α. Itaconate is produced from citrate and displays antibacterial function. Anti-inflammatory macrophages are characterized by the expression of ARG1, FIZZ1, SOCS1, CD206, Adenosine receptor (A2R), and the decoy IL1RII and by the production of cytokines such as TGF-β, IL-10, IL-4, IL-13, IL-8, IL-1Ra, and VEGFA. Their profile is mainly controlled by the activity of the transcription factors STAT6, GATA3, PPARγ, and LRX. Metabolically, these cells display enhanced OXPHOS metabolism, fatty acid oxidation, glutaminolysis, tryptophan catabolism with release of kynurenine, and synthesis of polyamines. AP-1, Activator proten 1; ARG1, Arginase 1; COX2, cicloxygenase 2; FIZZ1, Found in inflammatory zone 1; iNOS, inducible Nitric Oxide Synthase; GATA3, GATA binding protein 3; HIF1α, Hypoxia-inducible factor 1-alpha; IFN-γ, Interferon gamma; LXR, Liver X receptor; MHC-II, major histocompatibility complex class 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PPARγ, Peroxisome proliferator-activated receptor gamma; SOCS1, Suppressor of cytokine signaling 1; SREBP-1, Sterol regulatory element binding protein 1; STAT, Signal transducer and activator of transcription; TNF-α, Tumor necrosis factor alpha; TGF-β, transforming growth factor beta; VEGFA, Vascular endothelial growth factor A.

the antioxidant glutathione (38). In the non-oxidative phase, intermediates from glycolysis are diverted for the synthesis of ribose-5-phosphate, a precursor of nucleotides, and amino acids.

Glycolysis is a crucial metabolic event for M1 macrophages and its inhibition affects many functions typical of their inflammatory phenotype, including phagocytosis, ROS production, and secretion of pro-inflammatory cytokines (29, 39, 40). Glycolytic metabolic adaptation relies on the activation of several transcription factors, among which HIF1α plays a key role in the commitment to glycolysis also under normoxic conditions (41).

In macrophages, two main signaling pathways culminate in oxygen-independent regulation of HIF1α transcription: the TLR/NF-κB (42) and AKT/mTOR (43–45) pathways. Several inflammatory signals, such as pathogen recognition through pattern recognition receptors (PRRs) or pro-inflammatory cytokines, converge in NF-κB activation, the master regulator of macrophage functions that regulates the expression of several genes, including HIF1α (46, 47). On the other hand, the AKT/mTOR pathway is triggered by growth factors, such as GM-CSF, and pathogen-sensing receptors, such as Dectin-1 or TLR4 (45, 48, 49). Interestingly, mTORC1 also increases the expression of genes involved in mitochondria biogenesis and oxidative metabolism, such as PPAR-γ and Yin Yang 1 (YY-1) (50). On this line, Akt kinases seem to regulate macrophage polarization in an isoform-specific manner: while Akt1 deletion promotes the M1 profile, deletion of Akt2 has opposite effects, resulting in amplification of M2 responses (44). In addition to these signaling pathways, in M1 cells HIF1α expression may also be stabilized by succinate coming from the TCA breakpoint at succinate dehydrogenase (SDH) (31) (see "Krebs cycle" section).

In macrophages, HIF1α acts as a metabolic and functional regulator of cell responses, regulating the expression of genes encoding for glycolytic enzymes, the glucose transporter GLUT1, as well as inflammatory mediators (41, 42, 47). The upregulation of GLUT1 is important for the glycolytic activity

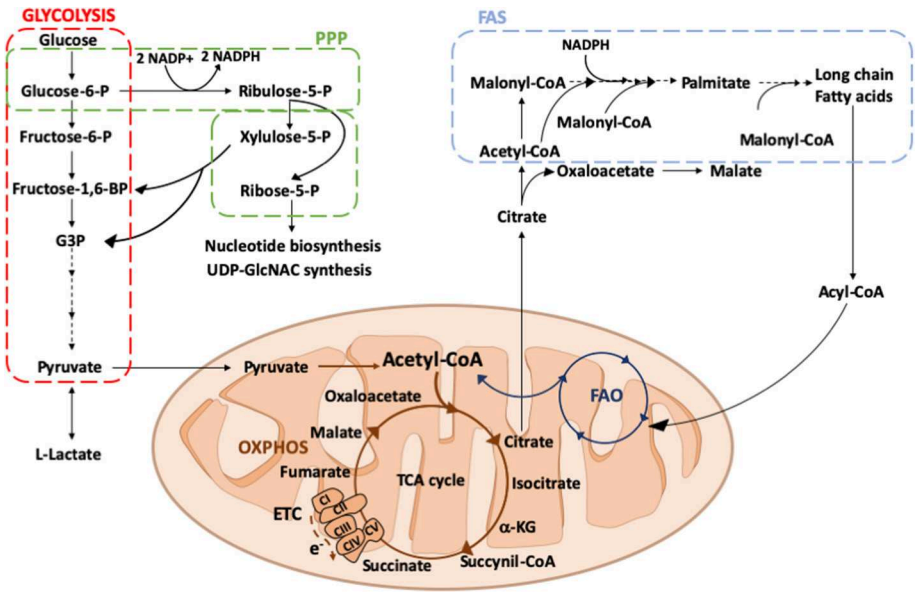

FIGURE 2 | Overview of glucose and fatty acid metabolism. Glucose is converted into pyruvate by glycolysis (red square), in the cytosol. Among the glycolytic intermediates, glucose-6P can be diverted into PPP (green square) sustaining NADPH and ribose-5P production that, in turn, are used for fatty acid or nucleotide and UDP-GlcNAC synthesis, respectively. In hypoxic conditions, pyruvate is preferentially reduced to lactate, whereas in normoxic conditions it is decarboxylated into acetyl-CoA within the mitochondria. Here, acetyl-CoA enters into the TCA cycle, providing reducing agents to the ETC to generate energy. Citrate, an intermediate of the TCA cycle, can be exported into the cytosol where it participates in fatty acid synthesis (FAS; light blue square). Fatty acids can be oxidized via FAO (dark blue square) within the mitochondrial matrix thus generating acetyl-CoA to replenish the TCA cycle. PPP, pentose phosphate pathway; ETC, electron transport chain; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation; FAS, fatty acid synthesis; FAO, fatty acid oxidation; Glucose 6-P, glucose 6-phosphate; Fructose 6-P, fructose 6-phosphate; Fructose-1,6-BP, fructose 1-6-biphosphate; G3P, glyceraldeyde 3-phospate; Acetyl-CoA, acetyl-Coenzyme A; α-KG, alpha-ketoglutarate; e-, electrons; CI, CII, CIII, CIV, CV, complex I, II, III, IV, V; UDP-GlcNAC, Uridine diphosphate N-acetylglucosamine.

of M1 macrophages as it facilitates rapid glucose uptake (29). Additionally, HIF1α supports the conversion of pyruvate into lactate by promoting the expression of two enzymes: the lactate dehydrogenase (51), which produces lactate from pyruvate, and the pyruvate dehydrogenase kinase (52, 53), which inactivates pyruvate dehydrogenase thus limiting pyruvate entering into the Krebs cycle. In M1 macrophages, in which OXPHOS is limited, the conversion of pyruvate into lactate is essential to restore NAD^+ and maintain flux through the glycolytic pathway.

Two additional points of the glycolytic flux regulation occur at the level of the 6-phosphofructo-2-kinase B (PFKFB) and the pyruvate kinase M2 (PKM2). M1 macrophages express predominantly the PFKFB3 isoform (53) which, if compared to the other isoforms, less efficiently catalyzes the conversion of fructose-2,6-bisphosphate in fructose 6-phosphate, enhancing the glycolytic flux. Moreover, M1 cells upregulate the isoform 2 of the pyruvate kinase (PKM2), which plays multiple roles in macrophage metabolism and polarization. Indeed, when highly expressed, PKM2 exists in an equilibrium of enzymatically inactive monomers or dimers and enzymatically active tetramers (53). The inactive enzyme translocates into the nucleus and, by binding to HIF1α, triggers the expression of HIF1α-regulated genes (53–56), whereas the enzymatically active tetramers are retained in the cytoplasm and promote glycolysis as well as M1 polarization (53).

As mentioned above, the oxidative steps of the PPP are crucial for macrophages: oxidation of glucose leads to the reduction of $NADP^+$ to NADPH, which is fundamental not only for NADPH oxidase and macrophage's killing activity but also for anti-oxidant defense mechanisms and fatty acid biosynthesis, required for prostaglandin production. Indeed, the oxidative PPP activity is prominent in M1 macrophages (31) and the knockdown of 6-phosphogluconate dehydrogenase (PGD), which converts 6-phosphogluconate into ribulose 5-P, generates a deficient pro-inflammatory response in macrophages during hypercholesterolemia (57). On the other hand, as expected, the non-oxidative branch of PPP is repressed in M1 macrophages (32). This occurs through the downregulation of sedoheptulose kinase (CARKL), a carbohydrate kinase-like protein that is involved in the conversion of sedoheptulose into sedoheptulose-7-phosphate (58). In line with this finding, overexpression of CARKL in macrophages results in defective M1 polarization and dampened inflammatory response (57, 58).

The role of glycolysis in M2 macrophage functions is more controversial. Several studies have shown that glycolysis is active in M2 cells and that its blockade with 2-deoxyglucose (2-DG), a well-established glycolysis inhibitor, may inhibit M2 polarization and functions (19, 59). On the other hand, more recent data suggest that glycolysis is not required for M2 differentiation, as long as OXPHOS remains intact (60). This suggests that M2 macrophages display a more flexible

metabolic activity since they can supply OXPHOS even in absence of glycolysis using glutamine (60). Another control point of glycolysis in M2 macrophages is represented by the selective expression of the glycolytic enzyme 6-phosphofructo-2-kinase B1 (PFKFB1), which much more efficiently catabolizes fructose-2,6-bisphosphate, an activator of glycolysis, to fructose-6-phosphate, lowering the glycolytic rate (61, 62). Finally, in the M2 phenotype, CARKL is upregulated, enhancing the non-oxidative steps of PPP, which can lead to ribose-5P production, necessary for nucleotide and UDP-GlcNAC synthesis (58). UDP-GlcNAC is required for N-glycosylation, which is essential for the modification of different cell surface proteins (i.e., CD206) abundantly expressed in M2 macrophages (32).

The metabolic differences between M1 and M2 cells impact on the ability of these cells to generate ROS. In a situation of coupled and efficient respiration, the amount of ROS produced by the electron transport chain (ETC) is kept under control and at low levels. In conditions of OXPHOS dysfunction, a significant leakage of electrons occurs, which, in the presence of oxygen, produces ROS (63, 64). This is the case of pro-inflammatory macrophages, in which polarization profoundly modifies the OXPHOS, leading to ROS production. Although CI and CIII are considered the main sites of mitochondrial ROS production, recent studies suggest that ROS are generated by reverse electron transport (RET) at CI of the ETC rather than CIII, in a situation of impaired OXPHOS (65). The evolutionary conserved signaling intermediate in Toll pathways (ECSIT), a TRAF6 target for ubiquitination and CI-associated protein, is a master regulator of ROS production and mitochondrial quality control in macrophages. In particular, after phagocytosis of bacteria, ECSIT triggers recruitment of mitochondria to the phagosome to produce ROS that activate NADPH oxidase to kill bacteria (63). Other than being a harmful byproduct of metabolism, cellular ROS have emerged as master regulators of cellular signaling through the activation of many redox-sensitive pathways [extensively reviewed in (66)]. In macrophages, ROS are known to regulate several functions, including phagocytosis, bacterial killing, and polarization into specific phenotypes. Mitochondrial ROS are known to sustain inflammation by mediating IL-6, TNF-α, and IL-1β cytokine secretion, through a mechanism involving mitochondrial ROS-dependent MAPK activation (67).

THE KREBS CYCLE

Once pyruvate is generated, it becomes oxidized through a series of reactions termed the Krebs or Tricarboxylic Acid (TCA) cycle (**Figure 2**). The continuous flux through this cycle utilizes acetyl CoA, deriving from the breakdown of carbon-based nutrients, to reduce NAD^+ and FAD to NADH and $FADH_2$, which are then oxidized leading to ATP production. M2-like macrophages are known to display a functional and intact TCA cycle, which is crucial to meet the ATP demand due to the high (UDP-GlcNAc requiring) glycosylation levels of lectin and mannose receptors necessary for M2 macrophage function (32). At variance with M2 metabolism, the increased flux of glycolysis displayed by M1 macrophages is accompanied by metabolic changes involving the Krebs cycle, which are not only important for anabolic or energetic purposes, but also for sustaining the inflammatory response (68–70). Indeed, in pro-inflammatory macrophages, the Krebs cycle is interrupted at several key points allowing signal metabolites citrate, succinate and itaconate to escape mitochondria and exert their regulatory role.

Citrate

Citrate production and conversion connects mitochondrial and cytosolic metabolism. It is produced in the Krebs cycle by condensation of oxaloacetate and acetyl-CoA, the latter deriving from glycolytic pyruvate or from the catabolism of fatty acids. Citrate is converted to isocitrate and then to α-ketoglutarate (αKG), through the activity of isocitrate dehydrogenase (IDH). However, citrate can also be exported into the cytosol in exchange with malate through the transport activity of the mitochondrial citrate carrier (CIC), also known as solute carrier family 25 member 1 (SLC25A1) (71, 72). Once in the cytosol, citrate displays a plethora of regulatory roles. It inhibits glycolysis, by acting directly on phosphofructokinase (PFK) 1 and 2 and, indirectly, on pyruvate kinase (PK) (73); it stimulates lipid synthesis, through the activation of acetyl-CoA carboxylase (ACC) (74), and gluconeogenesis, through the activation of fructose-1,6-bisphosphatase. Cytosolic citrate is also a substrate of ATP-Citrate lyase (ACLY), producing acetyl-CoA and oxaloacetate (72). Oxaloacetate can be converted to malate by malate dehydrogenase (MDH). Malate can be transported back into the mitochondrial matrix in exchange with citrate through CIC (75) or can lead to pyruvate through the NADPH producing-malic enzyme (76). Through ACLY activity, cytosolic citrate positively regulates protein and histone acetylation (77).

Because of its major role in controlling cell metabolism, citrate plays a crucial role in sustaining macrophage inflammatory response (**Figure 3**). M1 macrophages are characterized by accumulation of citrate due to two main transcriptional changes, such as downregulation of IDH (32) and upregulation of the mitochondrial citrate carrier CIC (71), leading to citrate withdrawal from mitochondria. CIC upregulation occurs in response to LPS, TNF-α, or IFN-γ stimulation. Both these events are responsible for the first interruption of the Krebs cycle and accumulation of citrate in the cytosol of M1 macrophages, which is crucial for NO, ROS, and prostaglandin E2 (PGE2) production (71, 78). Pharmacological or genetic targeting of CIC in human macrophages results in decreased levels of these inflammatory mediators (79), suggesting that citrate export supports fatty acid synthesis (FAS) on which PGE2 synthesis relies as well as the reduction of $NADP^+$ to NADPH, necessary for NO and ROS production, by means of the activity of the malic enzyme. CIC acetylation is required to additionally boost mitochondrial citrate export in macrophages activated in conditions of glucose deprivation; in this manner, the NADPH demand, that cannot rely on PPP, may be met through the NADPH–producing conversion of citrate into 2-ketoglutarate, catalyzed by the cytosolic $NADP^+$-dependent IDH (IDH1) (80).

Another crucial role of citrate is in providing, through conversion to acetyl-CoA, the acetyl moiety for the acetylation

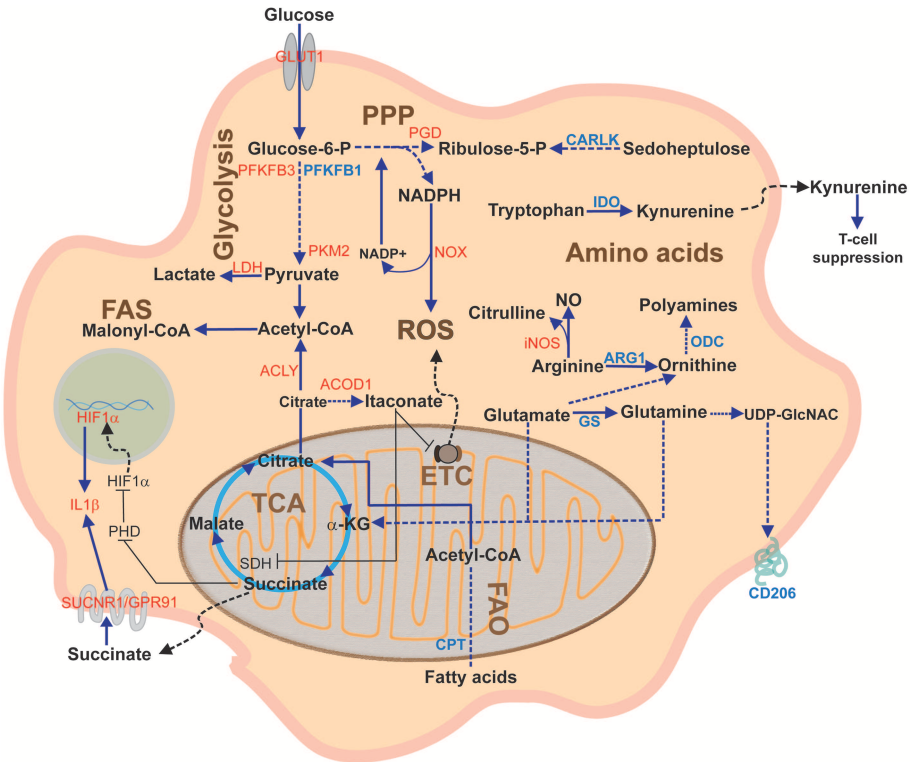

FIGURE 3 | Overview of macrophage metabolic pathways. This diagram depicts the main macrophage pathways during classical and alternative polarization and their components. In red, proteins upregulated in pro-inflammatory (M1) activation; in blue, proteins upregulated during anti-inflammatory (M2) activation. α-KG, alpha-ketoglutarate; ACLY, ATP citrate lyase; ARG1, arginase1; CARLK, *carbohydrate kinase-like* protein; CPT, carnitine palmitoyl transferase; ETC; Electron Transport Chain; FAO Fatty acid oxidation; FAS, Fatty acid synthesis; GS, glutamine synthetase; GLUT1, glucose transporter 1; IDH, Isocitrate dehydrogenase; IDO, indoleamine dioxygenase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; NO, nitric oxide; NOX, NADPH oxidase; ODC, ornithine decarboxylase; PGD, phosphogluconate dehydrogenase; PHD, prolyl hydroxylase; PPP, Pentose phosphate pathway; PFKFB3, phosphofructokinase fructose 2,6-biphosphatase B3; PKM2, pyruvate kinase M2; ROS, Reactive Oxygen Species; SDH, Succinate dehydrogenase; SUCNR1, succinate receptor 1; TCA, Tricarboxylic acid cycle or Krebs cycle.

of proteins, which is known to regulate protein function at multiple levels (81). Protein acetylation requires the presence of acetyl-CoA in different cellular compartments and it relies on the activity of ATP citrate lyase (ACLY), which converts citrate in acetyl-CoA. Similarly to CIC, ACLY is upregulated in M1 macrophages (78) and its activity regulates the expression of many genes through histone acetylation (82). Although no specific studies address the role of ACLY in regulating epigenetic changes in M1 macrophages, many enzymes and proteins are known to be affected by acetylation (83), among which NF-κB (84), IL-6, and IL-10 (85, 86). In M2 macrophages ACLY is regulated by the Akt-mTORC1 axis, leading to histone acetylation and induction of some M2 genes (87). However, a recent study has shown that polarization of human macrophages toward an M2 phenotype does not require ACLY (88).

Itaconate

Itaconate is produced from cis-aconitate in the Krebs cycle in classically activated macrophages (89, 90). This occurs through a strong upregulation of the enzyme aconitate decarboxylase 1 (ACOD1), originally called immune-responsive gene 1 protein (IRG1) (91). In this pathway, cis-aconitate is withdrawn from the Krebs cycle to produce this metabolite. Interestingly, upregulation of ACOD1 has been reported not only in cell lines and murine M1 macrophages, but also in septic patients (92).

The well-known anti-bacterial properties of itaconate rely on its ability to inhibit the bacterial isocitrate lyase and its bactericidal properties against gram-positive and gram-negative bacteria (93–95). In addition, itaconate may play a role in immunomodulation, suppression of inflammation and tolerance (96).

Itaconate was shown to inhibit SDH, leading to accumulation of succinate in LPS activated macrophages [(27–35); **Figure 3**], and this was associated to reduced mitochondrial respiration, ROS production, proinflammatory cytokine release, and inflammasome activation (96). The mechanism by which itaconate induces these metabolic and functional changes in macrophages was recently elucidated by Mills et al.: itaconate contributes to stabilize the levels of the anti-inflammatory transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), which targets genes involved in anti-inflammatory and anti-oxidant response (97). Itaconate mediates a post-translational alkylating modification on –SH groups of Kelch-like ECH-associated protein 1 (KEAP1), causing its fast degradation.

Since KEPA1 targets NRF2 for proteasomal degradation (98), its itaconate-mediated degradation allows NRF2 to translocate to the nucleus, leading to transcription of genes involved in protection against stress-induced cell death and oxidative stress. Concomitantly, NRF2 suppresses the expression of genes encoding IL-1β and IL-6 (99).

The chemical features of itaconate, particularly its electrophilicity, make the molecule reactive toward the cysteine groups of glutathione and proteins. It has been speculated that itaconate could trigger the electrophilic stress response (ESR), by modifying –SH residues of proteins and depauperating the cell from glutathione (100). It is then conceivable that other targets, besides the KEAP1–NRF2 axis, that are known to sense ESR, could be influenced by itaconate.

With respect to M2 macrophages, the role of itaconate has not been clearly elucidated. When M2 macrophage differentiation is impaired, IRG1 expression increases and itaconate accumulates in macrophages (101), probably as a compensatory effect. Furthermore, itaconate has been identified as a key player in the microRNA miR93, IRF9, IRG1 axis during macrophage polarization. In particular reduction of itaconate levels favors M2-like polarization in macrophages and this might be ascribed to the release of the SDH brake improving OXPHOS flux (102).

Succinate

Succinate is an intermediate of the Krebs cycle produced from succinyl-CoA. It is the substrate of SDH, which is part of Complex II of the mitochondrial respiratory chain. SDH-mediated oxidation of succinate into fumarate is coupled to reduction of ubiquinone (UQ) to ubiquinol (UQH2). When high amounts of succinate are oxidized to fumarate in conditions of no ATP production, electrons flux in the opposite direction toward complex I, leading to reverse electron transport (RET). This associates to a significant release of ROS, which can activate HIF1α in M1 macrophages (65, 103).

In addition to its role as metabolic intermediate, succinate works as a signaling molecule in many ways. Succinate is transported into the cytosol by the activity of the dicarboxylate carrier (DIC), also known as solute carrier family 25 member 10 (SLC25A10) (72). Succinate influences HIF1α stability by inhibiting prolyl hydroxylases (PHDs), a class of αKG-dependent dioxygenases that regulate HIF1α stability in an oxygen-dependent manner, thus blocking HIF1α degradation in the presence of oxygen [(31); **Figure 3**].

High cytosolic succinate levels favor post-translational lysine succinylation on proteins, a process that profoundly modifies protein functions since it alters their charge and structure (104, 105). In the case of pyruvate kinase M2, succinylation promotes its translocation into the nucleus, where it interacts with HIF1α to boost IL-1β transcription (106). In sirtuin 5-deficient mice, pyruvate kinase M2 hyper-succinylation has been described as a strategy to sensitize mice to experimental colitis due to the increased IL-1β production (106).

Succinate exerts signaling roles also acting at the extracellular level. During inflammation, succinate is released by inflammatory macrophages and can accumulate into the extracellular milieu (107), as observed in murine ischemic or hypoxic tissue (108–110), inflammation of the central nervous system (111), as well as in biological fluids of rheumatoid arthritis patients (112). Once outside the cell, it can bind to the succinate receptor SUCNR1/GPR91, a G-protein–coupled cell surface sensor for extracellular succinate (113) expressed in many cell types, that is known to be activated in diabetic retinopathy (108), diabetic renal disease (114), hypertension (113, 115), and atherothrombosis (116). Interestingly, macrophages express GPR91 and, in response to inflammatory signals like LPS, they activate a GPR91-mediated signal transduction that sustains the proinflammatory phenotype and leads to IL-1β production [(117, 118); **Figure 3**]. This represents a novel mechanism by which succinate fuels inflammation in a autocrine manner to sustain and amplify the inflammatory response (118). Interestingly, in an *in vivo* model of experimental autoimmune encephalomyelitis (EAE), GPR91 expressed by transplanted neural stem cells exerted a protective role against neuroinflammation which was mainly due to their scavenging effects and reduction of the succinate levels in the cerebrospinal fluid (111).

AMINO ACID METABOLISM IN THE INNATE IMMUNITY

Amino acid availability is essential to mount a proper immune response. During inflammatory or immune reactions, amino acid deficiency may result in defective immune cell migration, division, maturation, and completion of effector functions. Macrophage adaptation to rapidly changing nutrient sources implicates exploiting amino acid catabolism to sustain activation and maintenance of their immune activity. Amino acid availability controls several pathways governing macrophage responses, including mTOR signaling and NO production. Moreover, altered amino acid metabolism can influence macrophage responses by generating catabolites with immunomodulatory properties. Finally, the metabolic competition or cross talk between host immune cells and pathogens may affect the evolution of an infection.

Arginine represents the best example of how a strict metabolic regulation can drive opposite phenotypes, depending on which metabolic pathway is engaged (**Figure 3**). Under pro-inflammatory stimuli, such as LPS, TNF-α, or IFN-γ, iNOS (also known as NOS2) is overexpressed, channeling arginine catabolism toward NO and citrulline production. NO production is functional to boost macrophage anti-microbial activity: NO spontaneously reacts with oxygen or ROS to produce reactive nitrogen and oxygen intermediates that lead to the formation of a variety of antimicrobial species (119). Most importantly, NO prevents M1 to M2 repolarization, since the blockade of iNOS gives to M1 macrophages the ability to repolarize into M2, when exposed to IL-4 after LPS + IFN-γ treatment (120). On the other hand, citrulline produced by iNOS is used by argininosuccinate synthase 1 to produce argininosuccinate, which is promptly broken to recover arginine and sustain NO production (121). In contrast to M1 macrophages, anti-inflammatory M2 macrophages overexpress ARG1 that produces

ornithine and urea from arginine catabolism. Ornithine is transformed by ornithine decarboxylase (ODC) to polyamines (putrescine, spermidine, and spermine) that control cell growth and are important for tissue repair. Interestingly, it has been recently reported that ODC limits M1 activation and macrophage anti-microbial activities by chromatin modification (122). Moreover, arginase competes with iNOS for arginine, and many pathogens exploit this by increasing expression of arginase and thus block NO production (123, 124). ARG1 activity in macrophages triggers an anti-inflammatory phenotype and reduces T-cell proliferation and cytokine production (117).

Although ARG1 and iNOS are competitively regulated by Th1 and Th2 cytokines and complex intracellular biochemical pathways, including negative feed-back loops and competition for the same substrate (125), simultaneous activation of ARG and NOS pathways occurs in myeloid cells licensed by the tumor (126). In tumor-infiltrating myeloid cells, L-Arg is metabolized by ARG1, ARG2, and iNOS. ARG and NOS co-activation within the same environment leads to production of several ROS and reactive nitrogen species (RNS) by the iNOS reductase domain at low L-Arg concentrations (127–131). Peroxynitrite produced by myeloid and tumor cells can nitrate tyrosine residues in the TCR and CD8 receptors, resulting in decreased recognition of peptide–MHC complexes (132) and T cell dysfunction (133); moreover, RNS can induce post-transcriptional modifications of chemokines and thus prevent intra-tumoral infiltration of antigen-specific T cells (134).

Tryptophan metabolism is a major mechanism of peripheral immune tolerance. In immune cells, the limiting step of tryptophan catabolism is mediated by indoleamine 2,3-dioxygenase (IDO) that converts tryptophan into kynurenine (**Figure 3**). Although IDO expression is induced by IFN-γ, TNF-α, or prostaglandins, macrophages are driven toward an M2 phenotype when IDO is overexpressed, and IDO silencing promotes a pro-inflammatory macrophage profile (135). Macrophages that express high levels of IDO may deplete extracellular tryptophan, thus affecting T-cell proliferation and functions (136, 137). TAMs, and sometimes tumor cells themselves, upregulate IDO and create an immunosuppressive microenvironment via at least two mechanisms: tryptophan depletion and accumulation of tryptophan catabolites, such as kynurenine, 3-hydroxyanthranilate, and quinolinate (137–139). From a mechanistic point of view, while tryptophan depletion inhibits rapid expansion of activated T cells, tryptophan-derived catabolites act as ligands of the aryl hydrocarbon receptors (AHR) (140). Kynurenine is a potent suppressor of T cell immunity: by stimulating AHR, it skews the differentiation of naive T cells toward $FoxP3^+$ regulatory T cells (Tregs), whereas it suppresses T_h17 cells differentiation (141).

In immune cells, glutamine is used for amino acid and nucleotide synthesis, NADPH and energy production, and many other biosynthetic pathways involved in cell proliferation and functions. Macrophages utilize glutamine at high rates and are dependent upon extracellular sources of the amino acid (76, 142). During macrophage activation, the different routes of glutamine consumption direct its role to promote either the M1 or M2 phenotype. Channeling of glutamine into the Krebs cycle is the main route to promote succinate synthesis in M1 macrophages, with the GABA shunt (a bypass of the TCA cycle in which glutamine is used for synthesis of glutamate, GABA, succinic semialdehyde, and eventually succinate) also playing a role (31). This is fundamental to stabilize HIF1α (33). On the other hand, glutamine metabolism drives M2 polarization by acting at multiple levels: (i) α-ketoglutarate generated from glutaminolysis is essential for M2 OXPHOS and FAO; (ii) α-ketoglutarate generated from glutaminolysis promotes an M2 phenotype by macrophage epigenetic reprogramming, involving demethylation of H3K27 on the promoters of M2-specific marker genes (143); (iii) α-ketoglutarate generated from glutaminolysis favors PHD activity and thus inhibits HIF1α expression; (iv) glutamine provides substrate for the UDP-GlcNAc synthesis (**Figure 3**). Indeed, the pathway for UDP-GlcNAc synthesis is upregulated in M2 macrophages and is essential for the glycosylation of different proteins expressed abundantly in M2 macrophages (32). Tracing experiments in M2 cells with ^{13}C- and ^{15}N-glutamine have shown that a third of all carbon in TCA metabolites and more than half of the nitrogen in UDP-GlcNAc derive from glutamine (32), providing further evidence for the essential role of glutamine metabolism in M2 differentiation of macrophages. Thus, M2 cells do not exclusively rely on glutamine uptake for their metabolism, but they induce glutamine synthesis from glutamate and ammonia via glutamine synthetase (GS). While GS is barely detectable in M1 macrophages, highly GS expression in M2 macrophages, particularly in response to IL-10, is fundamental for the acquisition of a M2-like phenotype (144). Indeed, GS inhibition skews IL-10 stimulated macrophages to a M1-like state, through a mechanism involving metabolic reprogramming (144, 145). Additionally, GS ablation in TAMs reduces M2 markers, such as ARG1 and CD206, and decreases tumor metastasis in mice (144, 145).

LIPID METABOLISM IN INNATE IMMUNITY

Cellular lipid metabolism comprehends several key enzymatic processes that lead to the synthesis or the degradation of lipids (cholesterol, fatty acids, and phospholipids, **Figure 2**). As specialized phagocytic cells, macrophages are capable to uptake different forms of lipids such as LDL, VLDL, and oxidized lipoproteins from both engulfed dying cells and microenvironment via phagocytosis, macropinocytosis, and scavenger receptor-mediated pathways (146). After that, all ingested lipids are processed by acid lipases within the lysosomes, leading to the generation of free fatty acids and cholesterol (147). Free fatty acids are subsequently transported into mitochondria, where they are converted by the FAO pathway into different products that continuously replenish the TCA cycle with acetyl-coenzyme A, or the ETC through the generation of NADH and FADH2 (148).

On the other side, if metabolically required, FAS is induced through mTOR signaling within the cytosol. Notably, the FAS pathway permits the generation of lipids by using different precursors of TCA cycle, glycolysis and PPP pathway (149).

Transcriptional regulation of lipid metabolism is tightly controlled by sterol receptor element binding protein (SREBP) and liver X receptor (LXR). In macrophages, SREBP-1a and LXRα are highly expressed and regulate cytokine release and cell responses (150, 151). LPS treatment increases macrophage SREBP-1a activity via NF-κB, and macrophages deficient in SREBP-1a fail to produce IL-1β upon LPS stimulation (152), thus connecting lipid metabolism and inflammasome activation in M1 macrophages (153). In contrast, M2 macrophages are characterized by LXR activation, which regulates cholesterol homeostasis and lipid synthesis (154). Overexpression or activation of LXRα dampens M1 responses and inflammation by inhibiting the activity of NF-κB and AP-1 (155, 156).

It is clear that differential induction of FAS and FAO elicits macrophage polarization toward the M1 and M2 profiles, respectively (**Figure 3**). FAS represents an important pathway for energy production and prostaglandin biosynthesis in M1 cells; moreover, the accumulation of malonyl-CoA, product of the first step of FAS, can induce post-translational modifications (malonylation) that modulate responses of pro-inflammatory macrophages. On the other side, the main sources of fatty acids in M2 derive from uptake via the scavenger receptor-mediated pathway (157) and through lysosomal lipolysis mediated by lysosomal acid lipase, all these pathways being up-regulated upon IL-4 stimulation (147). M2 macrophages rely on fatty acid uptake and oxidation, which are supported by STAT6, the PPARγ (158) and its co-activator 1 (PGC1) (159). Indeed, M2 polarization could be prevented by inhibiting FAO, using a pharmacological approach targeting the carnitine palmitoyltransferase (CPT) system which mediates fatty acid translocation within mitochondria (160, 161). However, it has been recently reported that genetic ablation of CPT2 does not prevent macrophage polarization toward M2 profile upon IL-4 stimulation, both *in vitro* and *in vivo* (162). Interestingly, a recent work highlights the involvement of glutaminolysis-derived α-ketoglutarate as a positive metabolic regulator of FAO (163), thus suggesting further connections between different metabolic pathways.

MACROPHAGE METABOLISM IN DISEASES

Alterations in macrophage polarization, function, and metabolic signature are present in various human diseases. In inflammatory diseases, such as sepsis, rheumatoid arthritis, and atherosclerosis, as well as in metabolic diseases, including obesity and diabetes, macrophages display prolonged or atypical M1 polarization; on the other hand, cancer growth is often associated with "M2-like" responses of TAMs (25, 164). A discussion of the role of macrophage metabolic adaptations in human diseases is not the focus of our review but here we briefly analyze some aspects of macrophage metabolic pathways in two conditions: obesity and cancer.

Among the immune cells that infiltrate obese adipose tissue, macrophages are functionally and numerically dominant (165). In the adipose tissue of lean mice, macrophages are 10–15% of cells, whereas they represent 45–60% of cells in the adipose tissue of obese animals (166). In addition to the difference in their numbers, adipose tissue macrophages in lean and obese animals exhibit distinct localizations and responses. Adipose tissue macrophages in lean animals have an alternatively activated (M2) phenotype, are anti-inflammatory and are uniformly dispersed throughout the adipose tissue, whereas adipose tissue macrophages of obese mice have a pro-inflammatory, classical (M1) phenotype and are primarily found in "crown-like" structures around dying adipocytes (167, 168). In lean adipose tissue, M2 macrophages have a crucial role in maintaining the insulin sensitivity of adipocytes via the secretion of interleukin-10 (IL-10) (158, 167), a regulatory cytokine that potentiates insulin signaling in adipocytes (165). By contrast, classically activated macrophages in obese adipose tissue secrete pro-inflammatory cytokines, which induce insulin resistance (165).

Fatty acids and TNF-α have been shown to induce M1 polarization in obesity (165) but also glucose, insulin, and obesity-induced hypoxia trigger macrophages toward a pro-inflammatory phenotype (169). Interestingly, in a study analyzing the metabolic signature in obese and normal-weight children, the serum concentration of glutamine was lower in obese children than in normal-weight ones (170). Moreover, a high ratio of glutamine to glutamate in the plasma is associated with a lower risk of diabetes mellitus (171). Because skeletal muscles participate in maintaining the concentration of glutamine in serum, the reduction in muscle mass in obese patients may account for reduced serum concentrations of glutamine (172). In addition, in the subcutaneous adipose tissues of obese patients there are higher concentrations of glutaminase and lower concentrations of glutamine synthase, compared with the lean subjects (173). Glutamine is known to promote M2 macrophage polarization (see above) and thus glutamine supplementation may represent a strategy to target macrophage polarization in obesity. Although a few studies have shown that therapeutic administration of glutamine is beneficial in obesity and diabetes, the direct effects of this metabolic targeting on macrophages is not proven (172).

As already discussed, the succinate receptor SUCNR1/GPR91 is known to play a role in several diseases including diabetic retinopathy (108), diabetic renal disease (114), hypertension (113, 115), atherothrombosis (116), neuroinflammation (111), rheumatoid arthritis (114), and metabolic dysfunctions (174). Interestingly, the concentration of succinate in plasma is higher in patients with type 2 diabetes than in non-diabetic individuals (174) and it is significantly associated with the body mass index (BMI) (175). Thus, targeting succinate and its receptors may represent an interesting therapeutic strategy to modulate macrophage responses and inflammation in several pathological contexts.

It is highly probable that the metabolic signature of TAMs depends on the surrounding microenvironment and may thus be different in different tissues. In general terms, and on the basis of the recent publications, TAMs seem to depend on glycolysis for their metabolic needs and produce lactate at high concentration (79, 176). Lactate (177), in turn, induces VEGF and ARG1, thus promoting a pro-angiogenic signature, and

potentiates glycolysis by activating the Akt/mTOR pathway. However, when cancer grows, hypoxia induces in TAMs up-regulation of REDD1 (regulated in development and in DNA damage response 1), which inhibits mTOR, and thus inhibits glucose uptake by macrophages and glycolysis. This is associated with an increased angiogenic response and formation of aberrant leaky vessels due to enhanced glucose availability for endothelial cells (178).

As mentioned above, TAM-produced lactate induces ARG1, which depletes arginine and is directly involved in TAM-induced immunosuppression. On the other hand, highly overexpressed cyclooxygenase in TAMs induces IDO expression, which depletes tryptophan thus suppressing T cell responses. Therefore, macrophage functions in the tumor microenvironment are regulated by a complex and interconnected reprogramming involving glucose, amino acid, and lipid metabolism.

CONCLUSIONS

Macrophage metabolic adaptations have been deeply analyzed during the last years and have emerged as critical factors regulating a variety of cell responses. Pathogen or inflammatory signals drive macrophage differentiation toward the acquisition of new functions by rapidly modulating the expression of key genes. Associated to this program is a remodeling of the metabolic pathways that sustains, from an energetic, biosynthetic, and regulatory point of view, its execution (**Figure 3**). Information from studies on inflammation-linked diseases is teaching us that pathological immune response might be underpinned by aberrant metabolic rewiring (145, 179, 180). Several metabolic products play important roles as signaling mediators, affecting not only macrophages but also neighboring cells, thus representing interesting targets for therapeutic strategies (181). To this aim, it is fundamental to understand the interplay between metabolism and immunity by dissecting the different metabolic reactions important for the acquisition of specific functions. On the other hand, it is becoming evident that the different metabolic pathways are strongly interconnected and that positive and negative feedback loops are involved in amplification or dampening of immune responses. Thus, as growing literature defines metabolic processes and pathways in macrophages, the ultimate goal is an integrated view of the metabolic networks regulating inflammation, immunity, and tissue responses to homeostasis perturbations.

AUTHOR CONTRIBUTIONS

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REVIEW
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Hijacking the Supplies: Metabolism as a Novel Facet of Virus-Host Interaction

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Viral replication is a process that involves an extremely high turnover of cellular molecules. Since viruses depend on the host cell to obtain the macromolecules needed for their proper replication, they have evolved numerous strategies to shape cellular metabolism and the biosynthesis machinery of the host according to their specific needs. Technologies for the rigorous analysis of metabolic alterations in cells have recently become widely available and have greatly expanded our knowledge of these crucial host–pathogen interactions. We have learned that most viruses enhance specific anabolic pathways and are highly dependent on these alterations. Since uninfected cells are far more plastic in their metabolism, targeting of the virus-induced metabolic alterations is a promising strategy for specific antiviral therapy and has gained great interest recently. In this review, we summarize the current advances in our understanding of metabolic adaptations during viral infections, with a particular focus on the utilization of this information for therapeutic application.

Keywords: virus, metabolism, rhinovirus, host-pathogen interaction, metabolome

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CELLULAR METABOLISM: THE NOVEL FRONTIER OF HOST–PATHOGEN INTERACTION

Viruses depend on the host cell to obtain the macromolecules and biosynthesis machinery required for their replication. In order to ensure the undisturbed supply of these elements, viruses have evolved a plethora of strategies to shape host-cell metabolism according to their specific needs. The simultaneous course of both the activation of host cell defense mechanisms and the high biomolecular turnover associated with virion production results in a highly anabolic cellular state. This is often accompanied by upregulation of the ingestion of an extracellular carbon source (e.g., glucose or glutamine) and a redirection of these carbon supplies to metabolic pathways crucial for viral replication, such as lipogenesis and nucleotide synthesis. However, not only do viruses shape host-cell metabolism in order to obtain supplies for virion production, but they also induce a reorganization of the cellular membrane and biosynthesis machinery, which is accompanied by alterations in lipid metabolism, as we shall explain later.

The first insights into the dependence of viruses on certain carbon sources were gained decades ago, when researchers focused on investigating the consequences of glucose or glutamine deprivation on viral replication (1–6). However, it was the availability of mass spectrometry (MS)-based analysis of the metabolome that enabled fast progress toward an in-depth understanding of the interaction between viruses and host-cell metabolism. Munger et al. pioneered the field in 2006, when

they showed that human cytomegalovirus (HCMV) not only was highly dependent on extracellular carbon but also induced a plethora of alterations in host-cell metabolism that are required for proper replication (7). MS-based assessment of the host cell metabolome and carbon flux has since then become widely available and has enabled the investigation of host–pathogen interactions in detail. Furthermore, the acquired knowledge on these processes has enabled the establishment of several antiviral strategies, and the exploitation of the novel metabolic insights in terms of therapy has only just begun. Herein, we review the recent progress made toward our understanding of the interactions between viruses and host-cell metabolism, and we will also elaborate on strategies that might result in targeted antiviral therapy.

GLUCOSE AND GLUTAMINE: VIRUS-INDUCED FEEDING OF THE TRICARBOXYLIC ACID CYCLE

Under homeostatic and aerobic conditions, cells maintain their energy production mainly by aerobic glycolysis, which is followed by feeding pyruvate into the tricarboxylic acid (TCA) cycle and subsequent utilization of reduced molecules in oxidative phosphorylation. However, under anaerobic conditions, pyruvate is converted to lactate, which is then eliminated by efflux from the cell. Aside from anaerobic conditions, this phenomenon can often be observed even under normal oxygen conditions, as was first described in cancer cells by Otto Warburg and has thus been termed the Warburg effect (8). Under these circumstances, the intermediates of the TCA cycle are mainly fed into anabolic processes, such as lipogenesis. Cells infected by certain viruses appear to adopt similar metabolic alterations in order to cope with the high anabolic demands of virion production. Nonetheless, there are highly unique patterns of virus-induced reshaping of host cell metabolic processes and the mode of manipulation appears to be different between DNA and RNA viruses.

DNA Viruses

Members of the *Herpesviridae* are probably the best-studied group of viruses in terms of their impact on cellular metabolism. Herpes simplex virus-1 (HSV-1) was among the first viruses for which a dependency on extracellular glucose was shown. Deprivation of glucose from the medium had detrimental effects on virion production, whereas glutamine appeared to be more dispensable for the replication of this virus (2). Later studies confirmed the dependency of HSV-1 on glucose, as the glycolysis inhibitor 2-deoxyglucose (2-DG) also impaired viral replication (4, 9). 2-DG is a glucose analog that impairs the function of phosphoglucose isomerase and thus results in both an inhibition of glycolysis and the processing of glucose toward the TCA cycle (in contrast to the more downstream inhibitor oxamate that inhibits only anaerobic glycolysis). Abrantes et al. found that HSV-1 increased the glucose uptake, lactate efflux, and ATP content of HSV-1 infected cells, which was accompanied by an activation and enhanced expression of phosphofructokinase-1, a rate-limiting enzyme in glycolysis (10). In contrast, using a metabolomic screening, Vastag et al. found that glycolysis was not markedly induced by HSV-1, and the virus instead triggered anaplerotic (glutamine-dependent) feeding of the TCA cycle and an enhancement of pyrimidine synthesis (11). A possible explanation could be that the uptake of glucose mainly shifted to nucleotide synthesis pathways rather than glycolysis or the TCA cycle, which was further underlined by the increase in pentose phosphate pathway intermediates in the metabolomic analysis and would explain the high susceptibility of this virus to nucleotide analog treatment (11).

HCMV, another important member of the *Herpesviridae* family, causes significant morbidity in immunosuppressed individuals (12–14). Since the metabolic alterations caused by this virus have already been extensively reviewed recently (15), we will only briefly discuss the main findings in order to better delineate the concepts of differential metabolic alterations by viruses. Early investigations had hinted toward a manipulation of host-cell metabolism by HCMV, where it was shown that glucose uptake was enhanced in infected cells (5). In the first metabolomic study of virus-infected cells conducted by Munger et al. the authors were able to show that metabolites from glycolysis, TCA cycle, and pyrimidine pathways were increased upon infection, which was accompanied by the upregulation of enzymes involved in these pathways (7). Further carbon flux analysis delineated how an increase in glucose uptake results in a fast processing through glycolysis and the TCA cycle toward fatty acid (FA) biosynthesis (16). The expansion of pyrimidine metabolite pools was found to be of particular importance for the correct glycosylation of viral proteins, as pyrimidine feeds into glycosylation pathways via UDP-sugars (17). Mechanistically, early HCMV gene expression was shown to be responsible for the changes in glycolytic flux and appeared to be dependent on Ca^{++} signaling, since calmodulin-dependent kinase kinase (CaMKK) inhibition abolished the HCMV-induced metabolic alterations (9). Subsequent research has highlighted a role of AMP-activated protein kinase (AMPK) in the replication cycle of HCMV, since this kinase is activated upon infection and its inhibition has detrimental effects on viral replication (18, 19). Since CaMKK is known to be upstream of AMPK, and blocking of CaMKK abolished the HCMV-induced AMPK activation, the authors proposed a CaMKK–AMPK axis in the mediation of HCMV's metabolic effects. Other groups have investigated the role of glucose transporters (GLUTs) in HCMV infection and found upregulation of GLUT4 expression, but downregulation of GLUT1, following infection (20). These changes in GLUT expression were later shown to be dependent on the carbohydrate-response element-binding protein (ChREBP), which is targeted in HCMV infection (21). Apart from the apparent need for adequate glucose supply, HCMV also depends on extracellular glutamine as a carbon source (22). Deprivation of glutamine from the extracellular medium dampened high-titer virus replication, which could be restored by the addition of TCA cycle metabolites, thus pointing toward an anaplerotic utilization of glutamine in HCMV infection (22). Recent research has additionally established a role of the viral protein U_L38 in the upregulation of both glucose

and glutamine (and other amino acid) consumption, which was mediated by the modulation of tuberous sclerosis complex 2 (TSC2) but was mTOR independent (23).

Epstein-Barr virus (EBV) causes infectious mononucleosis, and its latent infection is associated with the development of various malignant diseases. Latently infected cells were found to enhance both glucose and glutamine uptake and to have deregulated glycolysis (24, 25). These changes were described to have been induced by EBV's latent membrane protein 1 (LMP1) and were associated with fibroblast growth factor receptor 1 (FGFR1) signaling (24, 25). Such metabolic alterations have been speculated to play a role in the long-term cancerogenic transformation of the latently infected cells (25).

Another virus of the *Herpesviridae* family, Kaposi's sarcoma-associated herpesvirus (KSHV), was also shown to broadly interact with host-cell metabolism in a quite sophisticated manner. Sanchez et al. found that glucose and glutamine were important for early viral replication and gene translation, respectively (26, 27). Furthermore, FA synthesis was shown to be crucial for optimal virus assembly and maturation (27, 28). Yogev et al. found that viral-encoded microRNAs were important for inducing the alterations in glucose metabolism, by repressing the expression of the metabolic regulator genes *EGLN2* (encoding Egl nine homolog 2) and *HSPA9* (encoding Stress-70 protein, mitochondrial), which then results in increased glycolysis and GLUT1 expression (29). Additionally, recent evidence suggests that KSHV-transformed cells critically depend on extracellular glutamine and asparagine to enable ɤ-nitrogen synthesis that fuels nucleotide synthesis (30). Accordingly, expression of enzymes engaged in glutamine metabolism including glutaminase, glutamate dehydrogenase 1, and glutamic-oxaloacetic transaminase 2 were needed to support cell proliferation in KSHV-transformed cancer cells (30). Supporting this evidence, the research group of Chandran was able to demonstrate that both *de-novo* and latent KSHV infection of endothelial cells and B cells induces glutaminase expression, which was found to be partly c-Myc dependent. Furthermore, the virus triggers extracellular glutamate secretion, the breakdown product of glutaminase-mediated enzymatic degradation of glutamine (31). The authors proposed that glutamate may act as an autocrine and paracrine growth factor during the course of KSHV-induced oncogenic transformation, as blockade of glutamate secretion or inhibition of metabotropic glutamate receptors attenuated KSHV-infected cell proliferation (31). Other important targets within the host cell that shape the KSHV anti-viral response and/or KSHV-induced cell proliferation include HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1) (32) and heme oxygenase-1 (33).

The group of Christofk has performed pioneering work toward our better understanding of adenovirus-induced host cell reprogramming and particularly in the mechanistic basis of virus–metabolome interactions. They were able to show that the viral product E4ORF1 localizes to the nucleus and binds the transcription factor Myc to induce the transcription of a number of glycolytic genes, resulting in enhanced glycolytic pathway activity and nucleotide production (34). Later, they showed how Myc regulated glutamine metabolism in adenovirus-infected cells and that glutaminase was a critical enzyme for adenovirus replication, which was also true for HSV-1 and influenza A (35). Coherently, inhibition of glutaminase by CB-839 impaired adenovirus, HSV-1, and influenza A replication (35).

All DNA viruses discussed so far induce glycolysis and/or increase glucose uptake in the course of infection. However, an exception to this is the vaccinia virus (VACV). Metabolomic studies have shown that although the virus does not affect glycolytic flux, it is highly dependent on glutamine as a carbon source for feeding into the TCA cycle (36, 37) (**Figure 1**). Further studies showed that the viral protein C16 might be responsible for these effects through the stabilization of hypoxia-inducible factor 1-alpha (HIF-1α) (38).

RNA Viruses

In contrast to the large DNA viruses discussed above, we found a markedly different mode of metabolism manipulation by the small RNA virus rhinovirus (RV), which belongs to the *Picornaviridae* family and is the causative agent of the common cold. Similar to other viruses, we found an enhancement of glucose uptake and the virus was dependent on both extracellular glucose and glutamine for optimal viral replication (39). However, the amplification of glucose uptake was detectable as fast as 1.5 h upon infection, which ruled out a transcriptional control of this process. Indeed, we found the enhanced uptake to be reversible by phosphoinositide 3-kinase (PI3K) inhibition, suggesting a role of this pathway in mediating RV's effects. In contrast to HCMV infection, we found upregulation of GLUT1 expression upon RV infection, whereas GLUT3 expression was unaffected (**Figure 2**). This is in line with the concept of PI3K-driven upregulation of GLUT1, likely to mediate RV effects. Metabolomic studies revealed increased levels of metabolites associated with glycogenolysis, a process that has not been described so far in the context of viral infections. Furthermore, we found an enhancement of lipogenesis and nucleotide synthesis. The deprivation of both glutamine and glucose from the medium impaired high-titer RV replication, and the early glycolysis inhibitor 2-DG potently inhibited viral replication and reversed the RV-induced alterations of the host cell metabolome. Thus, our findings underline the potential of metabolism as a target of antiviral therapy (39).

As stated above, glutaminase is of pivotal importance for influenza virus replication (35). Furthermore, the influenza virus was shown to depend on extracellular glucose, and viral replication could be impaired by treatment with the glycolysis inhibitor 2-DG (6). In contrast, recent work has found that 2-DG has detrimental effects on survival in an *in vivo* influenza infection, which was attributed to an unregulated unfolded protein response in the absence of glucose (40). These findings are particularly intriguing, as they show how a metabolism-targeting intervention that is effective on the cellular level might still be detrimental when applied systemically and could thus affect a plethora of different cell populations. These considerations need to be taken into account when designing metabolism-targeting antivirals and deciding on their route of administration.

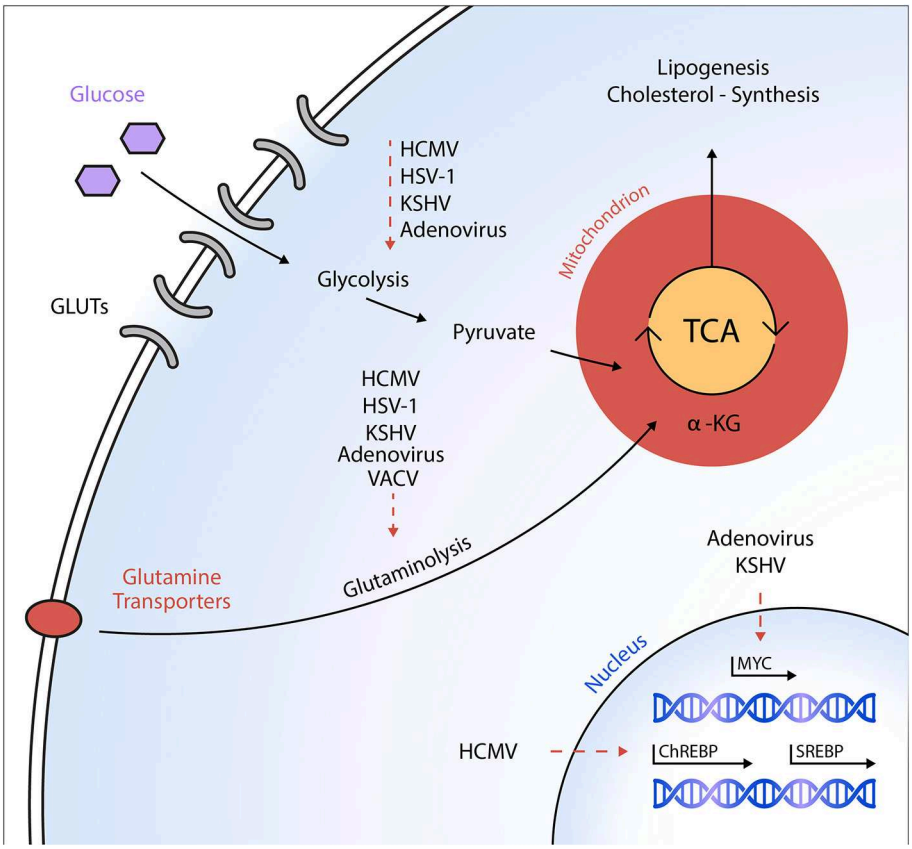

FIGURE 1 | Schematic overview of metabolic targets of DNA viruses. Different DNA viruses activate specific anabolic metabolic programs in host cells to ultimately support viral replication and virion maturation. Dashed arrows indicate a virus-mediated activation of the respective metabolic pathway or an activation of the transcription factor, respectively. HCMV, human cytomegalovirus; HSV-1, herpes simplex virus-1; KSHV, Kaposi's sarcoma-associated herpesvirus; VACV, vaccinia virus; GLUT, glucose transporter; ChREBP, carbohydrate-response element-binding protein; SREBP, sterol regulatory element-binding protein; α-KG, α-ketoglutarate; TCA, tricarboxylic acid cycle.

Several viruses of the *Flaviviridae* family were shown to be potent modulators of host-cell metabolism. Zika virus was shown to modulate metabolism differently in human and mosquito cells; that is, whereas the infection resulted in enhanced glucose utilization through the TCA cycle in human cells, glucose utilization shifted toward the pentose phosphate pathway in mosquito cells (41). These differences resulted in a reduction of nucleotide triphosphates and AMPK-dependent cell death in human cells (41). Dengue virus (DENV) stimulates and requires glycolysis for optimal replication (42), which was found to be mediated by the induction of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the virus's non-structural protein NS1 (43). The distantly related hepatitis C virus (HCV) was also shown to increase glucose demand and enhance glycolysis in infected cells (44, 45). Interestingly, HCV appears to have evolved several strategies to target host cell glycolysis, where it was shown that the NS5A protein interacts with hexokinase 2 to increase the glycolytic flux (45), and the HCV-regulated microRNA 130a enhances the activity of pyruvate kinase, another key enzyme in glycolysis (46).

Recently, there has also been great progress in the elucidation of the metabolic requirements for human immunodeficiency virus (HIV) replication. Hollenbaugh et al. were among the first to study the metabolic alterations in HIV-1-infected cells by means of metabolomics (47). Intriguingly, they were able to show that HIV-1 induced marked changes depending on the infected cell type; that is, whereas $CD4^+$ T cells exhibited increased glucose uptake and metabolite pools in the TCA cycle, the opposite was found for infected macrophages (47). Subsequent investigations have confirmed the increase in glucose uptake by infected $CD4^+$ T cells (48–53). These alterations were shown to be accompanied by increases in the expression of the glucose transporters GLUT1 (51–53), GLUT3 (53), GLUT4 (53), and GLUT6 (53), and also by increases in the expression of the key glucose-processing enzyme hexokinase 1 (HK1) (53, 54). Furthermore, phospholipase D1 (PLD1) was found to be a crucial regulator of the HIV-1-induced metabolic alterations in $CD4^+$ T cells (48). PLD1 further induced the activation of c-Myc, resulting in the activation of a transcriptional program that led to enhanced glucose uptake and nucleotide biosynthesis. Consequently, the pharmacologic inhibition of PLD-1 led to

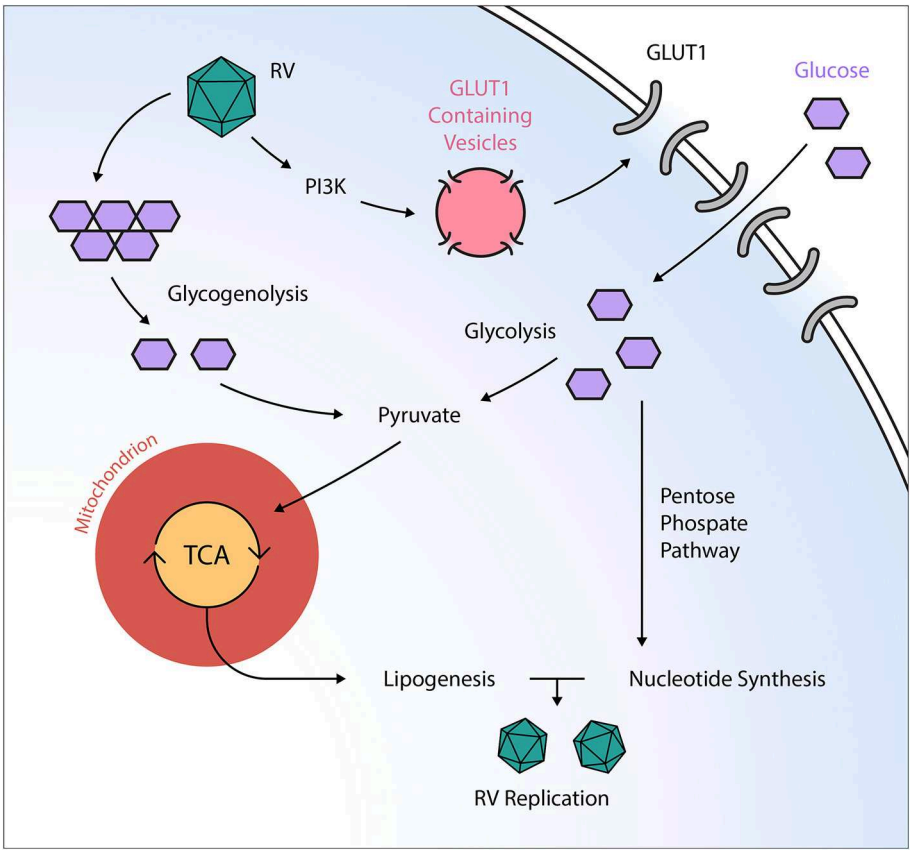

FIGURE 2 | Post-transcriptional manipulation of the host cell metabolism by the RNA virus rhinovirus. Rhinovirus (RV) orchestrates an anabolic reprogramming of the host cell metabolism: RV induces PI3K-dependent trafficking of GLUT1-containing vesicles to the cell membrane, ultimately leading to increased glucose uptake. Subsequently, RV increases both glycolysis and glycogenolysis, providing TCA intermediates needed for anabolic lipogenesis. Additionally, RV infection activates the pentose phosphate pathway, resulting in elevated nucleotide levels that support viral replication. GLUT, glucose transporter; PI3K, phosphatidylinositol 3-kinase; RV, rhinovirus; TCA, tricarboxylic acid cycle.

a reduction in HIV-1 replication (48). Apart from glucose, glutamine concentrations were found to be elevated as well in HIV-1-infected $CD4^+$ T cells, which was accompanied by increases in the levels of glutaminase (55). Studying the differences between the alterations induced by HIV-1 and HIV-2, Hollenbaugh et al. found that although both viruses induced similar changes in infected macrophages, there were differences observed in the levels of quinolinate, a tryptophan pathway component (56). In another important study, Hegedus et al. found marked differences between primary T cells and cell lines infected with HIV-1, thus underlining the importance of the cell system when studying cellular metabolomics (49).

Taking these findings together, we can see that most viruses have evolved strategies to alter central carbon supply pathways, such as glucose or glutamine consumption, and these manipulations were shown to be vital for high-titer virus replication. Apart from this, virion production requires a re-orchestration of the entire biosynthesis machinery, a process that usually involves a reorganization of many parts of the cellular lipidome, as we review in the next section.

VIRAL CONTROL OF FATTY ACID METABOLISM

Apart from the alterations mentioned above, the FA synthesis machinery of the host cell has proven vital for viral genome replication, virion production and morphogenesis. Several viruses induce the formation of phosphatidylinositol 4-phosphate/cholesterol-enriched membranes to build viral replication complexes (VRCs) at the interface of the host endoplasmic reticulum (ER). Accumulation of sterols at the VRCs of RNA viruses allows for the production of secluded membranes that contain an optimal environment for viral replication and shield virus nucleic acids from immune surveillance (i.e., cytosolic pattern recognition receptors) (57, 58). Formation of the VRCs critically depends on reprogramming of the host's sterol synthesis via recruitment of the phosphatidylinositol-4 kinase III beta and oxysterol-binding protein (PI4KB–OSBP) axis, and disruption of cellular cholesterol homeostasis impairs viral replication (59–63). Apart from this apparent need for an adequate sterol supply, viruses

transactivate and co-opt enzymes engaged in *de novo* lipid synthesis and in the enzymatic modification of intracellular FAs.

Generally, several carbon sources can be used as substrates for FA or cholesterol synthesis, with the most important one being citrate derived from the TCA cycle. Citrate is carried across the mitochondrial membrane and cleaved into acetyl-CoA in the cytosol. Acetyl-CoA is then carboxylated by acetyl-CoA carboxylase (ACC) to yield malonyl-CoA. FA synthase (FASN) catalyzes the production of palmitic acid (C16:0) from cytosolic acetyl-CoA and malonyl-CoA in the presence of NADPH. The palmitic acid can then be further processed by elongases and desaturases into more complex FAs for use in the synthesis of cell membranes, storage in lipid droplets, or the palmitoylation of host and viral proteins. For sterol biosynthesis, two units of acetyl-CoA are metabolized to form acetoacetyl-CoA, which then enters the mevalonate pathway. On the other hand, FAs can be metabolized by catabolic beta-oxidation to yield high amounts of ATP.

Several key metabolic transcription factors activate the transcriptional program of anabolic lipid and sterol metabolism. Among those, sterol regulatory element-binding proteins (SREBPs) represent the most important family of transcription factors that transactivate lipogenic genes in order to increase FA and cholesterol syntheses. Under homeostatic conditions, SREBPs are synthesized in an inactive form and are attached to the ER membrane. Upon intracellular sterol shortage, SREBPs are proteolytically cleaved, whereupon they translocate to the nucleus and bind to sterol response elements (SREs). This binding of the activated SREBP to SRE DNA motifs governs the transcriptional control of key lipogenic metabolic enzymes, such as FASN and ACC (64–66).

DNA Viruses

As discussed above, the first evidence of metabolic reprogramming upon HCMV infection was provided by Munger et al. Metabolic flux and MS analyses revealed that HCMV infection induces a glucose flux, which directly fuels FA synthesis (7, 16). In 2011, Munger's research group was able to show that HCMV infection facilitates the mTOR-dependent proteolytic cleavage of SREBP2 (67, 68). Other research groups provided additional evidence that SREBP1 cleavage is also required for optimal metabolic reprogramming toward lipogenesis to enable high-titer HCMV replication (69, 70). In those studies, the inhibition of SREBP proteolytic cleavage and of the downstream targets of SREBP-induced lipogenesis (e.g., ACC and FASN) impaired HCMV replication (16, 67, 69). Additionally, HCMV infection induces the expression of FA elongases (ELOVLs), which in turn leads to the accumulation of long-chain and very-long-chain FAs (VLCFAs) (68, 71) that are shuttled toward viral envelope production (71, 72). Among the family of FA elongases, ELOVL7 is increased more than 150-fold upon HCMV infection in an mTOR/SREBP-dependent manner (68). Remarkably, inhibition of ELOVL7 impairs HCMV replication, and this effect can be rescued upon ELOVL7 overexpression or VLCFA supplementation (68, 71). Additionally, HCMV-infected cells upregulate low-density lipoprotein receptor-related protein 1 (LRP1) in a SREBP-dependent manner, and interference with LRP1 disturbs the intracellular cholesterol availability (73). Besides this control of lipogenesis on a transcriptional level, HCMV can also directly increase ACC activity (67).

As mentioned above, KSHV infection induces a transformation of the host cell's glucose, glutamine, and fatty acid metabolism (27, 28). While infection-induced glycolysis and glutaminolysis prove essential for early steps of KSHV infection including genome replication, fatty acid synthesis appeared not to be involved in those processes. Instead, fatty acid synthesis is critical for virion assembly and the maturation of infectious particles, since KSHV-infected cells cultured in the presence of an ACC1 inhibitor produced only non-infectious intracellular virions (27). Other evidence provided by the same research group suggests that lipogenesis is required for KSHV survival and latent infection (28) (**Figure 1**).

RNA Viruses

Likewise, perturbations of cellular lipid metabolism have proven vital for the *Flavivirus* replication cycle. Both DENV and West Nile virus (WNV) are known to be highly sensitive to the inhibition of ACC or FASN (74–78) as well as to interferences with cholesterol uptake (79), homeostasis (80), and biosynthesis (81, 82). An urgent need for *de novo* lipogenesis as well as changes in the intracellular lipid distribution and accumulation of unsaturated FAs have also been proposed to be essential prerequisites for DENV type 2 (DENV2) infection (75, 83). Gullberg et al. identified stearoyl-CoA desaturase 1 (SCD1, which catalyzes the rate-limiting step in the formation of unsaturated FAs) as a critical target that regulates the composition of intracellular membranes to induce a favorable microenvironment for optimal DENV2 replication and to sustain a high rate of infectious particle release (83). The pharmacologic inhibition of SCD1 interrupted the generation of monounsaturated FAs, such as oleic acid (C18:1) or palmitoleic acid (C16:1) (69, 83, 84), which consequently affected an optimal lipid membrane composition and membrane fluidity, leading to decreased viral replication efficiency in DENV2-infected cells. This detrimental effect of pharmacologic SCD1 inhibition on DENV2 replication has been expanded to several other *Flaviviridae* members, including four DENV serotypes, Yellow Fever Virus (YFV), Zika virus, and Japanese encephalitis virus (JEV) (83, 85). Therefore, the inhibition of ACC or FASN or that of more downstream lipid-modifying enzymes such as SCD1 may guide future therapies against *Flavivirus* infection (86, 87). Substantiating these findings, several groups have observed temporal changes in numerous lipid species, especially phospholipids, upon HCV infection (44), and a critical need for *de novo* ACC- and FASN-mediated FA synthesis to fuel viral replication (44, 88–90). More recently, metabolomic profiling revealed that unsaturated long-chain FAs, such as oleic acid (C18:1), specifically accumulated upon HCV infection, and that the accumulation of unsaturated FAs may influence the membrane composition and fluidity (84, 91, 92). Hofmann et al. demonstrated that the inhibition of FA elongases or desaturases restricted HCV replication (91). In their study, treatment with an inhibitor of Δ6-fatty acid desaturase (FADS2) impaired HCV virion production possibly through changes in the intracellular membrane composition,

virion assembly, and morphogenesis (91, 92). Likewise, a liver-specific SCD1 inhibitor has been proposed for anti-HCV therapy following its proven efficacy in mice (84, 92–94). Similarly, inhibition of FASN with C75 reduced HCV replication *in vitro* (88). Other evidence suggests that currently used anti-HCV agents like ribavirin inhibit lipogenesis as a side effect, which may contribute to their antiviral properties (95, 96). Mechanistically, ribavirin suppresses the expression of lipogenic genes such as SREBP-1c, FASN, and SCD-1 in a retinoid X receptor α- and CCAAT/enhancer-binding protein α-dependent manner (95, 96). Statins, another class of drugs used in a wide number of patients due to their cholesterol- and lipid-lowering properties, exhibited an inhibitory effect on HCV replication probably due to the inhibition of the rate-limiting step of the mevalonate pathway, 3-hydroxy3-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase) (97, 98).

Lipidomic analysis has also broadened our understanding regarding the metabolic reprogramming that ensues upon human coronavirus (HCoV) (99) and Middle East respiratory syndrome coronavirus (MERS-CoV) infections (99, 100). Yan at al. observed a striking rearrangement of the cellular lipid profile indicated by an accumulation of FAs (both saturated and unsaturated FAs) and phospholipids upon HCoV infection. The authors claimed that the *Coronaviridae* specifically fine-tuned the host lipid profile to achieve optimal viral replication (99). These findings were corroborated by a recent study that identified the pharmacologic targeting of SREBP (with the specific inhibitor AM580) as a promising means to inhibit MERS-CoV infection in multiple cell types *in vitro* and *in vivo* (100). Inhibition of the proteolytic processing of SREBP by AM580 caused the inhibition of several post-viral-entry steps, including reduced intracellular lipid droplet formation, reduced double membrane vesicle formation, and reduced palmitoylation of viral proteins (100), which potentially mirror the observations and conclusions made earlier by Yan et al. (99). Importantly, treatment with AM580 also restricted SREBP-dependent lipogenesis in influenza H1N1-infected cells, which resulted in the decreased palmitoylation of the surface glycoprotein hemagglutinin and ultimately impaired H1N1 replication (100).

Similarly, elevated levels of multiple long-chain mono- and polyunsaturated FAs have been associated with RV infection (39, 101). As discussed earlier, our group has recently shown that RV induces PI3K-dependent glucose uptake that feeds anabolic lipogenesis in primary human fibroblasts and HeLa cells (39). Another group recently confirmed our findings, using lipidomic technologies in primary human bronchial epithelial cells at different time points during a single replicative cycle of RV infection (i.e., ranging from 2–6 h post infection) (101). In accordance with our data, Ngyuen et al. observed an accumulation of FAs with long acyl chains in infected cells as compared with uninfected controls, as well as dynamic changes in the desaturation status of FA pools within the host cell. As a proof of concept, they treated the cells with several inhibitors of enzymes engaged in FA synthesis, elongation, and modification (including C75, an inhibitor of FASN), which resulted in a reduction in RV replication (101). Similarly, inhibition of FASN with a novel potent inhibitor (TVB-3166) decreased the replication of RV, respiratory syncytial virus (RSV), and human parainfluenza virus 3 (HPIV 3) (102). Confirming the essential need of FASN during viral replication, those observed effects could be rescued upon addition of exogenous palmitic acid (102). Altogether, both the upstream interference in the glucose flux (using 2-DG) (39) and the downstream inhibition of lipogenesis (101, 102) can serve as new therapeutic targets for treating RV- or RSV-induced respiratory infections.

During Chikungunya virus (CHIKV) infection, the FASN-mediated increase in the cellular lipid pool results in the increased palmitoylation of the virus's non-structural protein NsP1 at three cysteine residues by zinc finger DHHC domain-containing palmitoyltransferases (103). The palmitoylation of NsP1 is critical for CHIKV replication since it orchestrates capping of the virus's RNA (104–106). Therefore, the CHIKV induction of anabolic lipid synthesis via FASN in host cells generates an adequate substrate supply for the proper functioning of intracellular palmitoyltransferases. Confirming these observations, the inhibition of FASN was shown to impair CHIKV replication, which could be rescued upon exogenous palmitic acid supply (103).

Last, an up to 5-fold induction of FASN was also observed upon HIV-1 infection, translating into increased intracellular palmitic, oleic, and stearic acid pools (107). Although the authors were not able to delineate how those *de novo*-synthesized lipids fuel HIV-1 replication, they did show that FASN was exclusively required during the late stage of the viral replication cycle. This indicates a role for FASN in HIV-1-mediated viral budding or in post-translational modifications of HIV-1 structural proteins, such as the Gag protein (107). In line with this, existing evidence has proposed an essential role for several post-translational lipid modifications of HIV-1 structural proteins (108–111).

Hence, the life cycle of most viruses is closely linked to the composition of the cellular lipidome that defines the viral and cellular membrane composition, macromolecule synthesis, and post-transcriptional modification of viral proteins. In order to ensure sufficient substrate supply to enable the optimal replication of viral particles, viruses exploit host transcription factors and co-opt several enzymes engaged in *de novo* lipid synthesis and processing. The aforementioned lines of evidence suggest that lipid-based antiviral strategies may guide future antiviral therapies. In particular, the inhibition of SREBP cleavage and the targeting of FA-modifying enzymes (e.g., FA elongases and desaturases) represent promising targets for broad-spectrum antiviral metabolic intervention. However, the *in vivo* relevance of virus lipid interactions has yet to be determined, and further studies are urgently needed to better understand these processes.

CONCLUSION AND OUTLOOK

We have elaborated on the various forms of virus interference with the host cellular metabolome. We have seen that although many of the induced changes follow similar patterns between different viruses, a distinct virus-specific fingerprint can nonetheless be found for each virus, which mirrors the needs of

the respective pathogen for specific molecular compounds in the process of its replication.

Notwithstanding, most of our knowledge on the field consists of phenotypic characterizations of the impact of the infection on central pathways in host-cell metabolism, whereas our understanding of the mechanistic basis for these changes is far more limited. As we have seen, viruses have developed strategies as diverse as the activation of cytosolic signaling [e.g., PI3K (39) and CaMKK1/AMPK (9, 18) activation] or transcriptional regulation [e.g., activation of Myc (34, 35), ChREBP (21), SREBP (67–70, 100)]. The current data point toward a dichotomy between DNA and RNA viruses when looking at their respective strategies for host cell manipulation; that is, whereas the transcriptional control of key metabolic pathways was found for several DNA viruses (21, 34, 35), RNA viruses appeared to shape host-cell metabolism via

TABLE 1 | Strategies for metabolism-targeting interventions against different viruses.

Virus	Compound	Target	Pathway	References
HSV-1	2-DG	Phosphoglucose-isomerase	Glycolysis	(4, 9)
	STO-609	CaMKK	Ca^{++}-sensing	(9)
	CB-839	Glutaminase	Glutamine metabolism	(35)
HCMV	STO-609	CaMKK	Glycolysis	(9, 19)
	Compound C	AMPK	Broad metabolic alterations	(18, 19)
	AICAR	AMPK	Broad metabolic alterations	(19, 112)
	2-DG	Phosphoglucose-isomerase	Glycolysis	(9)
KSHV	Oxamate	Lactat-dehydrogenase	Anaerobic glycolysis	(27)
	BPTES	Glutaminase	Glutamine metabolism	(27)
	TOFA	ACC1	Fatty acid metabolism	(27, 28)
VACV	BPTES	Glutaminase	Glutamine metabolism	(36)
	TOFA	ACC1	Fatty acid metabolism	(37)
	C75	FASN	Fatty acid metabolism	(37)
RV	2-DG	Phosphoglucose-isomerase	Glycolysis	(39)
	C75	FASN	Fatty acid metabolism	(101)
	TVB-3166	FASN	Fatty acid metabolism	(102)
RSV	TVB-3166	FASN	Fatty acid metabolism	(102)
HCV	MK8245	Stearoyl-CoA desaturase-1	Fatty acid metabolism	(93)
	SC-26196	Fatty acid Δ-6-desaturase	Fatty acid metabolism	(91)
	C75	FASN	Fatty acid metabolism	(88)
	CP640186	ACC	Fatty acid metabolism	(93)
	Ribavirin	SREBP-1c, FASN, stearoyl-CoA desaturase-1	Fatty acid metabolism	(95, 96)
	Statins	HMG-CoA reductase	Cholesterol synthesis	(97, 98)
DENV	C75	FASN	Fatty acid metabolism	(76)
	Cerulenin	FASN	Fatty acid metabolism	(76)
	MK8245	Stearoyl-CoA desaturase-1	Fatty acid metabolism	(85)
	A939572	Stearoyl-CoA desaturase-1	Fatty acid metabolism	(83)
HPIV 3	TVB-3166	FASN	Fatty acid metabolism	(102)
ZIKA	MK8245	Stearoyl-CoA desaturase-1	Fatty acid metabolism	(85)
WNV	C75	FASN	Fatty acid metabolism	(77)
	Cerulenin	FASN	Fatty acid metabolism	(77)
	TOFA	ACC	Fatty acid metabolism	(74)
JEV	MK8245	Stearoyl-CoA desaturase-1	Fatty acid metabolism	(85)
CHIKV	C75	FASN	Fatty acid metabolism	(103)
	Cerulenin	FASN	Fatty acid metabolism	(103)
YFV	A939572	Stearoyl-CoA desaturase-1	Fatty acid metabolism	(83)
Adenovirus	CB-839	Glutaminase	Glutamine metabolism	(35)
HIV	VU0359595	PLD-1	Glucose metabolism, nucleotide synthesis	(48)
	Fasnall	FASN	Fatty acid metabolism	(107)
MersCoV	AM580	SREBP	Fatty acid metabolism	(100)
Influenza A	AM580	SREBP	Fatty acid metabolism	(100)
	CB-839	Glutaminase	Glutamine metabolism	(35)

post-transcriptional modifications (39), which are in line with the pace of the respective replication cycles.

As summarized in **Table 1**, our knowledge on the specific alterations induced by a given virus has resulted in numerous strategies to target viral replication with high efficacy in cell culture and *in vivo* models. However, because many of the established targets for metabolism-manipulating antivirals are central enzymes in cellular metabolism, future research will have to elaborate on whether the mentioned strategies can be translated into clinical applicability without causing major harm to unaffected host cells. Here, 2-DG in particular appears to be a promising compound, given its very well-established and favorable side-effect profile. Undoubtedly, further research in this dynamic area will help deepen our understanding of this interaction and might result in additional ways to impair viral replication by means of metabolic intervention. For instance, there are still major blind spots, particularly in our understanding of the mechanistic basis of RNA virus-induced alterations in cellular metabolism. Furthermore, there has been little research on the role of pattern recognition in the context of the above-mentioned adaptations. Additionally, many of the findings reported herein were generated in the context of highly specific cellular models, and differential modulations in different target cells (e.g., proliferating T cells) might result in adverse observations. We still have limited knowledge on the role of metabolism in the pathogenesis of a plethora of pathogenic viruses, which requires further research. Insights into these and other questions will help us to greatly advance our understanding of this crucial host–pathogen interaction and might sharpen our therapeutic arsenal to target this viral Achilles' heel.

AUTHOR CONTRIBUTIONS

KM and GG wrote the article. JS and GZ carefully revised the manuscript and provided critical intellectual input. All authors agreed to the final version of the article.

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OPINION
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Immunoregulatory Interplay Between Arginine and Tryptophan Metabolism in Health and Disease

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INTRODUCTION

Over evolution, some amino-acid catabolic pathways have become critical checkpoints in immunity (1–3). The associated immunoregulatory effects rely on the depletion of specific amino acids in the microenvironment and/or generation of biologically active metabolites (4). Consumption of L-arginine (Arg) by arginase 1 (ARG1) represents a well-known immunoregulatory mechanism exploited by M2 macrophages (5) and myeloid-derived suppressor cells (MDSCs) (6–8) in tumor settings. ARG1 is also expressed by human neutrophils (9). Indoleamine 2,3-dioxygenase 1 (IDO1)—a powerful immunosuppressive enzyme catalyzing the first, rate limiting step in L-tryptophan (Trp) catabolism—depletes Trp and produces immunoregulatory molecules collectively known as kynurenines (10–13). High IDO1 expression and catalytic activity occur in dendritic cells (DCs)—professional antigen presenting cells—in response to interferon-γ (IFN-γ) (8, 10, 11). Unlike ARG1, IDO1 is also endowed with non-enzymatic signaling activity in DCs that, in the presence of transforming growth factor-β (TGF-β) in microenvironments, leads to durable immunoregulatory effects (14, 15). In conventional DCs (cDCs), a relay pathway—marked by the sequential activation of ARG1 and IDO1—promotes a potent immunoregulatory phenotype (8, 16, 17). In this setting, spermidine, i.e., a polyamine produced downstream of the ARG1-dependent pathway (18), is capable of triggering IDO1 phosphorylation and signaling, and thus may represent the critical molecular interconnection between the two enzymes (8, 16). Here, we discuss the possible protective vs. pathogenetic roles of the interplay between IDO1 and ARG1 in reprogramming immune cell functions in neoplasia and autoimmune diseases.

THE ARG1 AND IDO1 INTERPLAY AS PHYSIOLOGIC IMMUNE CHECKPOINT

As all biological processes, immune responses rely on both energy-consuming and energy-producing pathways (19). The availability of specific substances and the immunological signature of the microenvironment directly control immune cell fate and functions. Pathogen-associated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs) molecules (recognized by pattern recognition receptors such as Toll-like receptors or TLRs) as well as amino acids, glucose, and fatty acids drive T-cell proliferation. Indeed, among immune cells, T lymphocytes are particularly dependent on nutrient availability and such feature (known as auxotrophy) has evolved as biological containment strategy that promotes the life-or-death decision (19).

By reducing the supply of indispensable amino acids, IDO1 and ARG1 directly suppress T cell proliferation and differentiation. The inadequacy of Arg and Trp substrates promotes a state of quiescence, whereby non-essential functions are temporarily quenched, including the cell cycle

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progression in the G_0-G_1 phase and the expression/activation of the TCR ζ-chain (2, 20, 21). IDO1 and ARG1 are indeed considered as physiological checkpoints ensuring a short-lived immunosuppression in normal pregnancies. In the placenta, DCs and extravillous trophoblasts highly expressing IDO1 and ARG1 secure a reversible T cell hyporesponsiveness and thus the survival of the fetus *in utero* (22, 23).

The activity of ARG1 and IDO1 translates not only into amino acid deprivation, but also in the production of metabolites endowed with several physiologic effects. L-kynurenine and spermidine, derived from Trp and Arg, respectively, are clear archetypes of non-inert byproducts that can influence immune and non-immune cell functions. In particular, L-kynurenine, by engagement of the aryl hydrocarbon receptor (AhR; a ligand-activated transcription factor), favors the differentiation of regulatory T (Treg) cells and induces IDO1 expression in DCs (24). On the other hand, the polycationic spermidine regulates cell growth and proliferation, and it affects several signal transducing pathways by interacting with ion channels, membrane receptors, and kinases (18).

Under specific conditions (as those dominated by TGF-β), Arg and Trp metabolic pathways are co-activated, thus potentiating the immunoregulatory phenotype of DCs and MDSCs (8, 25). The intimate relationship between ARG1 and IDO1 is allowed by spermidine, which activates the non-enzymatic functions of IDO1 and thus reprograms the cDC toward a long-term, immunoregulatory phenotype. More specifically, through Src kinase activation, spermidine induces the phosphorylation of IDO1, which, in turn, behaves as signaling molecule, promoting activation of the non-canonical pathway of NF-κB and induction of TGF-β1 and IDO1 expressions (3, 8). Contrary to spermidine, the small molecule nitric oxide (NO; derived from the Arg breakdown catalyzed by NO synthase) negatively regulates Trp metabolism, as it directly binds the heme prosthetic group and thus blunts the enzymatic function of IDO1 (26). However, besides this effect that would dampen IDO1-mediated immunosuppression, high levels of NO can combine with superoxide anion thus generating reactive nitrogen species that compromise both the activity and migration of T cells at the tumor site (27). Of note, it has been recently shown that AhR can sustain intracellular polyamines production at least in neoplastic conditions (28). However, whether such positive modulation belongs to a physiologic, bi-directional regulation program, where Trp metabolites and/or IDO1 itself affect ARG1 functions, has not been investigated yet.

ARG1 AND IDO1 IN NEOPLASIA

Difference in the metabolism of normal and cancer cells underlie the quest for more specific and less toxic therapies than those currently used. Tumor development is conditioned by genetic changes in malignant cells, immunological tolerance, and immunosuppression (29). At the initial stages of carcinogenesis, the immune system is capable of anti-tumor activity; however, cancer progression compromises the action of T helper type 1 (Th1)/Th2/Th17 lymphocytes via Treg cells, tumor-associated macrophages (TAMs), and MDSCs, resulting in immunosuppression and loss of reactivity to tumor antigens (30, 31). Recently, much attention has been dedicated to the influence of Arg and Trp metabolic pathways on both tumor cell growth and host's immune antitumor response. Arg is essential for the maturation of the TCR ζ-chain, and its deprivation impairs T cell ability to activate tumor immunity. MDSCs deplete Arg because they express high levels of ARG1, and their number increases 4–10 times depending on the type of cancer. For these reasons, in cancer immunotherapy studies, the effects of both deprivation and supplementation of Arg have been tested, the former on the assumption that tumors may be Arg auxotrophic, and the latter in an effort to counteract the detrimental effect of ARG1-competent, tumor-associated MDSCs on the host antitumor response. Overall, seemingly contradictory results were found in such oncological therapies based on the deprivation or supplementation of Arg, and those results are not easily reconciled (29). In particular, the high efficacy of subtracting Arg to Arg auxotrophic tumors may hardly explain *per se* the global protective effect of this maneuver, in that most tumors may ultimately activate the arginine-succinate synthetase (ASS1) pathway that enables synthesis of Arg from citrulline. The recent finding of a supportive influence of ARG1 on IDO1-dependent tolerogenesis (8)—which would impair host antitumor responses—suggests that it is not the Arg subtraction to the tumor that matters so much as the impairment of ARG1's supportive role in allowing full expression of the IDO1 mechanism in suppressing antitumor responses. In fact, $ARG1^+$ MDSCs, obtained by cell incubation with medium derived from mouse melanoma cells, can condition DCs to acquire an IDO1-dependent, immunoregulatory phenotype *in vivo* via production of polyamines (8, 16). Therefore, these data would sustain the existence of an immunosuppressive cross-talk mechanism between distinct cells present in tumor stroma and expressing ARG1 and/or IDO1 (32). The mechanisms whereby IDO1 acts as an immunosuppressant are multiple, and they are detailed elsewhere (2, 10, 11, 33).

There are, however, clinical settings where pharmacological administration of Arg resulted in cytoreductive effects in patients with Arg non-auxotrophic tumors (29). Paradoxical as it seems, this effect could again be explained by the relationship between ARG1 and IDO1 in immune cells. Increased ARG1 activity might lead to IDO1-dependent Trp starvation in cancer cells. Because Trp is an essential and the rarest of all amino acids, this likely results in an overall proteostatic action that affects fast-growing tumors, as discussed elsewhere in detail (3).

With specific regard to Arg auxotrophy, this phenomenon takes place in certain tumors and is caused by the silencing of ASS1 or arginine lyase genes. Those tumors are characterized by an intrinsic chemoresistance and thus a poor prognosis. Nevertheless, on a positive note, Arg auxotrophy theoretically favors the treatment of these tumors with Arg-degrading enzymes. Among the most frequently applied Arg-degrading agents are arginine deiminases (ADI) from bacteria. The antitumor effects of ADI derived from different bacteria have been extensively studied *in vitro* and *in vivo* [for review,

see (34)]. Mycoplasma-derived ADI-PEG20 is the one most commonly used and is under clinical investigation as a single agent therapeutic as well as in combination with other chemotherapeutic compounds. Mechanistically, ADI reduces metabolic activity in tumor cells, contributing to autophagy, senescence, and apoptosis in Arg auxotrophic cells (34). Although clinical trials are promising, the development of resistance after initial treatment is challenging, as illustrated above. Furthermore, an ADI interference within the tumor microenvironment is to be considered. Again, non-specific subtraction of the substrate for ARG1 may indirectly affect the host response to the tumor via effects on IDO1.

Another important issue in cancer is the expression of Arg and Trp transporters in tumor and immune cells. Among Arg carriers (cationic amino acid transporters or CATs), the most important appear to be CAT1, which is constitutively expressed in several tissues, and CAT2B, normally inducible by inflammatory cytokines (35). CAT1 is often overexpressed by tumor cells, and event that can favor tumor growth. In an experimental model of prostate cancer, CAT2B, which allows a rapid transport of Arg into the cell, is expressed at higher levels in tumor-infiltrating as compared to peripheral MDSCs (36). Moreover, the upregulation of CAT2 is coordinated with the induction of both NOS2 and ARG1, thus further favoring Arg uptake by MDSCs at the tumor site. Subsets of human melanoma cells are also characterized by very high levels of CAT2B expression, possibly due to the secretion of inflammatory mediators by the tumor cells themselves (35). Overexpression of Trp carriers (mainly, LAT1/CD98 and SLC6A14) is also involved in the increased proliferation and chemoresistance of several tumor cell types (37). Because SLC6A14 is a broad specific amino acid transporter that can also transfer Arg and its expression can be upregulated by IDO1 (by a mechanism not identified yet) (38), the "doors" for the cell entrance of Arg and Trp may represent suitable cancer drug targets capable of interfering with both ARG1 and IDO1 pathways (39).

Therefore, new insight is definitely needed into the molecular mechanisms underlying the antitumor effects of Arg starvation in both host and tumor, which might facilitate the refinement of IDO1 inhibitory approaches in cancer immunotherapy.

ARG1 AND IDO1 IN AUTOIMMUNITY

The use of checkpoint inhibitors in tumor immunotherapy is frequently accompanied by the development of autoimmune diseases (40), suggesting that the exploitation of immune checkpoint molecules could be a valid therapeutic means in autoimmunity (4, 41). Because both ARG1 and IDO1 act as immune checkpoint mechanisms in neoplasia, their functional "alliance" in specific immune cells could be remarkably effective in controlling adaptive immunity toward auto-antigens.

IDO1 is defective in DCs of non-obese diabetic (NOD) mice (42), an experimental model of human autoimmune diabetes (type 1 diabetes or T1D), and maneuvers aimed at enhancing its expression and activity will exert therapeutic effects in prediabetic and also overtly diabetic animals (43, 44). In T1D patients, a significantly reduced IDO1 expression can be observed in peripheral blood mononuclear cells (PBMCs) (17) and in pancreatic β cells (45), normally producing insulin. In PBMCs, the defect can be corrected by tocilizumab, a blocker of the interleukin 6 (IL-6) receptor, which inhibits the IL-6–dependent, IDO1 proteasomal degradation (17). In T1D, although its expression and function in immune cells remains unclear, endothelial ARG1 induces the vascular dysfunction associated with hyperglycemia (46). Moreover, administration of difluoromethylornithine (DFMO), a potent inhibitor of polyamine production, protects NOD mice from the development of diabetes.

In experimental models of rheumatoid arthritis (RA), an inflammatory/autoimmune disease of the capsule surrounding joints, lack of IDO1 expression reduces the time to develop a more severe disease (47). Moreover, the protective effects of interferon-α rely on the activation of a TGF-β/IDO1 axis in plasmacytoid DCs (48). Although $ARG1^+$ M2 macrophages contribute to resolve arthritis inflammation in mice (49), ARG1 activity may be responsible for subclinical endothelial dysfunction also in RA patients (50). Interestingly, methotrexate, an immunosuppressive drug widely used in RA, greatly inhibits the synthesis of polyamines in lymphocytes of RA patients (51).

A definitely clearer picture is emerging in autoimmune neuroinflammation. Administration of 3-hydroxyanthranilic acid (3-HAA; a Trp metabolite of the kynurenine pathway) (52) or of an orally active synthetic derivative thereof (53) ameliorates neuroinflammation and paralysis in mice with acute experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS). Moreover, 3-HAA–treated DCs express higher levels of TGF-β and induce the generation of Treg cells (52). Conversely, administration of 1-methyltryptophan (1-MT), a standard inhibitor of IDO1, exacerbates the clinical course of EAE (54, 55). In leukocytes infiltrating the spinal cord of untreated mice, IDO1-expressing cells exhibit the same morphology as activated macrophages/microglia (54). VCE-004.8, a semisynthetic cannabinoid, protects from EAE, possibly by upregulating ARG1 in macrophages and microglia (56). A $Lewis^X$ trisaccharide of schistosome eggs reduces EAE severity by a TLR-mediated mechanism that enhances both ARG1 and IDO1 expression in $CD11b^+Ly\text{-}6C^{hi}$ inflammatory monocytes (57). Expression and activity of ARG1 and IDO1 are significantly reduced in PBMCs from MS patients as compared to healthy control subjects (58). Spermidine, the polyamine produced downstream ARG1, protects from autoimmune-directed demyelination of neurons in acute EAE (59). The effect appears to be related to an immunosuppressive function acquired by $ARG1^+$ macrophages, since (i) their depletion or the administration of an ARG1 inhibitor abolishes spermidine therapeutic activity *in vivo* and (ii) the polyamine induces ARG1 in macrophages (59).

Therefore, although in both T1D and RA the pathways of Arg and Trp metabolism do not seem to be properly interlinked (and this may require cautions when attempting immunotherapies potentiating both ARG1 and IDO1), in MS, the pieces of evidence, when put together, would suggest that the induction

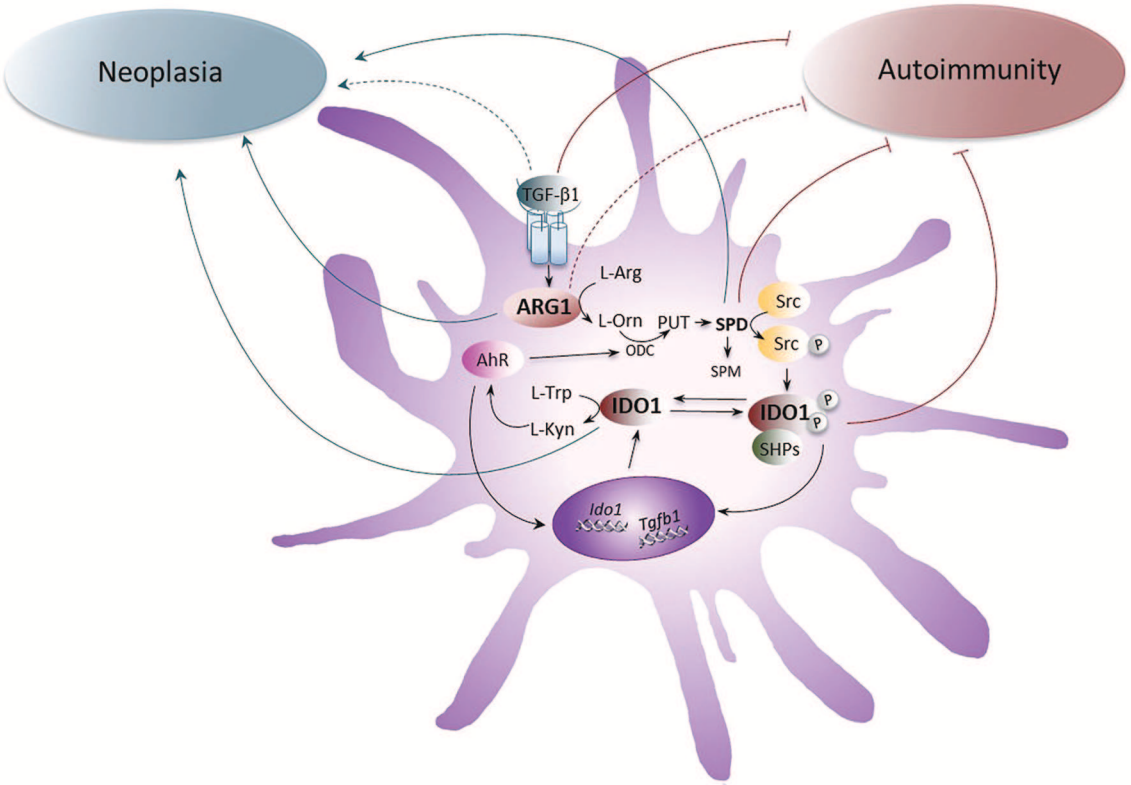

FIGURE 1 | The role of ARG1 and IDO1 in neoplasia and autoimmunity. The up-regulation of ARG1 activity, induced by the cytokine TGF-β, transforms L-arginine (L-Arg) into L-ornithine (L-Orn), which is further metabolized by ornithine decarboxylase (ODC) into polyamines (PUT, putrescine; SPD, spermidine; and SPM, spermine). SPD, through the activation of the Src kinase, promotes the phosphorylation of IDO1 and thus favors the initiation of immunoregulatory signaling events in DCs. Once phosphorylated, IDO1 recruits tyrosine phosphatases (SHPs) and promotes a signaling pathway that upregulates the expression of genes coding for IDO1 and TGF-β, thus creating a self-sustaining circuitry responsible for the maintenance of immune tolerance over the long-term. Moreover, IDO1 catalyzes the conversion of L-tryptophan (L-Trp) into L-kynurenine (L-Kyn), which activates the aryl hydrocarbon receptor (AhR). AhR further induces IDO1 expression in DCs and sustains the production of polyamines by up-regulating ODC. Whereas the pathogenetic and protective role of TGF-β, SPD, and IDO1 in neoplasia and autoimmunity, respectively, have been demonstrated, the role of ARG1 has been unclear and would require further investigations. Gray arrows indicate the pathogenetic effects of IDO1, ARG1, SPD, and TGF-β1 receptor signaling in neoplasia and brown arrows indicate the putative protective effects of IDO1, SPD, ARG1, and TGF-β1 receptor signaling in autoimmune diseases. Dotted lines are for molecules whose role is still unclear.

of the immunosuppressive interplay between ARG1 and IDO1 would represent a valid therapeutic objective.

CONCLUSIONS AND PERSPECTIVES

In neoplasia, both ARG1 and IDO1 are often overexpressed, either singly in tumor cells themselves (IDO1) or in association (i.e., both enzymes) in MDSCs and DCs, and they contribute to the impairment of the host anti-tumor immunity. However, the effect of Arg starvation on tumor cells may dampen their proliferation and therefore ARG1 inhibition as therapeutic strategy may have some caveats (**Figure 1**). In the majority of autoimmune disorders, the bulk of data would suggest that IDO1, expressed by either DCs or macrophages, stands out as an effective immune checkpoint molecule. In contrast, more often than not, ARG1 appears to be more pathogenetic than protective, possibly owing to the enzyme capacity to subtract Arg for NO production, which can be necessary for the resolution of damages induced by autoimmunity (4, 60). However, in autoimmune neuroinflammation, the available cues would indicate that both ARG1 and IDO1, expressed by macrophages and/or DCs, act as immune checkpoint molecules in EAE and that spermidine, i.e., the molecular connection between the two enzymes in a physiologic setting (8), exerts significant therapeutic effects on its own. Therefore, further investigations on Arg metabolism in neoplasia and autoimmune disorders and its possible cross-talk with IDO1 are needed for a full understanding of its role, protective vs. pathogenetic.

AUTHOR CONTRIBUTIONS

GM, AI, MA, PP, and UG equally contributed in writing the manuscript. UG supervised the final form.

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REVIEW
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Dynamic Metabolic State of Tissue Resident CD8 T Cells

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In the past years, there have been significant advances in the understanding of how environmental conditions alone or in conjunction with pathogen invasion affect the metabolism of T cells, thereby influencing their activation, differentiation, and longevity. Detailed insights of the interlinked processes of activation and metabolism can contribute to major advances in immunotherapies. Naive and memory T cells circulate the body. In a quiescent state with low metabolic demands, they predominantly use oxidative phosphorylation for their energy needs. Recognition of cognate antigen combined with costimulatory signals results in a proliferative burst and effector molecule production, requiring rapid release of energy, achieved via dynamically reprogramming metabolic pathways. After activation, most T cells succumb to activation induced cell death, but few differentiate into memory T cells. Of note, some memory T cells permanently occupy tissues without circulating. These, tissue resident T cells are predominantly CD8 T cells, maintained in a metabolic state distinct from naïve and circulating memory CD8 T cells with elements similar to effector CD8 T cells but without undergoing proliferative burst or secreting immune mediators. They continually interact with tissue cells as part of an immune surveillance network, are well-adapted to the tissues they have made their home and where they may encounter different metabolic environments. In this review, we will discuss recent insights in metabolic characteristics of CD8 T cell biology, with emphasis on tissue resident CD8 T cells at the epithelial barriers.

Keywords: T cells, metabolism, tissue resident cells, CD8 T cell, intraepithelial lymphocyte (IEL)

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INTRODUCTION

T lymphocytes, especially CD8-expresssing cytotoxic T cells, play a critical role in immune responses to intracellular microorganisms and cancer cells. Naive CD8 T cells are present in the circulation and secondary lymphoid organs (SLOs), where they encounter dendritic cells (DCs) presenting antigens. Naïve T cells may survive for a significant time, only rarely undergoing cell division. One of the underpinning concepts of immunity is the clonal expansion of T cells upon activation. After recognition of cognate antigen via the T cell receptor (TCR) in the context of appropriate co-stimulatory signals, CD8 T cells undergo rapid expansion and traffic from SLOs to the tissues. They differentiate into effector T cells, gaining cytotoxic activity characterized by the ability to release perforin and granzymes. In addition, they can secret large amounts of cytokines, such as TNF and IFN-γ (1). The majority of effector cells generated die by apoptosis, but a small population remains and develops into memory CD8 T cell subsets. Memory T cells were thought to circulate and pass through SLOs, similar to naïve T cells, but in larger antigen-specific numbers and with the intrinsic ability to respond more rapidly to reencountered antigens. These memory T cells

are now referred to as central memory T cell (T_{CM}) after the recognition that effector memory T cells (T_{EM}) circulate through the SLOs as well as the tissues (2). Although initially all memory T cells encountered in tissues were considered T_{EM} cells, more recent work has established a dedicated population of tissue resident memory T cells (T_{RM}), which do not circulate through the SLOs and provide a first line of tissue defense at the place of initial antigen encounter (3).

In the past few years it has become evident that CD8 T cell function, differentiation and numeric presence is dependent on nutrient availability, uptake, and processing (4). During different stages of CD8 T cell activation, cells go through dynamic alterations in their metabolic capacity and substrate use. These metabolic changes impact the cells bioenergetic and biosynthetic demands related to substrate uptake, mitochondrial function, and protein and lipid synthesis, ultimately influencing cell division, differentiation and effector capacity (5). In this review, we will discuss the recent findings shedding light on the intertwined relation between metabolic pathways and T cell biology, with focus on CD8 T cells, especially those that have taken residence in peripheral tissues.

METABOLISM OF NAIVE CD8 T CELLS

Metabolic demands of antigen inexperienced naive T cell are low. Their quiescent state needs to maintain the ability for base level proliferation only and metabolic activity largely serves to support cell migration and survival upon cells moving through the blood stream, lymph, and SLOs. Antigen inexperienced T cells use oxidative phosphorylation (OXPHOS), which generates an estimated 96% of energy needs (6). OXPHOS is the main source of energy in most eukaryotic cells, most efficiently obtaining energy in the form of adenosine triphosphate (ATP) by oxidizing nutrients using specialized enzymes in the mitochondria (7). OXPHOS can use a variety of substrates such as glucose, amino acids, and fatty acids, converted to acetyl-CoA, which enters the tricarboxylic acid cycle (TCA) cycle.

The quiescent metabolic status of naive CD8 T cells is not a default setting due to the cells lack of receiving any activating signals, but is actively maintained. Naïve T cells receive constant signals from cytokines such as IL-7, critical in sustaining basal levels of nutrient transporters, like GLUT1 for glucose uptake, and expression of anti-apoptotic proteins (Bcl-2), required for the long term survival of naïve T cells (8, 9). In addition to obtaining energy from glucose, naive T cells can oxidize lipids, such as oleate and palmitate (10).

GLYCOLYSIS IN EFFECTOR CD8 T CELLS

The activation of T cells rapidly switches the metabolic programmes from OXPHOS toward aerobic glycolysis, PPP, and glutaminolysis. The shift in metabolism is associated with a change in metabolic transcriptome, with mammalian target of rapamycin (mTOR), hypoxia-inducible factor 1 (HIF1), and c-MYC amongst the most prominent factors with the ability to rewire cell metabolism. In T cells, the mTOR pathway upregulates nutrient uptake (especially amino acids), activates glycolytic pathways and promotes cap-dependent translation. Activated CD8 T cells deficient in mTOR, or CD8 T cells treated with mTOR inhibitor rapamycin, become anergic, cannot proliferate and are incapable of metabolic reprogramming during activation (11). Molecular mechanisms by which mTOR influences T cell metabolism and differentiation are discussed elsewhere (12). In activated CD8 T cells, HIF1 upregulates aerobic glycolysis by promoting the transcription of the enzyme pyruvate dehydrogenase kinase 1 (Pdk1) and lactate dehydrogenase A (Ldha) (13). Another transcription factor required for the increase of glycolysis and glutaminolysis in activated CD8 T cells is the transcription factor c-Myc, transcriptionally regulating GLUT1 expression levels. Deletion of Myc abrogates activation induced proliferation and effector function of CD8 T cells *in vitro* and *in vivo* (14, 15).

Glycolysis is a highly conserved metabolic pathway that, independent of oxygen, converts glucose via a series of enzymatic reactions in the cytosol of cells into pyruvate (16). Despite its name, glycolysis does not solely use glucose, most monosaccharides can be converted into pyruvate. Pyruvate can be transported into the mitochondria and oxidized to generate acetyl-CoA. Alternatively, pyruvate remains in the cytosol and is converted into lactate. Lactate production was thought to occur as a consequence of anaerobic glycolysis, when the coenzyme nicotinamide adenine dinucleotide (NAD) required for glycolysis can be in short supply, but it can be produced as part of aerobic glycolysis (Warburg effect). Lactate is produced upon high-energy demands, such as T cell activation, possibly because of limited availability of NAD. Limited NAD availability may result in a switch to lactate production, which itself supplies additional NAD for continued glycolytic flux. Importantly, the production of lactate does not reduce the amount of pyruvate used for OXPHOS and both aerobic glycolysis and OXPHOS pathways are increased during cell activation (15, 17).

The importance of glycolysis for cytotoxic T cell function was shown using the glycolysis inhibitor 2-deoxyglucose (2DG), resulting in defective T cell cytotoxic capacity and selective reduction of the expression of key effector molecules, including IFN-γ and granzymes (18, 19). Of importance, enzymes involved in glycolysis can make direct contributions to T cell function. Increasing glycolysis capacity upon T cell activation result in the engagement of cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in catalyzing the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate, releasing it from binding to IFN-γ, thereby enabling its translation by human and mouse CD8 T cells (17, 20).

The reason for lactate production remains uncertain, but the energy needs may be acutely high so that the ATP production from rapid glycolysis alone is more efficient, possibly due to limited amounts of NAD+ required in the respiratory chain (21). Lactate can be oxidized back to pyruvate to be used for OXPHOS in some organs, such as muscle and brain, or can be converted to glucose via gluconeogenesis in the liver to be release back into the circulation. The latter would have the potential to sustain or control high-energy demand processes such as immune responses via the liver and its systemic glucose

level maintaining capacity (22). In addition, lactate can have direct immune- and cell-modulating properties (23, 24). Lactate can inhibit the motility of T cells, arresting them at the site of inflammation, thereby focussing the T cell response (25). The latter may contribute to chronic inflammatory disorders, although CD8 T cell cytolytic function is also inhibited by lactate, possibly acting as a safeguard to prevent immunopathology.

Aerobic glycolysis rapidly generates biosynthetic precursor molecules, can function under otherwise adverse hypoxic or acidic microenvironments, entraps T cells at inflammatory sites and may provide systemic control via blood glucose levels (22, 26). Hence, glycolysis may provide several advantages during T cell activation and inflammation and even contribute to immune resolution.

OXPHOS IN EFFECTOR CD8 T CELLS

Activation of CD8 T cells does not result in a complete shift from mitochondrial respiration to aerobic glycolysis. OXPHOS levels increase and remain an important ATP contributor to provide the full complement of factors needed for cell proliferation of activated T cells. The increased emphasis on aerobic glycolysis during CD8 T cell activation and parallel increase of OXPHOS may enable other substrates, such as fatty acids and glutamine, to enter the mitochondria to fuel the TCA cycle (14, 15, 27) (**Figure 1**). T cell activation in the absence of glucose significantly weakens T cell proliferation and function, but this can be partly rescued by supplying pyruvate or galactose. This highlights that mitochondrial respiration remains important in the process of T cell activation. Cells grown in galactose are forced to respire and do not use aerobic glycolysis, generate ATP at a slower rate and produce less IFN-γ compared with cells activated in the presence of glucose (17).

OXPHOS is accompanied by a production of mitochondrial reactive oxygen species (mROS), which increases during T cell activation. mROS plays a role in the activation and subsequent nuclear localization of nuclear factor of activated T cells (NFAT), responsible for the transcription of the T cell growth factor interleukin (IL)-2. In line with this, T cells from Uqcrfs-deficient mice (complex III subunit 5) show decreased activation and diminished clonal expansion upon *in vivo* antigen encounter (28). Furthermore, increased expression of lymphocyte expansion molecule (LEM), which positively regulates the expression of the mitochondrial electron transport chain complex, controlling the activity of OXPHOS proteins and mROS production, resulted in increased CD8 T cell proliferation and function (29). The removal of LEM reduced CD8 T cell proliferation and reduced levels of mROS. Inhibition of mROS in LEM protein overexpressing mice was sufficient to reduce CD8 T cell proliferation and cytotoxicity, confirming the importance of mROS in CD8 T cell activation.

METABOLISM OF CD8 MEMORY T CELLS

Although memory formation underpins immune protection and is the basis for vaccination success, how memory T cells are formed is not well-understood. However, their long-term maintenance and ability to respond swiftly upon TCR ligation is, at least in part, due to rewiring of their metabolic pathways. Interestingly, memory formation and improved effector function are often associated with suboptimal T cell activation or metabolite availability, such as reducing mTOR activity and decreased glucose or oxygen levels (12, 30–33). Adenosine monophosphate-activated protein kinase (AMPK) restrains mTOR activity, thereby reducing glycolysis and promoting transition from CD8 effector to memory T cell (34, 35).

There are several different characteristics in metabolic make up between CD8 memory T cells compared to naive and effector CD8 T cells (**Table 1**). CD8 memory T cells show an enhanced mitochondrial OXPHOS capacity that can provide extra energy required for the proliferative burst. CD8 memory T cells also possess greater mitochondrial mass compared to CD8 effector T cells (36). Although primarily found in non-lymphoid tissues (37), CD8 T_{EM} cells show increased uptake of blood glucose through the glucose transporter-1 (Glut-1) and rely on glycolysis as well as OXPHOS to meet their energy demands (36, 38). As such, although basic energy requirements are reduced, CD8 T_{EM} cells seem metabolically wired in similar fashion as effector T cells. CD8 T_{CM} cells on the other hand, predominantly depend on OXPHOS for both their differentiation and maintenance. They take up lipids and glucose from blood, for lipid synthesis, and use FAO as originally described for all memory CD8 T cells (39, 40).

LIPID METABOLISM IN CIRCULATING CD8 MEMORY T CELLS

CD8 memory T cells depend less on glycolysis and mainly rely on oxidative phosphorylation and lipid metabolism (36). A role for fatty acid metabolism in CD8 memory T cells was first suggested in mice that lack tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which show similar CD8 T cell activation and expansion but defective CD8 memory T cell generation (34). Although TRAF6 is implicated in several pathways, in CD8 T cells it stimulates AMPK while inhibiting mTOR signaling, thereby increasing fatty acid oxidation (FAO) (41). Upon activation T cells acquire extracellular glucose, which is used to synthesize lipids, can be stored in lipid droplets and subsequently used in FAO (39, 40, 42–44) (**Figure 1**). Furthermore, memory CD8 T cell development depends on cell intrinsic activity of lysosomal acid lipase A (LIPA) to mobilize fatty acids for FAO (39). The role of lipids stored in droplets during the effector phase remains unknown. Processes of lipolysis and autophagy can degrade lipids for substrate use in metabolic processes (45). Under optimal conditions, such as in SLOs, lipolysis may not be critical. The appearance of lipid droplets *in vitro* takes place within the first 24 h after activation (44). However, upon nutrient deprivation, such as encountered in tissues during inflammation or in the tumor environment, autophagy and lipolysis could become an important contributor to energy demands.

Although FAO is important for memory formation, established memory T cells contain few lipid droplets compared

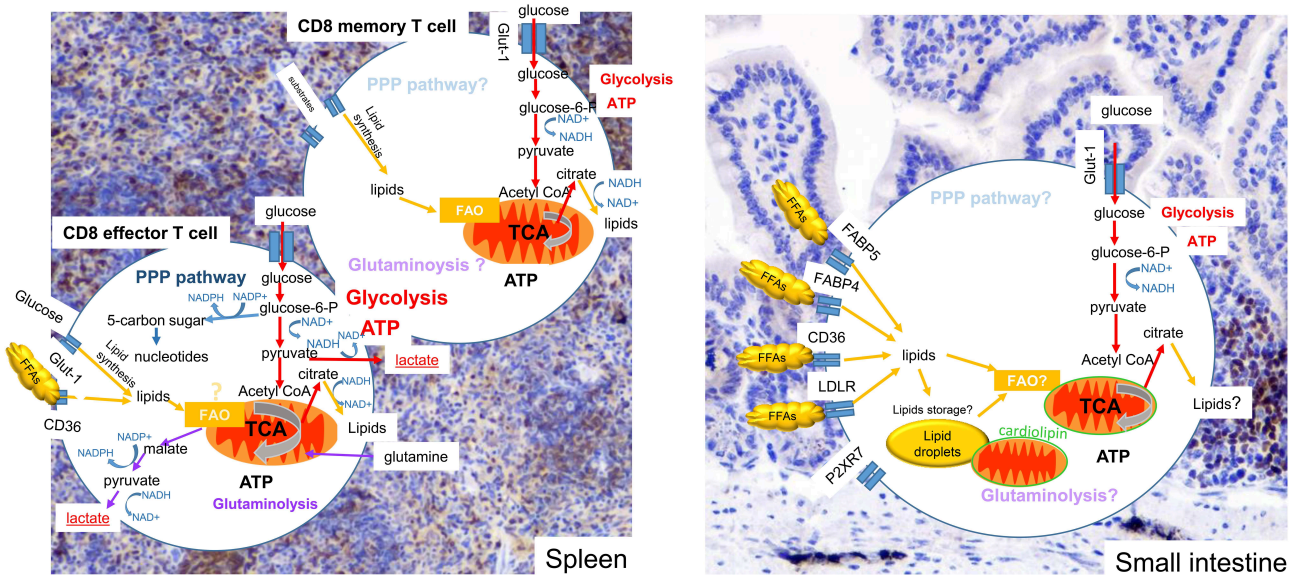

FIGURE 1 | Metabolism of CD8 T cells in SLOs and small intestine. Subtypes of CD8 T cells in different tissues display more or less dependence on particular metabolic pathways and substrates to promote cell survival, activation ability and function. Colored arrows show pathways reported to be used in the cell type indicated, pale text indicates pathways that might be used but have not been clearly defined. **(A)** Effector CD8 T cells in SLOs engage OXPHOS and glycolysis. Pyruvate is mainly converted to lactate, but remains an important metabolite for the TCA cycle. Metabolizing glucose through the PPP can benefit the generation of nucleotides and NADPH, used for lipid synthesis. In addition, glutaminolysis may also be engaged. Memory CD8 T cells use OXPHOS, but also use metabolites provided by glycolysis. **(B)** Tissue resident CD8 T cells (T_{RM}) engage OXPHOS and glycolysis. There are increasing indications that OXPHOS is dependent on lipids, either obtained from the local environment or released from internal lipid droplet stores. Furthermore, mitochondria of T_{RM} cells at the epithelia have are more numerous, but have an altered cardiolipon composition, contributing to a poised activation state curtailing OXPHOS potential. The importance of glycolysis for T_{RM} cells remains unknown.

with effector T cells (39, 44). Memory T cells are not known to increase the uptake of lipids from their surroundings, but can use alternative sources such as glycerol, to generate lipids (43, 46) (**Figure 1**). This suggests that the increased potential of memory T cells for OXPHOS is not explained by FAO, confirm recent results using carnitine palmitoyltransferase I (CPT1)-deficient cells, which cannot generate acetyl-CoA from long chain fatty acids (47).

TISSUE RESIDENT CD8 T CELLS

In addition to circulating memory T cells, a more recent subtype of memory T cells, called resident memory T cells (T_{RM}) has been described. T_{RM} cells are memory T cells that do not circulate and are predominantly found in non-lymphoid tissues (48, 49), although they have been reported in SLOs (50). In mice, the epidermis, forming the top layer of the skin, is home to specialized T cells, expressing TCRγδ. This population develops during embryogenesis and homes to the epidermis. They have a type 1 immune profile with the ability to produce IFN-γ. The small intestine are another tissue forming a large interfaces between the environment and the body and endowed with a specialized population of CD8 T cells that occupying the very top layer of the tissue, the intraepithelial lymphocyte (IEL) compartment. Similar to the epidermal compartment, innate-like CD8 T cells occupy the murine small intestine early in life, predominantly specialized TCRγδ CD8αα homodimer-expressing T cells with a type 1 immune profile, which home specifically to the IEL compartment. In humans, the IEL compartment is mainly composed of TCRαβ T cells with γδ T cells reported in the minority (51). Induced IELs or CD8 T_{RM} cells intercalate with innate-like small intestinal and skin IELs, predominantly expressing TCRαβ and the CD8αβ heterodimer (52, 53). The population of CD8 T_{RM} possess a distinct genetic signature compared with circulating CD8 T cells and are often defined by cell surface expression of CD69, CD103, and CD49a (54). The expression of CD49a and CD103 is indicative of cell interactions with collagen and E-cadherin epithelial tissues (54). Expression of CD69 together with expression of NK cell inhibitory receptors (CD244), and high levels of granzyme B marks CD8 T_{RM} cells as semi-activated T cells (53).

T_{RM} cells are metabolically highly active, continuously scanning the tissues for invasion using migration and long dendrite-like protrusions (52, 55), in line with their semi-activation status. Yet, their development is more in line with CD8 T_{CM} cells than T_{EM} cells (35). T_{RM} cells protect the host through rapid responses upon re-exposure to previously encounter pathogen as well as contribute to immune responses against newly encountered microorganisms via bystander activation (3, 56). Activation of CD8 T_{RM} cells influences the surrounding

TABLE 1 | CD8 T cells subset with their identifiable markers, effector, and metabolic status.

Subset of CD8 T cells	Naive	Circulating memory ($T_{cm/EM}$)	Effector	Tissue resident
Identity markers	$CD44^{1\circ}$ $CD62L^{+}$ $CCR7^{+}$ $CD103^{-}$ $CD69^{-}$	$CD44^{hi}$ $CD62L^{+/-}$ $CCR7^{+/-}$ $CD103^{-}$ $CD69^{-}$	$CD44^{hi}$ $CD62L^{-}$ $CCR7^{-}$ $CD103^{-}$ $CD69^{+}$ (early)	$CD44^{hi}$ $CD62L^{-}$ $CCR7^{-}$ ($CD49a^{+}$), $CD103^{+}$, $CD69^{+}$
Effector status				
• Immune function	Surveillance SLO	Surveillance of SLO/SLO + tissues	Search and kill infected or tumorigenic cells of all tissues	Surveillance of tissues previously infected
• Cell status	Quiescent	Quiescent	Active	Poised
• Secretory vesicles (Grzm)	Absent	Low	High	High
• Cytotoxicity (TCR/CD28)	TCR + CD28	TCR + CD28	TCR	TCR or cytokines
Metabolic status				
• Status	Quiesent	Primed	Active	Arrested
• Nutrient uptake	Basal	Basal	High	High(?)
• SRC	High	High	Low	Low(?)
• Glycolyisis	Basal	Basal	High	Basal(?)
• OXPHOS	Basal	Basal	High	Basal
• Lipid metabolism	+	++	++	++
• Glutaminolysis	–	–	+	?
• Biosynthesis	Minimal, no net growth	Synthesis/oxidation balance, no cell proliferation	High, Cell growth, proliferation	No net growth/cell proliferation

The table highlights the main characteristics, in common and setting apart, the CD8 T cell subsets; naïve, effector, circulating memory, and tissue resident memory.

tissue cells, such as a number of broad acting antiviral and antibacterial genes (57), increasing local defenses and decreasing susceptibility to invasion. Release of inflammatory molecules such as IFN-γ, results in the recruitment of additional myeloid and lymphoid cells, maturation and migration of local dendritic cells and activation of natural killer cells (58). In addition to sentinels of microbial invasion, T_{RM} cells bridge the adaptive immune system with the innate immune system (57, 58).

The integration of CD8 T_{RM} cells within tissues involves adaptation to the local environment compatible with their role in clearing potential pathogens, which necessitate changes in T cell metabolism for their maintenance as well as functional potential. A prime feature of T_{RM} cells is their ability to deeply penetrate tissues and their long-term maintenance within it. T_{RM} have to adapt to the new environment of periphery, which can differ in oxygen levels, nutrient availability, acidity, competition for resources in comparison to the environment of lymphatic organs (59, 60).

CD8 T_{RM} cells, not circulating CD8 T cells, express high levels of the purinergic receptor P2RX7, triggered by extracellular nucleotides, associated with tissue damage and exported by activated T cells (35, 61, 62). P2RX7 is involved in the establishment, maintenance and functional properties of CD8 T_{CM} and T_{RM} cells (35). Although tissue damage and stress have been associated with the activation of tissue integrated CD8 T cells, engagement of P2RX7 can result in the selective cell death of CD8 T_{RM} cells. The balance between activation and cell death is carefully orchestrated and context dependent, low concentrations are able to activate T cell and high concentrations result in cell dead (63). However, P2RX7 can be activated directly by ATP, or indirectly via NAD-dependent ADP-ribosylation by the ecto-ADP-ribosyltransferase ARTC2.2. The concentration of NAD thereby lowering the threshold of ATP concentrations required inducing apoptosis. This may be part of protecting tissues from aberrant immunity in the absence of cognate antigen reencounter since TCR stimulation of CD8 T_{RM} cells reduces P2RX7 expression and susceptibility to cell death. Recent data highlight the importance of both receptors in studying tissue resident T cells, the isolation process resulting in tissue damage and release of ATP and NAD, can causing reduced T_{RM} cell viability and compromises functional assessment (62, 64).

Aberrant regulation of IELs can compromise barrier function and increase susceptibility to infection and immunopathology (44, 65), which can ultimately contribute to inflammatory bowel disease and psoriasis (66, 67). These findings underscore the physiological significance of tissue integrated CD8 T cells in tissue homeostasis and disease. Due to different environmental conditions and their semi-activation status, the metabolic wiring of T_{RM} cells has to be adjusted, the details of which may depend on the host tissue. T_{RM} cells posse some similarities with T_{CM} and T_{EM} cells. We will here discuss some of the recent finding concerning T_{RM} cells and their metabolism.

CD8 T_{RM} CELLS AND LIPID METABOLISM

Lipids are, in comparison to SLOs, abundant at epithelial barriers where T_{RM} cells persist (59, 60). IELs, in skin and intestine, have adapted to a lipid-rich microenvironment. Early on, it was recognized that intestinal T_{RM} cells express required surface molecules to obtain lipids from the extracellular space, including low density lipid receptor (LDLR), ApoE, scavenger receptor CD36, and fatty acid biding proteins (FABP) 4 and 5

(**Figure 1**), suggesting an important role for lipid metabolism (68). FABP proteins are involved in FFAs fatty-acid influx and transfer from cytosol to mitochondria for the purpose of β-oxidation (69). In addition to regulating fatty acid influx, in macrophages FABP4 is involved in the nuclear factor-κB (NF-κB) pathway and stimulates pro-inflammatory effector function such as production of cytokines and inducible nitric oxide synthase (iNOS). Furthermore, FABP4 reduces cholesterol ester accumulation via inhibition of peroxisome proliferator-activated receptor-γ (PPAR-γ) pathways and is involved in integrating lipid signals to organelle responses, especially the endoplasmic reticulum (ER) (70). FABP5 is highly expressed in epidermal cells, but is found in many organs. Its function is to enhance lipolysis (71). Due to compensation mechanisms, combined deletion for FABP4/5 show a much stronger phenotype. Adipocytes and macrophages from double deficient mice have an altered lipid profile, in favor of shorter-chain fatty acids (C14). These changes result in higher glucose uptake, AMPK activity, and fatty-acid oxidation (72).

How skin CD8 T_{RM} persist and function in a lipid rich environment remained elusive (59, 60). More recently, Pan et al. (43), using a mouse model of cutaneous immunization with Vaccina virus, showed that CD8 T_{RM} cells in the skin adapt to utilize lipid metabolism using free fatty acids (FFA) obtained from the surrounding microenvironment for their endurance as well as effector function. Activation of CD8 T_{RM} cells fosters a transcriptional program that features notable increased expression of molecules facilitating exogenous FFA uptake and storage. Compared with naïve and circulating memory CD8 T cell subsets, sCD8 T_{RM} cells, in mouse and human, were able to express high levels of FABP4/5 and CD36 and lipoprotein lipase (LPL) (43). T cell specific deletion of FABP4/5 showed an impairment in FFA uptake in CD8 T_{RM} cells, limiting OXPHOS potential and reducing the survival of skin CD8 T_{RM} cells but not circulating memory CD8 T cells (43).

Intestinal CD8 T_{RM} cells show a similar transcriptional programme to skin T_{RM} cells, with pathways involved in FFA and cholesterol ester synthesis increased compared with naive and memory CD8 T cells (44, 68). Furthermore, intestinal IELs store accumulated FFA in lipid droplets (44), from which FFA can be made available for FAO via autophagy or via mitochondria tethered to the lipid droplet (73). These observations suggested a reliance of IELs on FAO. However, the accumulation of lipids is a characteristic of activated T cells and does not appear unique to IEL (36, 39, 44). The conditions under which FFA are made available and used for FAO remain unclear with IELs performing basal OXPHOS without additional capacity upon mitochondrial uncoupling (43, 44, 55). Yet, short-term culture of skin IELs with FFA or intestinal infection with *Salmonella* does result in a modest increase in OXPHOS (43, 55). The data suggests that the trigger that makes available FFA from lipid droplets is likely the same that releases additional OXPHOS potential in T_{RM} cells.

Transcriptomic analysis of IEL during intestinal *Salmonella Typhirum* challenge compared to steady state IELs revealed, once more, increased expression of genes involved in metabolism (55). *Salmonella* infection in the small intestine resulted in increased aerobic glycolysis, glucose uptake, as well as OXPHOS by IELs, similar to effector CD8 T cells (17, 36). In addition, IELs altered their immunosurveilance behavior upon infection, suggesting that the IEL semi-activation status can be further enhanced upon microbial encounter. In support of this, the use of the glycolysis inhibitor 2DG as well as mTOR inhibitor rapamycin resulted in increased *salmonella* burden and invasion. Although 2DG treatment would target many cells involved in the respond to enteric infection (74), GLUT1-deficient innate-IELs revealed the requirement for glycolysis in IELs. However, upon *Salmonella* infection a proliferative response was not detected (55). Collectively, IELs store large amounts of energy in the form of lipids, the signals resulting in the release of these remain unknown, but in line with effector T cells, IELs require both glycolysis and OXPHOS for their effector functions.

CD8 T_{RM} CELLS AND MITOCHONDRIA

Lipid droplet associated mitochondria are biochemically distinct from non-associated cytoplasmic mitochondria, with the later primarily using pyruvate as a substrate (75). These recent data could explain the observations that FAO and fatty acid synthesis seem to take place at the same time within memory T cells (39), but individual mitochondria can only perform one or the other. Interestingly, mitochondria in brown fat associated with lipid droplets, were shown to have reduced FAO capacity compared with cytoplasmic mitochondria. This suggest they are involved in lipid storage under steady state conditions and, upon exposure to the environmental cue of cold, initiate FAO (75).

Mitochondria actively contribute to T cell activation and circulating T cell memory formation, their fission and fusion determining energy production (76). Furthermore, P2RX7 is involved in metabolic function via stimulation of AMPK in CD8 effector T cells, increasing glucose and fatty acid uptake and OXPHOS, and by promoting mitochondrial fusion and reorganization, affecting the development of CD8 T_{CM} and CD8 T_{RM} cells (35). Unexpectedly, detection of mitochondria using mitotracker dyes, often equated to represent mitochondrial mass, or nonyl acridine orange, which binds to cardiolipins, in T cells, suggested very low levels of mitochondria to be present in IELs (innate-like as well as T_{RM} cells) compared with circulating CD8 T cells, with reduced mitochondrial membrane potential and ROS production (44). These observations were at odds with the active scanning behavior of barrier IELs, as well as their high expression level of P2RX7, the absence of which results in reduced OXPHOS potential but similar aerobic glycolysis (35, 77). Electron microscopy analysis revealed increased numbers of mitochondria to be present in IELs compared with circulating CD8 T cells, albeit of a smaller average size (44). Although the exact binding properties of dyes remains elusive, it suggested marked changes in mitochondria of CD8 T_{RM} cells at epithelial barriers.

IEL mitochondria were found to have an altered cardiolipin make up, enriched in longer and more unsaturated species. Additional experiments using T cells deficient in Tafazzin, an enzyme involved in cardiolipin metabolism, indicated that changes in cardiolipins in line with circulating T cells are

required for IEL activation (44, 78). Failure to alter the cardiolipin makeup restricts swift IEL proliferation and effector function, reducing microbial containment capability resulting in an increased microbial burden. In addition, the data also suggested that other changes in IELs may contribute to the absence of mitochondrial detection and possibly energy capacity, since changes in cardiolipin composition did not explain the absence of Mitotracker dye staining under steady state conditions. Whether mitochondria in IELs directly associate with lipid droplets and if their detection is masked by their subcellular location remains to be determined. These findings uncovered an alternative mechanism of mitochondria to control cellular activity, which appear particular to epithelial-resident CD8 T cells.

CD8 T_{RM} AND AhR METABOLISM

Lipid metabolism may involve a factor shared between epithelial T cell subsets, critical for the maintenance of IELs but not expressed in circulating T cells, the arylhydrocarbon receptor (AhR) (52, 65, 79, 80). AhR has been linked with cholesterol biosynthesis in hepatocytes as well as attenuating the expression of key fatty acid synthesis genes (81). The transcriptional activity of AhR is the result of ligand engagement in the cytosol (82). The absence of AhR results in alterations in intestinal microbial composition and acute sensitivity to intestinal injury, in line with the role of IEL in controlling the microbiota and regulating epithelial cell turnover and wound repair (65). The identity of the ligand remains unknown, but can include substances derived from food, light, and microorganism (65, 83, 84). However, AhR ligands are lipophilic and likely enriched in lipid-rich tissues. The transcriptional activity of AhR involves the production of metabolic enzymes, cytochrome P450 of the first family (Cyp1), including in T cells (85), involved in the metabolism of polyunsaturated fatty acids and arachidonic acid.

Although, the functional roles of FABP4/5 remain to be defined, their activity may be juxtaposed to that of AhR, the activity of which can dampen psoriasis-like symptoms (66). Since lymphocytes expressing AhR are enriched in tissues, besides CD8 T_{RM} cells, ILC3 and T_H17 cells, it is tempting to speculate that the AhR system provides a specific advantage in the tissue environment, not required in SLOs. Whether AhR is involved in the assistance of specific metabolic pathways generating energy or protection from metabolic factors encountered in tissues, or generated because of specific metabolic pathways, remains to be determined.

CD8 T_{RM} AND SYSTEMIC METABOLISM

In addition to IELs primary role to provide a first line of defense against invading microorganisms and tissue homeostasis, recent data suggests a potential role in systemic metabolism. In mice deficient in integrin-β7, which can pair with integrin-α4 (forming CD49d) or αE (forming CD103), immune cell homing to tissues is reduced. Intergin-β7-deficient mice lack natural IEL and are metabolically hyperactive (86). Consequently, these animals are resistant to obesity, hypertension, diabetes, and atherosclerosis when fed a high fat and high sugar diet. IELs express the glucagon-like peptide-1 receptor (GLP-1R) (87). IEL function, release of cytokines and antimicrobial factors, depends on the expression of GLP-1R, its absence resulting in dysregulated intestinal gene expression, an altered microbiota composition, and enhanced sensitivity to colitis, similar to AhR-deficiency with a link to psoriasis (88). GLP-1R, binding GLP-1, is known to be expressed on pancreatic β-cells and brain, its stimulation controlling blood glucose levels and appetite. Its stimulation converting ATP to cyclic adenosine monophosphate (cAMP), reducing the activation and function of IELs, but not that of circulating CD8 T cells (87).

Recent data indicates that the GLP-1R pathway in IELs can determine systemic metabolic capacity, whereby GLP-1 is released from enteroendocrine L-cells in gut epithelium (86). GLP-1 release is increased by sugars and bile acids in the intestinal lumen and in response to neuronal stimulation and inflammation (89). These data suggest that IELs also function as a metabolic and gut-health rheostat, their activity determined by the nutritional and inflammatory state of the organism, maintaining tolerance at the intestinal barrier when symbiotic bacteria produce carbohydrates and bile acids. If GLP-1 levels are limited to reach the blood stream, IEL capture will reduce the availability to stimulate β-cells and the release of insulin, increasing blood glucose levels and activity, as well as to the brain, increasing appetite. The physiological role of this process requires further scrutiny. Since inflammatory signals stimulate L-cell GLP-1 release (89), this could inhibit IEL activity and potentially aggravate intestinal inflammation. Although of potential benefit in times of scarcity, current food composition and ready availability may be detrimental to health.

CONCLUSIONS

In recent years, T cells metabolic characteristic in relation to their activation stage, differentiation and function have been more closely studied. In CD8 T cells, there are clear differences between the metabolic pathways used between naïve, memory and effector cells (**Table 1**). In addition, between the three identified memory T cell subsets, T_{EM}, T_{CM}, and T_{RM} cells, there are communalities and differences affecting cell development, maintenance and function. How the development of these memory subsets are fine-tuned, with initial differences between T_{RM} cell development compared to T_{EM} and T_{CM} cells reported, remains incompletely understood. CD8 T_{RM} cells have characteristics of effector T cells, with increased expression of transcripts for proteins involved in metabolism and effector proteins such as granzymes, active cellular migration, as well as uptake of FFA and storage in lipid droplets, but without active proliferation or secretion of effector molecules such as IFN-γ. The positioning of T_{RM} cells in diverse tissues would suggest that tissue-specific adaptations might be required for their long-term maintenance and specific function. Yet, the transcriptional make up of CD8 T_{RM} cells in different tissues is largely similar (90, 91). Lipids are recognized to be

an important substrate and FAO as important source of energy for CD8 T_{RM} cells, but the signals resulting the bioavailability of FFA stored in lipid droplets remain to be discovered. Upon activation, CD8 T_{RM} cells appear to use similar metabolic pathways compared with effector CD8 T cells, using OXPHOS and glycolysis. However, maintenance of T cells sets CD8 T_{RM} cell apart, with the high levels of P2RX7 increasing susceptibility to cell death, the expression of AhR critical for survival and the altered cardiolipin composition and mitochondria activity of those CD8 T cells residing at the top layers of the skin and intestine.

The biochemical analysis of T_{RM} cells has been hampered due to the difficulties in harvesting sufficient cell numbers and the inability to culture these cells, requiring constant interactions with tissue cells. Their important role in providing immediate protection against microbial invasion as well as tissue homeostasis and their role in systemic metabolism and pathological conditions, combined with technological advances enabling more sensitive cellular and biochemical analysis, will contribute important new discoveries in the coming years.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Ataxin-3 Links NOD2 and TLR2 Mediated Innate Immune Sensing and Metabolism in Myeloid Cells

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The interplay between NOD2 and TLR2 following recognition of components of the bacterial cell wall peptidoglycan is well-established, however their role in redirecting metabolic pathways in myeloid cells to degrade pathogens and mount antigen presentation remains unclear. We show NOD2 and TLR2 mediate phosphorylation of the deubiquitinase ataxin-3 via RIPK2 and TBK1. In myeloid cells ataxin-3 associates with the mitochondrial cristae protein MIC60, and is required for oxidative phosphorylation. Depletion of ataxin-3 leads to impaired induction of mitochondrial reactive oxygen species (mROS) and defective bacterial killing. A mass spectrometry analysis of NOD2/TLR2 triggered ataxin-3 deubiquitination targets revealed immunometabolic regulators, including HIF-1α and LAMTOR1 that may contribute to these effects. Thus, we define how ataxin-3 plays an essential role in NOD2 and TLR2 sensing and effector functions in myeloid cells.

Keywords: Nod2, TLR2, metabolism, innate immnuity, ataxin 3

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INTRODUCTION

Pattern recognition receptors (PRRs) recognize foreign antigen to direct innate and adaptive immune responses against invading pathogens (1). Polymorphisms in the PRR nucleotide-binding oligomerization domain-containing protein 2 (NOD2) represent the strongest genetic risk factor for the inflammatory bowel disease Crohn's (CD), and thus this bacterial sensor is the focus of particular research interest (2–4). NOD2 recognizes muramyl dipeptide (MDP), the largest fraction of peptidoglycan, that is present in the cell walls of all bacteria (5). Subsequent activation of NF-κB and MAPK pathways via interaction with receptor-interacting protein kinase 2 (RIPK2) results in an array of immune responses, such as production and regulation of pro-inflammatory cytokines (6), and modulation of T-cell function (7–9). NOD2 also directs autophagy, which is important both for bacterial clearance and MHC class II antigen presentation (10). Importantly, NOD2 signaling is intimately linked with that of toll like receptor TLR2, with both responding to ligands derived from the same bacterial component, peptidoglycan. Although the precise mechanisms of cross-regulation are not well-understood, both NOD2 and TLR2 activate separate upstream signaling cascades to recruit the same NF-κB and MAPK pathways, and are typically thought to act in a synergistic fashion (11). CD patients harboring NOD2 polymorphisms display loss-of-function for induction of NOD2

and NOD2/TLR2 effector signaling factors (12, 13). In contrast, gain-of-function mutations of NOD2 have been associated with other inflammatory disease, such as Blau syndrome and early-onset-sarcoidosis (EOS).

In recent years it has become clear that cross-talk between metabolic and immune pathways is central to the regulation of host defense (14). Immune cells undergo significant metabolic reprogramming during the immune response, both as a result of changes in the metabolic microenvironment induced by inflammation, and in response to immune triggering. This interplay appears of particular importance to dendritic cells and macrophages and controls core processes including differentiation (15). However, while the importance of PRR activation in directing metabolic pathways that impact on immune effector function is now well-established, how NOD2 and TLR2 influence myeloid metabolism is unclear. Here, following a phosphoproteomic screen of NOD2 and TLR signaling we identify a deubiquitinase essential for metabolic reprogramming and innate effector function in myeloid cells.

RESULTS

NOD2 and TLR2 Stimulation Leads to Ataxin-3 Phosphorylation Mediated by RIPK2 and TBK1

We identified ataxin-3 as one of the most differentially phosphorylated proteins on NOD2/TLR2 stimulation through a quantitative phosphoproteomic screen in monocyte derived dendritic cells (moDCs) from healthy human donors (**Supplementary Table 1**). Ataxin-3 is a deubiquitinase (DUB) (16) that is required for non-selective autophagy and that is linked to neurodegenerative disease (17–19).

We first validated this result by immunoblotting phospho-enriched samples for ataxin-3 (**Figure 1A**). Both NOD2 and TLR2 stimulation alone led to ataxin-3 phosphorylation; this effect was enhanced on dual stimulation of NOD2 and TLR2. It was also observed to a lesser extent following stimulation of TLR4, TLR7, and TLR8 (**Figure 1A**). NOD2 mediated phosphorylation of ataxin-3 was examined in greater detail. A time course experiment demonstrated that ataxin-3 phosphorylation was maximal 30 min after MDP stimulation (**Figure 1B**). While NOD2 is the only known receptor for MDP, the absolute requirement for NOD2 in the MDP stimulated phosphorylation of ataxin-3 was investigated. We downregulated expression of NOD2 in THP-1 cells using short hairpin RNAs (shRNA) targeting *NOD2* (**Figure 1C**). Reduction of ataxin-3 phosphorylation on MDP exposure was observed in NOD2 knockdown cells (**Figure 1D**). Next, given the central importance of RIPK2 in NOD2 signaling (20, 21), the requirement of RIPK2 for phosphorylation of ataxin-3 by NOD2 was investigated. NOD2-RIPK2 inflammatory signaling can be potently and selectively inhibited by the clinically relevant kinase inhibitor Ponatinib, that functions by blocking RIPK2 autophosphorylation and ubiquitination (22). moDCs were treated with Ponatinib prior to stimulation with MDP or PAM_3CSK_4 or both, with phosphorylation of p38 used as a positive control for the inhibitor. As expected, inhibition of RIPK2 blocked NOD2 induced phosphorylation of p38, but had no effect on induction by TLR2, which signals to p38 via a MyD88 pathway which is independent of RIPK2 (23). Inhibition of RIPK2 led to complete inhibition of NOD2 induced phosphorylation of ataxin-3, and significant abrogation of the synergistic NOD2/TLR2 signal in both cell types (**Figure 1E**). Recent evidence suggests that tank binding kinase 1 (TBK1) may represent a novel but important kinase in the NOD2/RIPK2 signaling cascade (24, 25) and MDP stimulation of the NOD2 receptor has been shown to induce TBK1 phosphorylation at S172 (24). Consequently, the requirement for TBK1 in NOD2/RIPK2 dependent phosphorylation of ataxin-3 was examined. We downregulated expression of TBK1 in THP-1 cells using short hairpin RNAs (shRNA) targeting *TBK1* (**Figure 1F**). Reduction of ataxin-3 phosphorylation on MDP exposure was observed in TBK1 knockdown cells (**Figure 1G**). The possibility that TBK1 might directly phosphorylate ataxin-3, as has been described for a number of other proteins including optineurin (26) and p62 (27), was explored using an *in vitro* kinase assay (**Figure 1H**). The expected autophosphorylation of TBK1 was demonstrated by a marginally higher molecular weight of the TBK1 band in samples containing both TBK1 and ATP. Importantly, a significant proportion of the ataxin-3 band was noted at a higher molecular weight in samples containing ataxin-3, TBK1 and ATP, consistent with ataxin-3 phosphorylation (**Figure 1H**). Notably, no change in migration of the ataxin-3 band was seen in samples containing ataxin-3 and TBK1 but not ATP, confirming the ATP dependency of this shift, consistent with phosphorylation. Finally, the phosphorylation site of ataxin-3 was sought, using liquid chromatography mass spectrometry analysis of endogenous ataxin-3 immunoprecipitated from THP-1 cells. A significant shift in mass/charge ratio, consistent with phosphorylation, was detected at a single peptide in the MDP/PAM_3CSK_4 stimulated sample only, corresponding to phosphorylation at serine 265 (**Figure 1I**). This residue has been described as a phosphorylation site in 12 separate large scale mass spectrometry (MS) screens of human primary cells and cell lines (28), and is highly conserved in placental bearing mammals (29), but there is no existing knowledge of its functional relevance. It is located in close proximity to the second ubiquitin interacting motif (UIM), suggesting that phosphorylation could affect specificity of DUB target, as has been described for neighboring serine residues 256/260/261 (30) (**Figure 1J**).

Taken together, this data shows that activation of NOD2/TLR2 signaling pathway induces phosphorylation of the DUB ataxin-3. TBK1 is required for the direct phosphorylation of ataxin-3 at serine 265 following NOD2/TLR2 activation.

Ataxin-3 Localizes With the Mitochondrial Cristae Protein MIC60 and Regulates the Expression of the Oxphos Machinery Components

To identify novel interacting partners of ataxin-3 in innate immune cells, endogenous ataxin-3 was immunoprecipitated in moDCs from healthy human donors, and subjected to mass

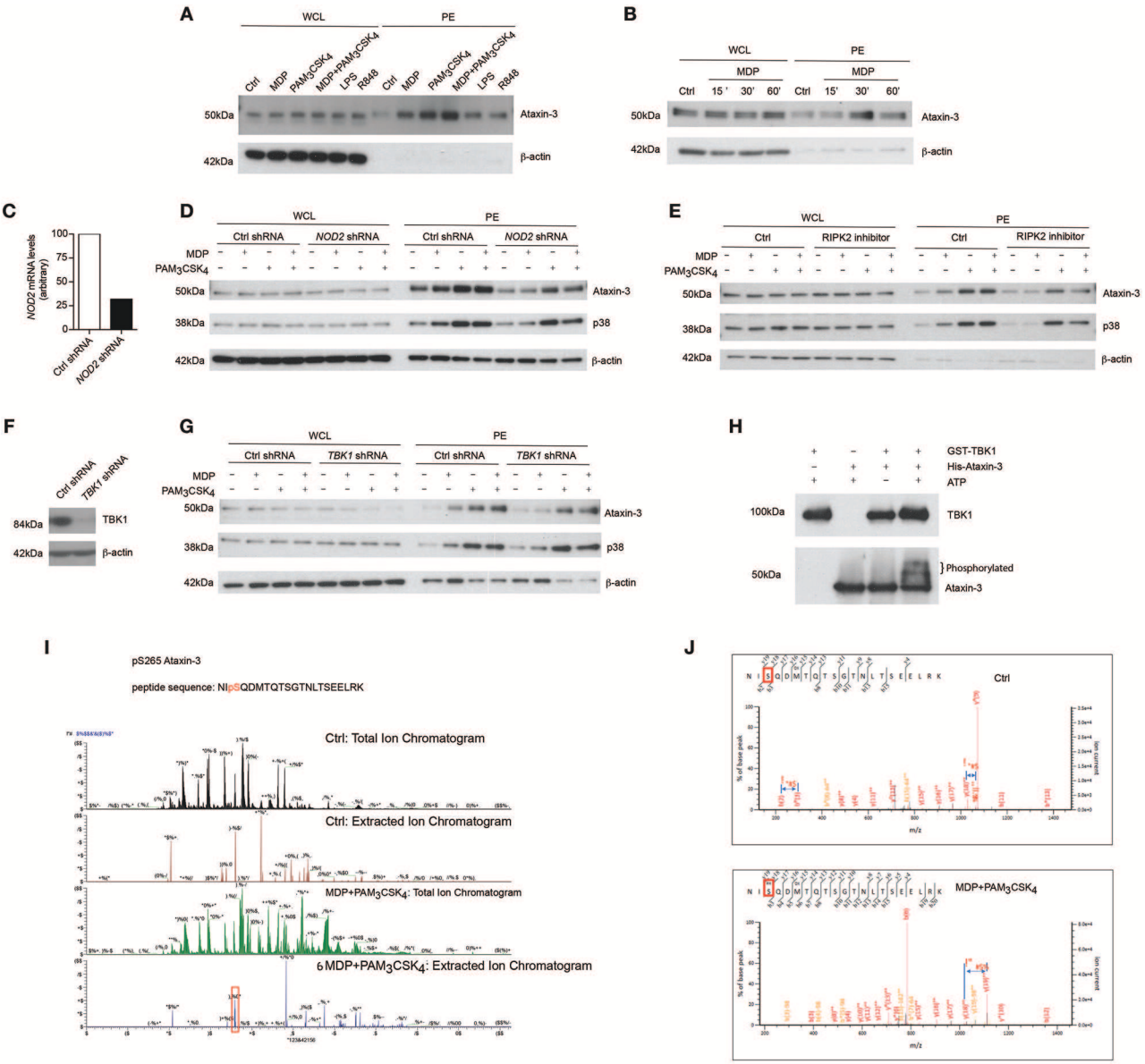

FIGURE 1 | NOD2/TLR2-mediated phosphorylation of ataxin-3. Immunoblot using antibodies against ataxin-3 and β-actin of whole cell lysates (WCL) and phosphoprotein enriched lysates (PE) from moDCs either **(A)** unstimulated or stimulated with the NOD2 ligand MDP 10 μg/ml, or the TLR2 ligand PAM_3CSK_4 1 μg/ml, or both, or the TLR4 ligand LPS 100 ng/ml or the TLR7/8 ligand R848 (Resiquimod) 1 μg/ml for 30 min or **(B)** unstimulated or stimulated with the NOD2 ligand MDP for 15, 30, or 60 min. **(C)** THP-1 cells were transduced with control or *NOD2*-targeting lentiviral shRNAs and analyzed for *NOD2* expression by qPCR analysis. **(D)** Immunoblot using antibodies against ataxin-3, p38, and β-actin of WCL and PE lysates from THP-1 cells expressing control or NOD2 shRNA and either unstimulated or stimulated with the NOD2 ligand MDP or the TLR ligand PAM_3CSK_4, or both, for 60 min. **(E)** Immunoblot using antibodies against ataxin-3, p38 and β-actin of WCL and PE lysates from THP1 cells which were pre-treated with the RIPK2 inhibitor Ponatinib 50 nM for 60 min and then left unstimulated or stimulated with the NOD2 ligand MDP or the TLR2 ligand PAM_3CSK_4 or both. **(F)** THP-1 cells were transduced with control or *TBK1*-targeting lentiviral shRNAs and immunoblotted to detect TBK1 expression **(G)** Immunoblot using antibodies against ataxin-3, p38 and b-actin of WCL and PE lysates from THP-1 cells expressing control or TBK1 shRNA left unstimulated or stimulated with the NOD2 ligand MDP or the TLR2 ligand PAM_3CSK_4 or both for 60 min. **(H)** Immunoblot using antibodies against TBK1 and ataxin-3 following an *in vitro* kinase assay of GST-TBK1 protein or His-ataxin-3 protein with ATP, or both GST-TBK1 and His-ataxin-3 with or without ATP which were incubated for 60 min at 30°C. **(I,J)** Identification of the phosphorylated serine residue (s265) in ataxin-3 with a characteristic increase in mass/charge ratio in the stimulated sample. All immunoblots are representative of at least two independent experiments.

spectrometry analysis. One of the most abundant proteins identified in the pull down was the mitochondrial cristae protein MIC60 (**Figures 2A,B**). The association between ataxin-3 and MIC60 was validated through immunoblot of immunoprecipitated ataxin-3 (**Figure 2C**). There appeared to be no change in abundance of MIC60 when moDCs were stimulated with MDP + PAM_3CSK_4 prior to immunoprecipitation of ataxin-3, suggesting that NOD2/TLR2 stimulation does not

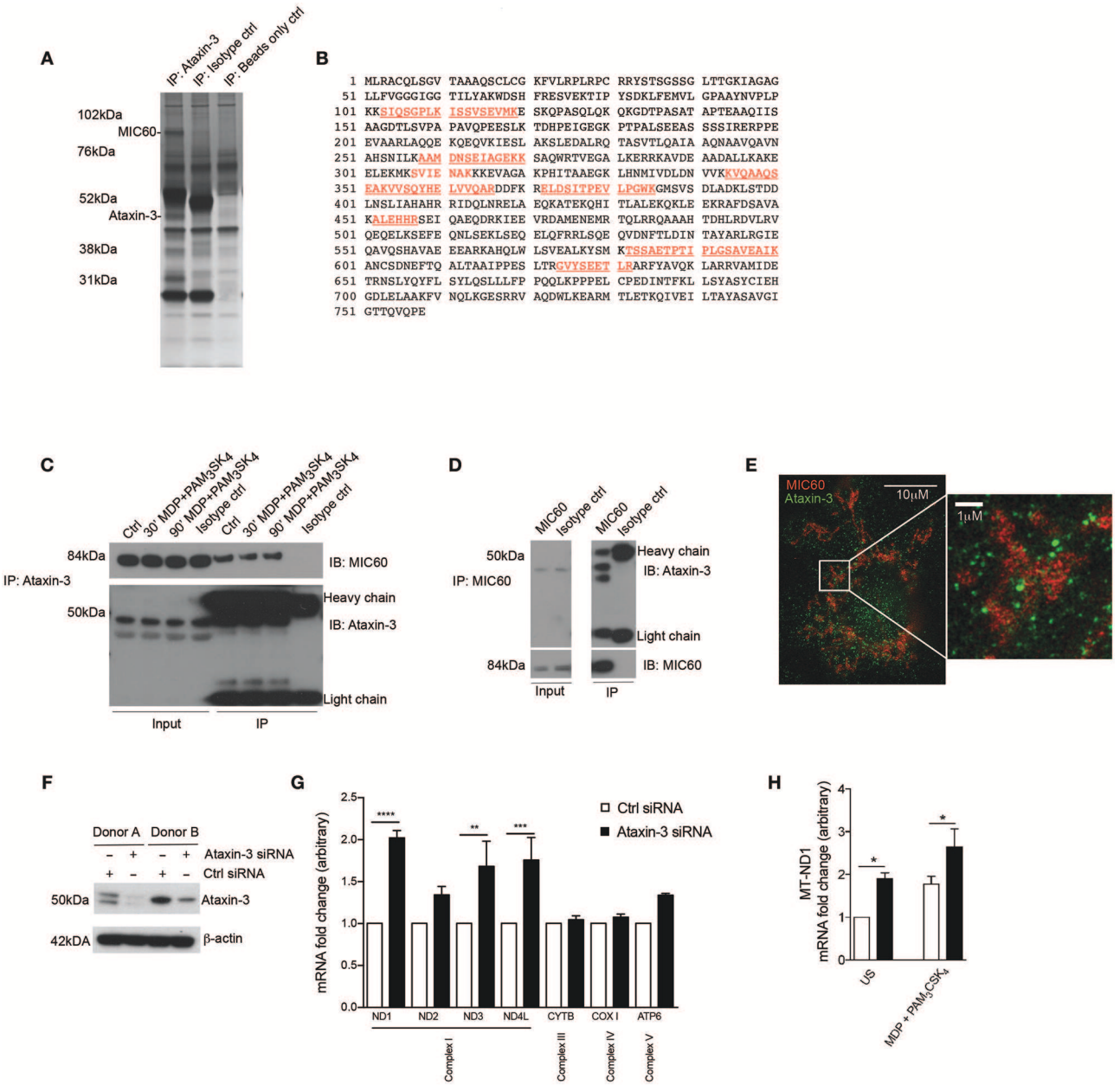

FIGURE 2 | Ataxin-3 associates with the mitochondrial protein MIC60 and regulates expression of the OXPHOS machinery components. **(A)** Silver stain of immunoprecipitation of ataxin-3 in moDCs, using ataxin-3 antibody, isotype control antibody, or immunoprecipitation beads only. **(B)** The sequence coverage of MIC60 identified following mass spectrometry analysis of the immunoprecipitation of ataxin-3 in moDCs, with the peptides in red indicating the identified peptides. **(C)** Immunoblot using antibodies against MIC60 and ataxin-3 of lysates where ataxin-3 has been immunoprecipitated from moDCs either left unstimulated or stimulated for 30 or 60 min with MDP + PAM_3CSK_4. **(D)** Immunoblot using antibodies against MIC60 and ataxin-3 of lysates where MIC60 has been immunoprecipitated from moDCs. **(E)** STED microscopy using antibodies against ataxin-3 and MIC60 in moDCs. **(F)** Immunoblot using antibodies against ataxin-3 and β-actin in moDCs following transfection with control or ataxin-3 siRNA. **(G)** RT-qPCR analysis of selected mitochondrial genome encoded transcripts following ataxin-3 depletion by siRNA in moDCs; $n = 3$, one way ANOVA $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ **(H)** RT-qPCR analysis of *MT-ND1* mRNA expression following ataxin-3 depletion by siRNA in moDCs subsequently left unstimulated or stimulated for 6 h with MDP + PAM_3CSK_4; $n = 3$, Student's paired *t*-test $^{*}p < 0.05$.

affect binding. To further confirm the association, MIC60 was pulled down, and an immunoblot for ataxin-3 performed (**Figure 2D**). Here, ataxin-3 appeared at two molecular weights, suggesting that either two separate isoforms bind to MIC60, or it is post-translationally modified. Finally, super-resolution assessment using stimulated emission depletion (STED) microscopy confirmed that ataxin-3 was situated in close proximity to MIC60 (**Figure 2E**).

MIC60 is the largest protein in the mitochondrial contact site (MICOS) complex, which is embedded in the mitochondrial inner membrane and acts as a key regulator of cristae junction formation and assembly of respiratory chain complexes which are required for oxidative phosphorylation (31). Additional specific roles for MIC60 include the import of proteins (32, 33) and regulation of mitochondrial DNA (mtDNA) transcription (34, 35). Consequently, we examined the effect of ataxin-3 knockdown in moDCS on expression of mtDNA genes that encode components of the oxphos machinery. moDCS from healthy human donors were found to express either a single or double isoform of ataxin-3 with approximately equal frequency, but all detectable isoforms could be efficiently knocked down (**Figure 2F**). Ataxin-3 depletion led to a 2-fold upregulation of NADH-ubiquinone oxidoreductase 1 (*MT-ND1*), with a statistically significant upregulation observed for mRNA expression of two other genes from Complex I, *MT-ND3* and *MT-ND4L* (**Figure 2G**). In comparison, genes encoding components of Complex III, IV and V were broadly unaffected. It is noteworthy that mtDNA transcription is tightly regulated due to its close links to oxphos, and thus 1.5- to 2-fold changes in mRNA expression level represent a potentially functionally significant alteration (36). The effect of NOD2/TLR2 stimulation was examined on *MT-ND1*, the most significantly affected gene. Stimulation led to a significant upregulation in mRNA expression in both the control and ataxin-3 depleted cells, but there was significantly greater ND1 in the ataxin-3 depleted cells following stimulation (**Figure 2H**). Finally, to understand if these effects on Complex I genes correlate with modulation of cytokine responses, we have assessed the expression of *IL8*, *IL1*β, *TNF*, *IL12B*, and *IL23A* in response to TLR/NOD2 signaling in human moDCS and found that ataxin-3 depletion using siRNA did not affect the levels of these cytokines (data not shown).

These results indicate a novel association between ataxin-3 and MIC60, a component of the MICOS complex involved in the regulation of mtDNA. We demonstrated that ataxin-3 regulates mtDNA by downregulating the expression of Complex I genes, an effect increased on NOD2/TLR2 sensing.

Ataxin-3 Is Important for Optimal Mitochondrial Respiration Following NOD2 and TLR2 Stimulation

We next investigated the functional relevance of the observed changes in the expression of Complex I genes. Using short hairpin RNAs (shRNA) targeting the ataxin-3 gene *(ATXN3)*, we downregulated ataxin-3 protein expression in THP-1 cells (**Figure 3A**). We next performed a real time analysis of oxidative phosphorylation to address the function of ataxin-3. We found that ataxin-3 depletion led to a significant reduction in all the key parameters of mitochondrial respiration assessed (**Figures 3B–F**).

We next determined whether NOD2/TLR2-mediated ataxin-3 phosphorylation stimulation affects mitochondrial respiration. Prolonged triggering of these PRRs led to an expected metabolic shift (37) with downregulation of oxidative phosphorylation. In the ataxin-3 depleted cells the level of oxphos remained significantly lower than in the control cells following stimulation (**Figures 3G–J**). Importantly, no significant difference in mtDNA copy number was found between the control and ataxin-3 depleted THP-1 cell line (**Supplementary Figure 1**), suggesting that there are no differences in mitochondrial mass or turnover through mitophagy to explain the observed changes.

Taken together, this data shows that in innate immune cells, ataxin-3 is required for optimal mitochondrial respiration and this effect is enhanced following its phosphorylation on NOD2/TLR2 stimulation.

Ataxin-3 Is Required for Mitochondrial ROS Production, and Is Necessary for Optimal Bacterial Killing

A key function of mitochondrial respiration in immune cells is the generation of mROS. This results from leakage of electrons, predominantly from Complex I and to a lesser extent from Complex III, which partially reduce oxygen to form superoxide (38). The effect of ataxin-3 depletion on mROS and total cellular ROS was therefore examined. Ataxin-3 depletion led to a significant reduction in mROS (**Figure 4A**). As expected, there was a corresponding decrease in total cellular ROS, to which mROS makes a significant contribution (**Figure 4B**). The ability of immune cells to upregulate mROS production on pathogen challenge is crucial to the immune response (39); importantly, ataxin-3 depleted cells also produced less mROS on NOD2/TLR2 stimulation (**Figure 4C**). mROS forms an important component of antibacterial responses, and is important for bacterial killing (39). To test the functional significance of the observed impairment of mitochondrial oxphos and mROS generation on NOD2/TLR2 triggering, we assessed the response of ataxin-3 depleted macrophages to *Salmonella* Typhimurium, a Gram-negative intracellular bacterium that is sensitive to ROS-dependent killing (39, 40). A gentamicin survival assay was undertaken. While bacterial invasion was unchanged, as evidenced by similar bacterial counts at 1 h post-infection, there was subsequently significantly greater bacterial survival in the ataxin-3 depleted cells that was maximal at 6 h (**Figure 4D**). There were no significant differences in cell viability between the two groups at any of the infection time points (**Supplementary Figure 2**), excluding differences in cell survival as a contributing factor in the bacterial killing deficit.

Taken together, this data shows that in macrophages ataxin-3 is required for mROS generation and this contributes to effective intracellular bacterial killing.

An Unbiased Ubiquitome Screen Reveals Novel DUB Targets of Ataxin-3 Following NOD2 and TLR2 Activation

To further define the role of ataxin-3 in NOD2/TLR2 signaling, we sought to define downstream DUB targets. Classically the ubiquitinated proteome, also termed the ubiquitome, was first enriched using His_6-tagged ubiquitinated conjugates under denaturing conditions. However, concerns exist over the impact of overexpressed modified His_6, which competes with

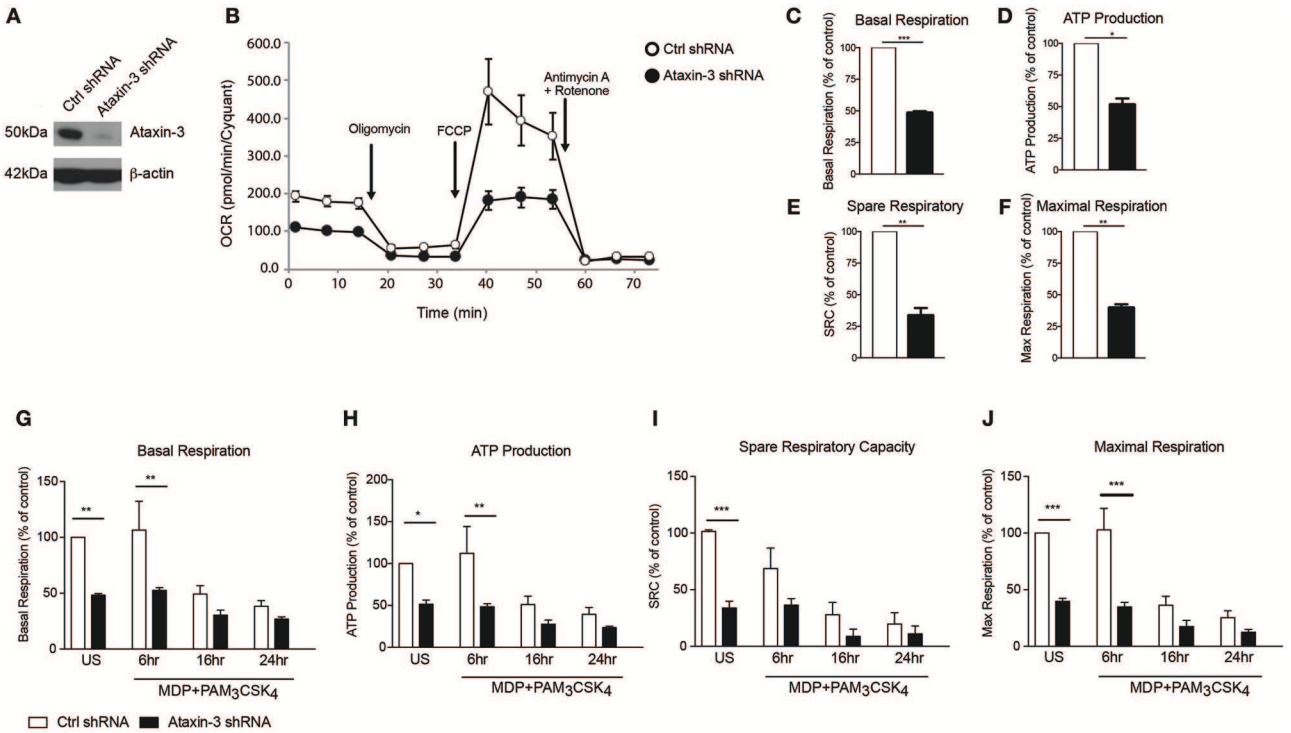

FIGURE 3 | Ataxin-3 is required for mitochondrial respiration in immune cells. **(A)** Immunoblot using antibodies against ataxin-3 and β-actin in THP1 cells following transfection with control or ataxin-3 shRNA. **(B)** Representative Seahorse sequential OCR measurements by Seahorse of control and ataxin-3 shRNA THP1 cells. **(C–F)** Seahorse analysis of key mitochondrial respiration parameters in control shRNA cells and ataxin-3 shRNA THP1 cells. $n = 3$, Student's paired t-test $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(G–J)** Seahorse analysis of key mitochondrial respiration parameters in control shRNA cells and ataxin-3 shRNA THP1 cells either left unstimulated or stimulated for 6, 16, or 24 h with MDP + PAM_3CSK_4. $n = 3$, one way ANOVA $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All OCR measurements were normalized to cell number.

endogenous ubiquitin, on the ubiquitome (41). An alternative method of enrichment, using high affinity ubiquitin traps has therefore been developed. These tandem ubiquitin binding entities (TUBEs) specifically recognize, bind to, and stabilize polyubiquitinated proteins, protecting them from degradation by DUBs or the proteasome. The enriched ubiquitinated proteins can then be analyzed by MS (41–43). The ability of TUBEs to preferentially recognize either K48 or K63 linked polyubiquitin provides a further advantage. Consequently, the use of a K63-TUBEs1 system which shows a 10-fold higher affinity for K63 linked chains, allows the selective enrichment of the K63 linked ubiquitome. This provides a specific means of purifying the K63 chains favored by ataxin-3 (44), and thus was employed as a strategy for defining novel DUB targets of ataxin-3 in immune cells.

The TUBEs2 system was used to enrich ubiquitinated proteins in ataxin-3 depleted and control THP-1 cells, either left unstimulated or stimulated for 1 h with MDP + PAM_3CSK_4. Samples from three biological replicates were then subjected to mass spectrometry analysis (**Figure 5A**). As a quality control prior to MS analysis, immunoblotting with an antibody against K63-linkage specific polyubiquitin demonstrated a marked increase in the levels of K63 ubiquitinated proteins in the ataxin-3 depleted cells, most notably at higher molecular weights, with NOD2/TLR2 stimulation leading to a separate shift in staining pattern (**Figure 5B**).

Mass spectrometry analysis identified 291 proteins as changing significantly between any condition when averaged across the three biological replicates. As ataxin-3 acts as a DUB, ataxin-3 depletion would classically lead to an accumulation of ubiquitinated targets and thus particular attention was paid to those proteins that showed an increase in abundance in the ataxin-3 depleted samples (**Supplementary Table 2**). However, as deubiquitination can also regulate protein stability, it is likely that a number of the proteins found to decrease in abundance on ataxin-3 depletion are also direct DUB targets of ataxin-3. Most notably, the immunometabolic regulator HIF1α (45) was found to be more abundant in the ataxin-3 depleted cells, suggesting that ataxin-3 may deubiquitinate HIF1α (**Figure 5C**). To specifically interrogate the importance of NOD2/TLR2 phosphorylation of ataxin-3 on DUB activity, the abundance of proteins in the ataxin-3 depleted cells was compared to the control cells following stimulation with MDP + PAM_3CSK_4 (**Supplementary Table 3**). Strikingly, a cluster of proteins related to metabolism were noted in the ataxin-3 depleted cells (marked in red on **Figure 5D**).

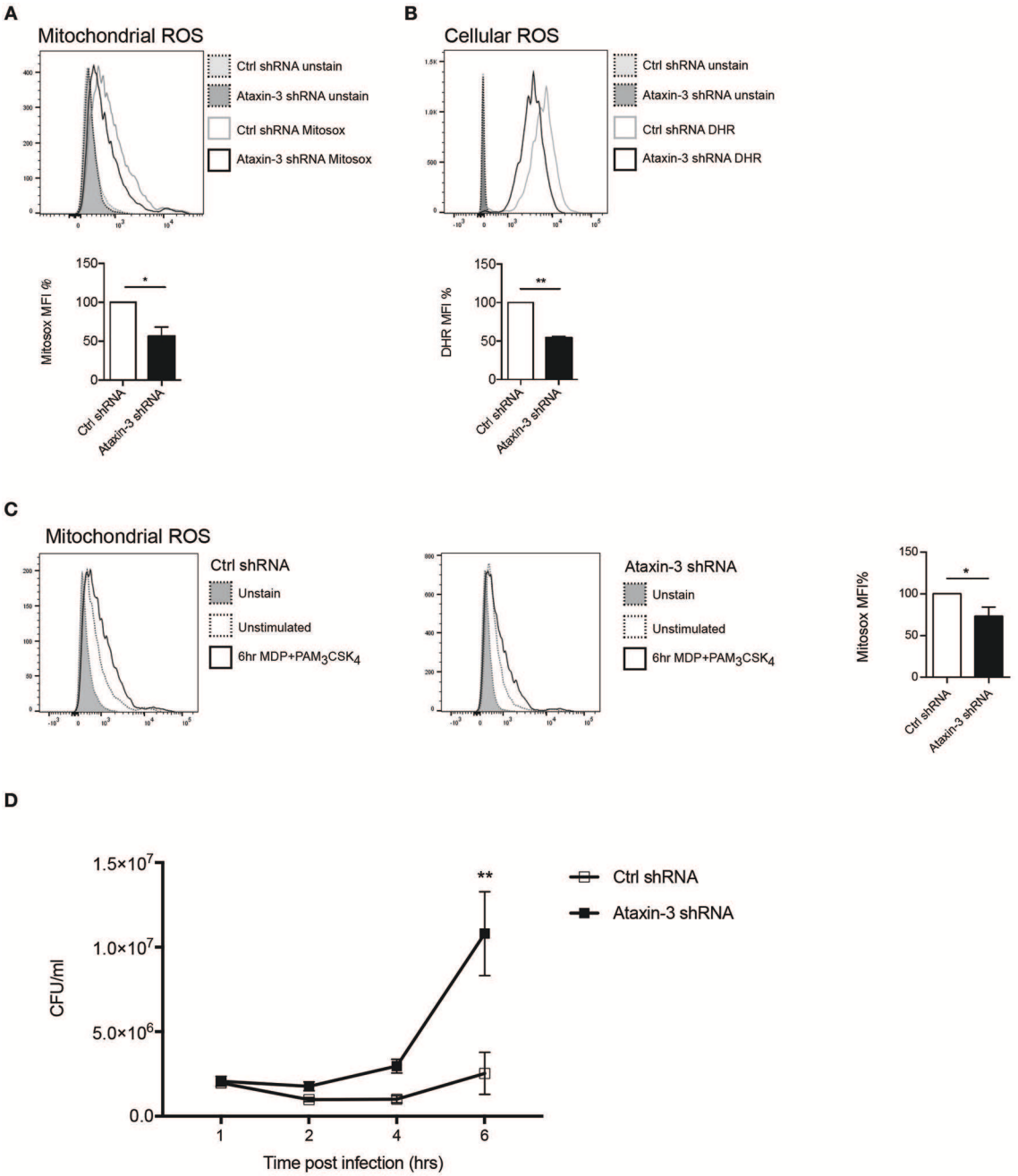

FIGURE 4 | Ataxin-3 is required for ROS production and bacterial killing. **(A)** FACS analysis of Mitosox to quantify mROS; $n = 3$ Student's paired *t*-test, $^{*}p < 0.05$. **(B)** FACS analysis of Mitosox of DHR to quantify total cellular ROS in control and ataxin-3 shRNA THP-1 cells. $n = 7$, Student's paired *t*-test, $^{**}p < 0.01$. **(C)** Mitosox to quantify mROS in control and ataxin-3 shRNA THP-1 cells either left unstimulated or following stimulation for 6 h with MDP + PAM_3CSK_4; $n = 4$, Student's paired *t*-test, $^{*}p < 0.05$. **(D)** Gentamicin survival assay of *Salmonella* Typhimurium infected ataxin-3 shRNA THP-1 cells compared to control. $n = 3$, one way ANOVA, $^{**}p < 0.01$.

Ataxin-3 Deubiquitinates HIF, PLD3, and LAMTOR1 Upon NOD2 and TLR2 Activation

A number of the proteins from the mass spectrometry analysis were selected for further validation. HIF1α was of particular interest given its central role in immunometabolism. Although classically described as part of the family of hypoxia-inducible factor regulators, mediating the cellular response to hypoxia (46), more recent work has demonstrated a broader role in regulation of the immune system. It is important for both the survival and function of cells of the innate immune system,

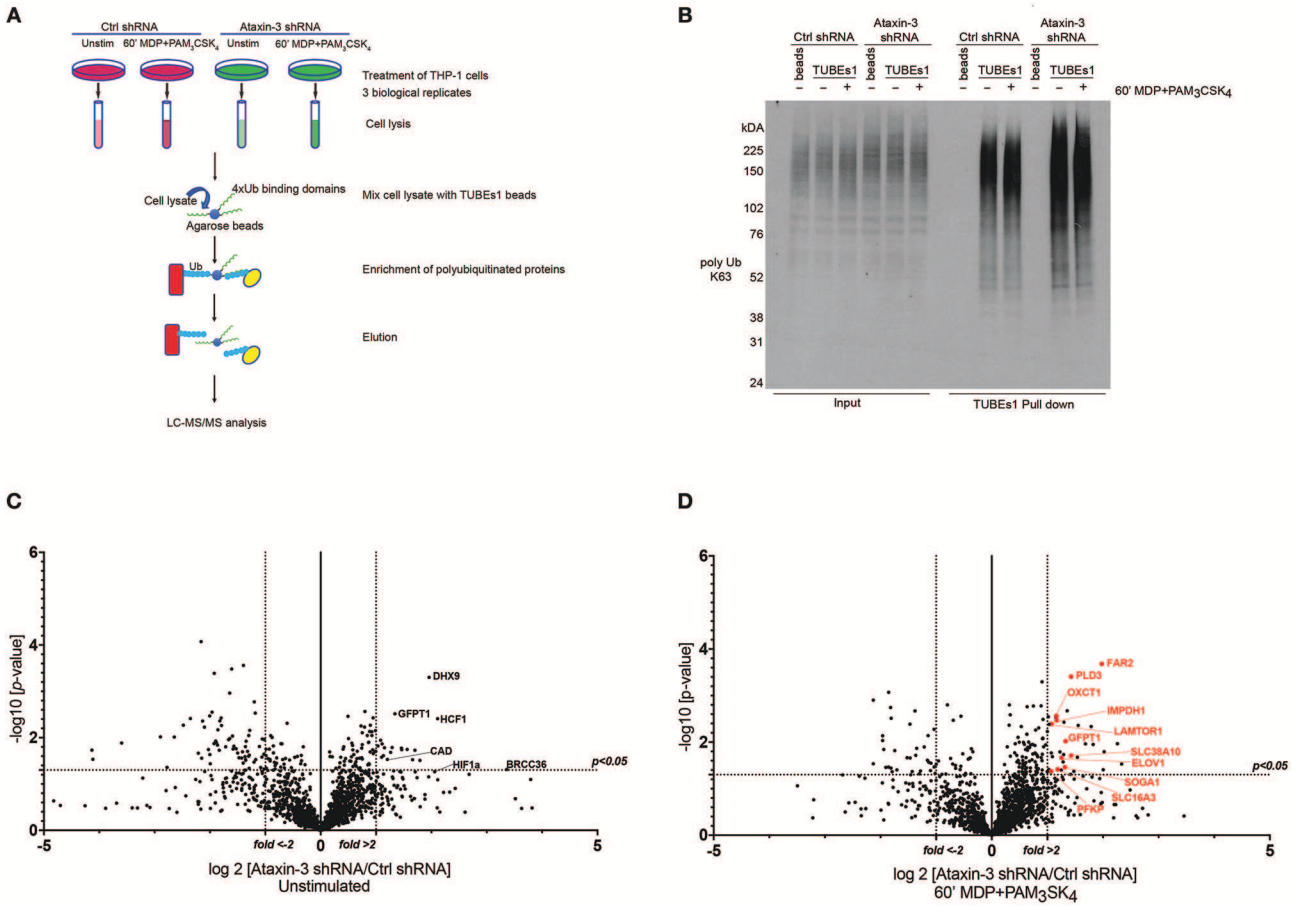

FIGURE 5 | Ubiquitome screen reveals novel DUB targets of ataxin-3. **(A)** Flow chart showing experimental protocol. Control and ataxin-3 shRNA THP-1 cells were left unstimulated or stimulated for 60 min with MDP + PAM_3CSK_4 prior to ubiquitin enrichment using TUBEs1 beads, with the lysates subsequently subjected to LC-MS/MS analysis. **(B)** Immunoblot using antibody against K63 linked polyubiquitinated proteins of whole cell lysates (input) and TUBEs1 enriched fractions or beads only control samples. Volcano plots comparing ubiquitinated protein expression in **(C)** unstimulated control and ataxin-3 shRNA THP-1 cells and **(D)** control and ataxin-3 shRNA THP-1 cells following MDP + PAM_3CSK_4 stimulation. The dotted lines on the y-axes represents a *p*-value of 0.05 (*t*-test), and on the x-axes represents fold change >2 or <-2.

through regulation of metabolic activation (47–49). HIF1αwas validated as a DUB target of ataxin-3 through immunoblot, with a significant increase in ubiquitinated HIF1α in the ataxin-3 depleted cells (**Figure 6A**). HIF1α was detected in the whole cell lysates of both control and ataxin-3 depleted cells in normoxia, as the proteasome inhibitor MG132 which reduces the degradation of all ubiquitinated proteins was added to the cell suspension 30 min before the end of all TUBEs experiments.

Phospholipase D3 (PLD3) was also intriguing as it has recently been shown to regulate inflammatory cytokine responses in response to TLR9 signaling by acting as an endonuclease (50). In humans, mutations confer an increased risk for the neurodegenerative diseases Alzheimer's (51) and spinocerebellar ataxia (52), with increasing evidence of the role of innate immune dysfunction in neurodegeneration (53). Importantly, polyglutamine repeat mutations in ataxin-3 are themselves associated with the spinocerebellar ataxia Machado-Joseph disease (18). Ragulator complex protein LAMTOR1 forms part of the Ragulator complex essential for amino acid sensing and activation of mTORC1 (54), but has also been linked to independent roles in lysosomal maturation (55) and M2 macrophage differentiation (56). PLD3 and LAMTOR1 were both validated by immunoblot (**Figures 6B,C**). Ataxin-3 depletion led to accumulation of ubiquitinated forms of both PLD3 and LAMTOR1. Notably, NOD2/TLR2 stimulation increased LAMTOR1 ubiquitination in the control cells, but there was markedly more LAMTOR1 ubiquitination in the stimulated ataxin-3 depleted cells (**Figure 6C**). This suggests that ataxin-3 modulates the ubiquitination of LAMTOR1 induced by NOD2/TLR2 stimulation.

To further study the interaction between LAMTOR1 and ataxin-3, an overexpression system was employed in the human cell line HEK293. HA-ataxin-3 and GFP-LAMTOR1 was overexpressed, together with a GFP-control and then IP of HA-ataxin-3 performed using an antibody against the HA tag. Ataxin-3 was found to bind directly to LAMTOR1 (**Figure 6D**). Next,

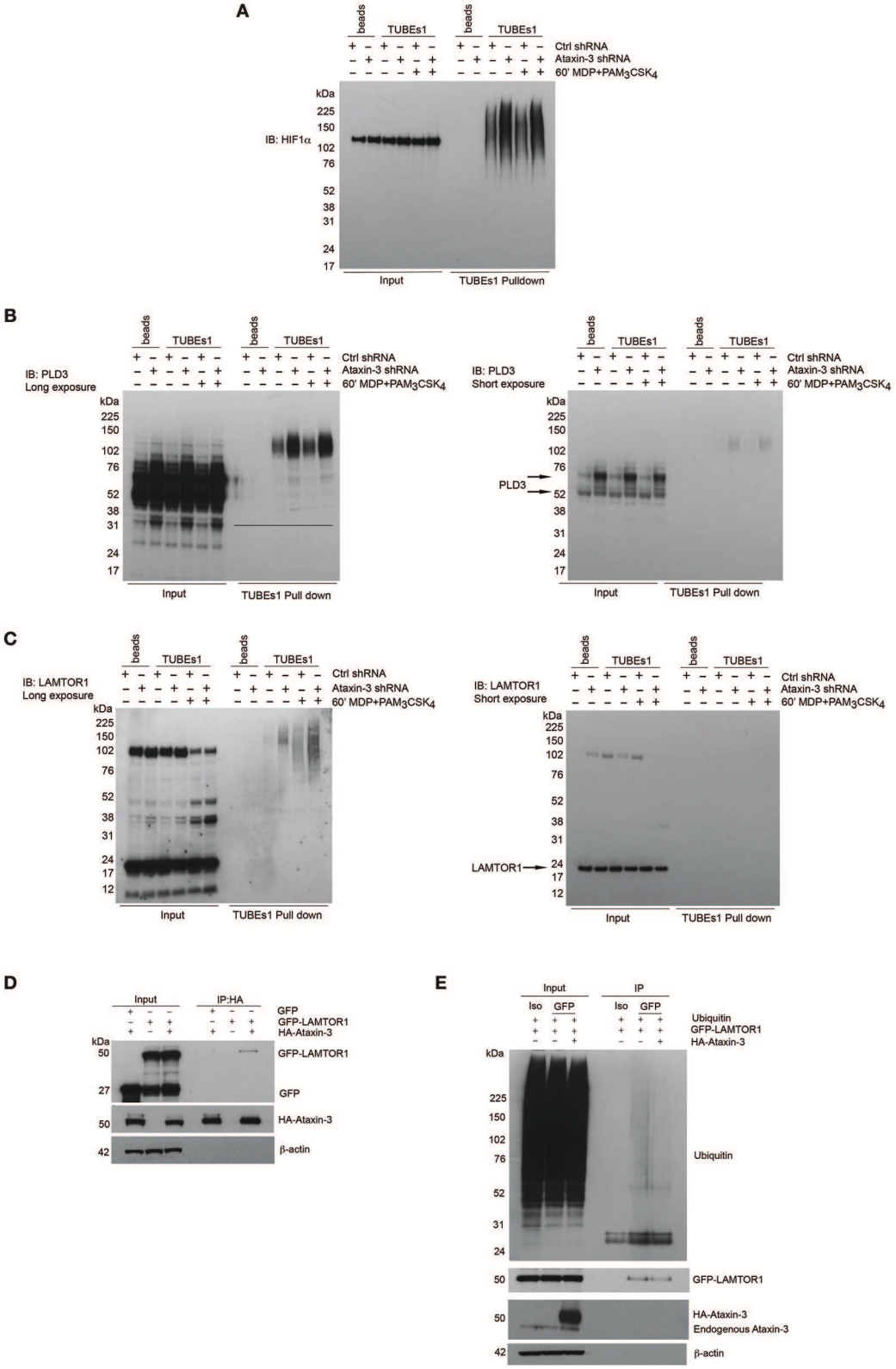

FIGURE 6 | Validation of novel DUB targets of ataxin-3. **(A)** Immunoblot using antibody against HIF1a in whole cell lysate (input) and TUBEs1 enriched fractions from control and ataxin-3 shRNA THP-1 cells left unstimulated or stimulated for 60 min with MDP + PAM_3CSK_4. Immunoblot using antibodies against **(B)** PLD3 and

(Continued)

FIGURE 6 | (C) LAMTOR1 in whole cell lysates (input) and TUBEs1 enriched fractions from control shRNA and ataxin-3 shRNA THP-1 cells left unstimulated or stimulated for 60 min with MDP + PAM_3CSK_4. **(D)** Immunoblot using antibodies against GFP, HA, and β-actin in input and HA-immunoprecipitated lysates, in HEK293 cells where GFP and/or GFP-LAMTOR1 and/or HA-ataxin-3 were overexpressed. **(E)** Immunoblot using antibodies against ubiquitin, GFP, ataxin-3, and β-actin in input and isotype control (iso) or GFP immunoprecipitated lysates where ubiquitin and GFP-LAMTOR1 were overexpressed with or without HA-ataxin-3. All immunoblots are representative of at least two independent experiments.

the finding that LAMTOR1 is a direct DUB target of ataxin-3 was confirmed. Ubiquitin, GFP-LAMTOR1 and HA-ataxin-3 were overexpressed in HEK293 cells and IP of GFP-LAMTOR1 performed using an antibody against the GFP tag. The level of ubiquitinated GFP-LAMTOR1 was found to be significantly less in cells where HA-ataxin-3 was co-expressed (**Figure 6E**).

Taken together, these results show that ataxin-3 modulates the ubiquitination of previously unidentified targets related to metabolism. This effect is enhanced on following phosphorylation of ataxin-3 on NOD2/TLR2 triggering.

DISCUSSION

In this study we demonstrate that NOD2 and TLR2 phosphorylates the deubiquitinase ataxin-3 at serine 265 through a signaling cascade involving RIPK2 and TBK1. Immunoprecipitation and MS analysis of interacting partners established an association with a core component of the mitochondrial MICOS complex MIC60. Ataxin-3 was subsequently shown to be necessary for optimal mitochondrial respiration and mitochondrial ROS generation in macrophages, an effect enhanced by its phosphorylation on NOD2/TLR2 triggering. In line with this, we found that ataxin-3 is required for optimal intracellular bacterial killing of *Salmonella* Typhimurium. Finally, we dissected the specific DUB role of ataxin-3 in an immune context through an unbiased MS screen of the ubiquitome. A preponderance of metabolism related proteins were discovered including HIF1α, phospholipase D3 and LAMTOR1, underlining a central role of ataxin-3 in immunometabolism.

Deubiquitinating enzymes (DUBs) represent specialized proteases which modify ubiquitin chains by cleaving the isopeptide bonds linking the ubiquitin C-terminus to a lysine side chain on the target protein. Modification of ubiquitination may alter cellular responses through regulation of target protein stability, or mediate signal transduction through non-degradative pathways including mediation of protein-protein interactions (57). Ataxin-3 is a small protein, consisting of 364 amino acids, that is ubiquitously expressed (58). At the N-terminus there is a catalytic Josephin domain, which acts as a protease that hydrolyses ubiquitin linkages and allows ataxin-3 to function as a DUB (59). A flexible C-terminal tail contains either two or three ubiquitin-interacting motifs (UIMs), according to the isoform (60). The UIMs mediate selective binding to ubiquitin chains, determining the type of chain that can be cleaved by the Josephin domain. Ataxin-3 shows a preference for cleavage of K63-Ub chains, although it is able to bind both K63 and K48 chains (44).

Ataxin-3 has been linked to neurodegenerative disease after unstable CAG repeat expansions in the ATXN3 gene were identified as the cause of spinocerebellar ataxia Type 3 (SCA3), also known as Machado-Joseph Disease, the most common autosomal dominant ataxia (18). Importantly, the expanded polyglutamine stretch results in more complex sequelae than a simple loss of protein function, and likely leads to a toxic gain of function through altered binding properties, aggregation and subcellular localization (61). Accumulating evidence suggests that ataxin-3 performs diverse cellular roles, including DNA repair (62, 63) and transcriptional regulation (64), regulation of protein quality through endoplasmic reticulum (ER) associated degradation (65) and aggresome formation (66), and beclin-1 dependent autophagy (17). A recent study provided a first link to immune regulation, demonstrating that ataxin-3 regulates Type 1 interferon antiviral responses through interaction with histone deacetylase 3 (HDAC 3) (67). The present study is the first to link ataxin-3 to PRR signaling and demonstrate its importance in mitochondrial respiration in macrophages, following the discovery that ataxin-3 associates with the mitochondrial protein MIC60.

MIC60 forms a core part of the MICOS complex, which is embedded in the mitochondrial inner membrane. It acts as a key regulator of mitochondrial inner membrane shape and organization. This is essential for cristae junction formation and assembly of respiratory chain complexes which are required for oxidative phosphorylation (31). MIC60 also appears to act independently of the MICOS complex, and has recently been implicated in regulation of mtDNA transcription (35). The mitochondrial genome encodes just 13 proteins, all essential components of oxphos complexes I, III and IV. In keeping with this known function of MIC60, we found that depletion of ataxin-3 led to specific upregulation of mtDNA transcripts encoding proteins required for Complex I, and this was further upregulated by NOD2/TLR2 stimulation. In addition, specific interrogation of mitochondrial oxidative phosphorylation through use of the Seahorse platform demonstrated that ataxin-3 depletion led to a particularly striking impairment in maximal respiration and spare respiratory capacity (SRC). SRC represents the extra mitochondrial capacity available within a cell to produce energy under conditions of increased work or stress and is important for cellular function and survival (68–70). Macrophages increase their SRC in response to bacterial infection to drive anti-microbial responses, and this is coordinated in part by modulation of the ETC Complexes I and II (71). In M2 macrophages, SRC is critical for their activation and prolonged survival, and clearance of the parasitic helminth (72). We found that ataxin-3 depletion impaired mROS production at both baseline and in response to NOD2/TLR2 stimulation, demonstrating the functional relevance of the observed mitochondrial respiratory impairment. Finally, we demonstrated the importance of ataxin-3 in intracellular killing

of the pathogen *Salmonella* Typhimurium, with PRR mediated mROS generation well-established as critical for destruction of this bacterium (39).

Fewer than 100 DUBs are thought to be responsible for regulating the ubiquitination of tens of thousands of proteins in a tightly regulated and sophisticated manner (57, 73). Hence defining the wide-ranging DUB targets of ataxin-3 is essential to decipher its functions. Ubiquitin signaling represents an indispensable mechanism of regulating both the innate and adaptive immune response, and is central to the NOD2 cascade (74). For the first time, this study undertook an unbiased screen of the ubiquitome in ataxin-3 depleted cells. Notably, a significant number of metabolism related proteins were found, further reinforcing the potential importance of ataxin-3 in cellular immunometabolism.

The discovery that HIF1α is a DUB target of ataxin-3 in macrophages is noteworthy, given the emergency of HIF1α as a key immunometabolic regulator. Work in a conditional HIF1 α knockout mouse, targeting the myeloid lineage, demonstrated the critical requirement of HIF1 α for inflammatory responses (75). This correlated with defects in glycolysis and metabolic activation, which is tightly regulated by HIF1 α The endolysosomal protein PLD3 was also validated as a further novel DUB target of ataxin-3. Although PLD3 contains two phosphodiesterase domains, and hence is classed as a member of the phospholipase diesterase (PLD) family which act to hydrolyse phospholipids, the phospholipase activity of PLD3 has not been definitively established. The importance of PLDS in immunity was recently demonstrated, with the discovery that it acts as a single-stranded acid exonuclease that breaks down ligands for the PRR TLR9, hence regulating TLR9 mediated inflammatory responses in collaboration with PLD4 (50).

The discovery in the present study that NOD2/TLR2 triggering leads to deubiquitination of LAMTOR1 by ataxin-3 is of particular interest. LAMTOR1, also known as p18, is a late endosome/lysosome membrane adapter protein that localizes to the lipid rafts of these organelles (76, 77). LAMTOR1 plays an essential role in the activation of the mTORC kinase complex in response to amino acid levels (54). Through a mechanism involving the lysosomal v-ATPase in the presence of amino acid sufficiency, LAMTOR1 forms a scaffold at the lysosomal membrane with LAMTOR2,3,4 and 5 (the pentameric Ragulator complex) for the Rag GTPase complex (RagAB/CD) (54, 78, 79). This leads to the recruitment and activation of mTORC1 which inhibits autophagy. The lysosomal v-ATPAse-Ragulator complex also activates another critical metabolic sensor, AMP-activated protein kinase (AMPK), which responds to falling energy levels by driving cellular catabolism programmes and downregulating anabolic pathways (80). Thus, this complex is able to respond to both energy/nutrient sufficiency and deficiency. Strikingly, of all cells, macrophages express the highest levels of the five Ragulator components, suggesting their importance in the immune response (81). Indeed, LAMTOR1 was recently found to be essential for the polarization of M2 macrophages both *in vitro* and *in vivo* in a knockout mouse model, by coupling metabolism to immunity (56).

The physiological importance of NOD2 and TLR2 in both the innate and adaptive immune response is well-established. The interplay between NOD2 and TLR2 has been well-characterized given the fact that they both respond to adjacent components of PGN found in the bacterial cell walls. The NOD2 signaling pathway amplifies TLR2 activation and both receptors synergize in the induction of cytokine production. *NOD2* variants confer the greatest single genetic risk factor for Crohn's disease disease (2, 3), yet significant gaps remain in our knowledge of how this receptor exerts its effects (82). Notably, despite the recent explosion of interest in the field of immunometabolism, almost nothing is known about how the synergistic effects of NOD2 and TLR2 signaling might intersect with metabolic pathways to modulate the immune response. Here, we defined the molecular and functional basis by which NOD2/TLR2 sensing links to ataxin-3 and, consequently, other immunometabolic factors. Future studies are required to provide novel prospects for modulating these pathways as new therapeutic strategies for inflammatory disorders.

MATERIALS AND METHODS

Cells

Human monocytes were purified from healthy donor peripheral blood mononuclear cells (PBMCs) by positive immunoselection with anti-CD14-conjugated MACS beads (Miltenyi Biotec). moDCs were obtained by culturing monocytes for 5 days with IL-4 and GM-CSF (Peprotech). Immature moDCs were harvested on day 5 of culture. The human THP-1 cell line was purchased from ATCC. Prior to use, THP-1 were differentiated by treatment with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) for 16 h.

Reagents and Antibodies

The following stains were used: MItosox Red M36008 (Invitrogen) and DHR 123 D23806 (Invitrogen). Antibodies include mouse anti-human ataxin-3 65042 1H9-2 (BioLegend), mouse anti-human MIC60 ab110329 (Abcam), mouse anti-human MIC60 ab137057 (Abcam), rabbit anti-human TBK1 #3504 D1B4 (Cell Signaling), rabbit anti-human RIPK2 #4142 D10B11 (Cell Signaling), rabbit anti-human p38 #9212 (Cell Signaling), rabbit anti-human LAMTOR1 #8975 D11H6 (Cell Signaling), rabbit anti-human PLD3 HPA012800 (Sigma), rabbit anti-human HA #3724 C29F4 (Cell Signaling). The secondary antibodies included: anti-rabbit HRP conjugate #7074 (Cell Signaling), anti-mouse HRP conjugate #7076 (Cell Signaling), goat anti-rabbit Alexa fluor 488 A-11034 (Invitrogen), goat anti-rabbit Alexa fluor 488 A-11029 (Invitrogen), goat anti-mouse Alexa fluor 568 A-11036 (Invitrogen), goat anti-rabbit Alexa fluor 568 A-11004 (Invitrogen). Beta actin HRP conjugate #5125 (Cell Signaling). For qPCR, the following Taqman primers were used (all ThermoFisher): NOD2 (Hs01550753_m1), RPLP0 (Hs99999902_m1), MT-ND1 Hs02596873_s1, MT-ND2, Hs02596874_g1, MT-ND3 Hs02596875_s1, MT-ND4L Hs02596877_g1, MT-CYB Hs02596867_s1, MT-CO1 Hs02596864_g1, ATP6, Hs02596862_g1. For overexpression

experiments, GFP-LAMTOR1 and HA-ataxin-3 were obtained from the University of Dundee.

Cell Stimulation

moDCs and THP-1 cells were left unstimulated or stimulated with 10 μg/ml MDP or 1 μg/ml PAM_3CSK_4 (Invivogen) or both at the indicated time points. In some experiments, other PRR ligands were used including LPS 100 ng/ml, and R848 1 μg/ml (Invivogen) or cells were treated with the small molecule inhibitor Ponatinib (50 nM) for 1 h.

Phosphoprotein Purification

Cells were harvested on ice and washed once with ice cold modified Hanks Buffered Saline (HBS) (20 mM HEPES pH 7.4, 150 mM NaCl in ddH_20). Cell pellets were lysed in Qiagen "Phosphoprotein lysis buffer" containing 0.25% (v/v) CHAPS with 1% (v/v) phosphatase inhibitor cocktail 3 (Sigma), protease inhibitor tablet (Qiagen) and the nuclease 0.0002% (v/v) Benzonase (Qiagen) at 4°C for 40 min, with vortexing every 10 min. Cell debris was removed by centrifugation of the lysate at 13,300 rpm for 30 min at 4°C. The clarified supernatant was then transferred to fresh pre-cooled tubes and protein concentration determined by BCA assay. The samples were then diluted in Qiagen "wash buffer" containing 0.25% (v/v) CHAPS to a concentration of 0.1 mg/ml. Aliquots of whole cell lysate and diluted whole cell lysate were kept for subsequent immunoblot. The phosphoenrichment columns were washed with 6 ml "wash buffer," before the diluted samples were loaded onto the columns. Following two further washes of the columns with 6 ml "wash buffer," the phosphoenriched fraction was eluted from the columns using Qiagen "elution buffer" containing 0.25% (v/v) CHAPS. Following concentration of the eluted fraction to a volume of 200–300 μl using 9 k molecular weight cut-off concentrator columns (Thermo Fisher) with centrifugation 13,000 rpm 30 min, protein concentration was measured by BCA. For mass spectrometry (MS) experiments, phosphoenriched lysates were stored at −80°C. Otherwise, whole cell lysate, diluted whole cell lysate and phosphoenriched lysates were processed for SDS-PAGE with NuPage LDS Sample buffer (Life Technologies) and 100 mM dithriothreitol (Sigma), followed by heating at 70°C for 5 min. Samples were then frozen at −80°C until immunoblotting was performed.

Immunoprecipitation

Samples were washed twice in ice cold HBS and then lysed in 1,000 μl lysis buffer for 30 min at 4°C with end over end mixing [Cell Signaling Lysis Buffer 20 mM Tris-HCL pH7.5, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3O_4, 1 μg/ml leupeptin supplemented with 1% (v/v) HALT protease inhibitor cocktail (Thermo Fisher) and 1% (v/v) Phosphatase inhibitor cocktail 2 and 3 (Sigma) and 1 mM PMSF (Cell Signaling)]. Lysates were clarified by centrifugation at 14,000 g for 15 min at 4°C, and the supernatant transferred to fresh Eppendorfs. Protein concentrations were calculated by BCA. Fifty μl of input lysate was heated with LDS/DTT and stored at −80°C for later immunoblot. Next, concentrations were adjusted to 1 mg/ml and 7.5 mg of protein was taken forward for IP for each condition. The appropriate antibody or isotype control antibody was added to the lysates followed by incubation with gentle end over end mixing at 4°C overnight.

The next morning, Protein G Dynabeads (Thermo Fisher) were washed once in lysis buffer, using a DynaMag2 magnet (Invitrogen) to separate the beads from solution, and 5 μl of beads per 1 μg of antibody was added to each sample. Samples were incubated with the beads with gentle end over end mixing for 2 h at 4°C. Following this, the supernatant was removed using a magnet to separate the beads, with 50 μl of the supernatant heated with LDS/DTT and stored at −80°C later immunoblot. The beads were washed 4 times in total with lysis buffer containing all protease and phosphatase inhibitors, with gentle end over end mixing for 5 min at 4°C for each wash. Elution of the beads was then performed by incubating the beads with pH 2.8 elution buffer (Pierce) with gentle end over end mixing for 30 min at 4°C. The eluate was collected and neutralized immediately with 1/10 volume of 1 M Tris-HCL pH 9. Elution and neutralization was performed a further two times to ensure complete elution. The eluate was stored at −80°C until used for downstream processing.

Tandem Ubiquitin Binding Entities (TUBEs) Ubiquitin Immunoprecipitation

Typically 2.5×10^7 THP-1 cells were used per condition. Thirty min before the end of the experimental conditions, samples were incubated with 10 μM MG132 (Sigma) for 30 min at 37°C before harvesting, washing once in ice cold PBS and lysing in 1 ml Ub-IP lysis buffer (50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche), 1% (v/v) phosphatase inhibitor cocktail 3 (Sigma), 20 μM MG132, 50 μM PR619 and 100 mM N-ethylmaleimide (Sigma). Cells were lysed for 30 min at 4°C with gentle end over end mixing. The lysate was clarified by centrifugation at 13,000 rpm for 20 min at 4°C, and the supernatant transferred to fresh Eppendorfs. Protein concentration was calculated by the Bradford assay and protein concentrations normalized to 2 mg/ml. Typically 2 mg of protein was taken forward for TUBEs IP. Fifty ul of input fraction was heated with LDS/DTT and stored at −80°C for later immunoblot. Samples were incubated with 40 μl TUBE1 agarose beads (LifeSensors), or control agarose beads (LifeSensors) for 4 h at 4°C with gentle end over end mixing. The beads were then centrifuged at 3,000 g for 3 min and washed with lysis buffer containing all inhibitors three times (each wash was performed for 5 min at 4°C with gentle end over end mixing), followed by two final washes with lysis buffer without SDS and Deoxycholate. Elution was then performed with 50 ul of pH 2.8 elution buffer (Pierce) for 30 min with gentle end over end mixing at 4°C, followed by immediate neutralization with 1/10 volume 1 M Tris-HCL pH 9. Elution and neutralization was performed three times in total. Samples prepared for later MS analysis were frozen at −80°C until further processing (100 of 150 μl total eluate). The

remaining 50 µl, and all eluate from other experiments were heated with 2 × LDS/DTT at 70°C for 10 min and stored at −80°C until immunoblot.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

100 µl of lysate was adjusted to 175 µl with ddH_20. All samples were then successively reduced and alkylated for 30 min with 5 mM dithiothreitol and 20 mM iodoacetamide, respectively. The proteins were then precipitated using chloroform-methanol precipitation and the pellet were solubilized in 6 M urea, 0.1 M Tris pH 7.8. The sample were diluted to 1 M Urea. The digestion was performed overnight at 37°C by adding 500 ng of trypsin. The peptides were desalted using a C18 cartridge (Waters). Briefly, the samples were conditioned with buffer A (1% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA) in water) prior to equilibration with buffer B (65% (v/v) acetonitrile, 0.1% (v/v) TFA in water). The acidified peptides were loaded onto the column, washed with buffer A and eluted with buffer B. The solution containing the peptides was dried with a speedvac and solubilised in 1% (v/v) acetonitrile, 0.1% (v/v) TFA in water for mass spectrometry analysis.

Peptides were analyzed with nano ultra-high performance liquid chromatography tandem mass spectrometry (nano-UPLC-MS/MS) using a Dionex Ultimate 3000 nanoUPLC, coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MS analysis was performed essentially as described previously (83). In brief, the data were acquired with a resolution of 120,000 full-width half maximum at mass/charge 200 with EASY-IC using the reagent ion source (202 m/z) for internal calibration enabled, Top speed precursor ion selection, Dynamic Exclusion of 60 s and fragmentation performed in Collision Induced dissociation (CID) mode with Collision Energy of 35.

Analysis of Mass Spectrometry Data

Label-Free Quantitative Analysis

The raw MS data was analyzed using Progenesis QI (Waters) and searched using Mascot 2.5 (Matrix Science). The search settings were as follows: trypsin with 1 miscleavage allowed, oxidation (M) and Deamidation (N, Q) were set as variable modifications and carbamidomethylation (C) as fixed modification. The data was searched against human protein sequences using the UPR_homoSapiens_20141015 (85,889 sequences; 33,866,397 residues) allowing a peptide mass tolerance of 10 ppm and a fragment mass tolerance of 0.05 Da.

Peaks Search for Phosphorylation

The raw MS data was analyzed in PEAKS Studio 7.5 (Bioinformatics Solutions Inc). The settings were the following: The database used was the swissprot human database was used for the proteins identification. The enzyme used for the search was trypsin allowing a maximum of 2 miscleavages. Fixed modifications: Carbamidomethyl (C); Variable modifications: Deamidated (N), Deamidated (Q), Oxidation (M), Phospho (STY). Ten ppm mass tolerance were allowed for the precursor ions and 0.05 Da was allowed for the fragment ions.

Mascot Search for Phosphorylation

The raw MS data was searched using Mascot with following settings. Enzyme: Trypsin; Fixed modifications: Carbamidomethyl (C); Variable modifications: Deamidated (N), Deamidated (Q), Oxidation (M), Phospho (ST), Phospho (Y); Peptide mass tolerance: ± 10 ppm (# 13C = 1); Fragment mass tolerance: ± 0.5 Da; Max missed cleavages: 1.

shRNA Lentiviral Transduction and siRNA Transfection

Short hairpin RNA lentiviral particles were produced and transduced following the RNAi Consortium (TRC) protocols. ShRNA containing pLKO.1 vectors targeting NOD2 (SHCLND-NM_022162), ataxin-3 (SHCLND-NM_004993), TBK1 (SHCLND-NM_013254) or non-Target shRNA Control Plasmid DNA were all obtained from Sigma (MISSION shRNA Plasmid DNA). In brief, HEK293T packaging cells growing in 6 cm well plate were transfected with a mix of 1 µg packaging vector (psPAX2), 0.4 µg envelope vector (pMD2.G) and 1.6 µg hairpin-pLKO.1 vector (SHC016 control or gene specific shRNA. Fugene-6 (Promega) was used as transfection reagent. Cell culture medium containing lentiviral particles (LVP) was collected 48 h later and passed through a 0.45 µm filter (Sartorius). Virus preparations were then concentrated by centrifugation at 30,000 rpm for 90 min. Viral particles were added to cultured THP-1 cells in R10 [Roswell Park Memorial Institute medium (RPMI-1640) (Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Sigma), 2 mM (1% v/v) L-glutamine (Sigma)] together with 8 µg/ml Polybrene (Sigma) to improve transfection efficiency. Following incubation for 3 h at 37°C, the cells were harvested, washed, and resuspended at 1×10^6 cells/ml in R10 media with antibiotics including puromycin (as selective antibiotic). After 10 days of continuous selection with puromycin, knockdown efficiency was assessed by immunoblot. Transfection of human dendritic cells was performed by electroporation of SMARTpool ONTARGETplus human ataxin-3 (ATXN3) or non-targeting siRNAs (Dharmacon). Cells were resuspended in the solution provided with the kit (Invitrogen) followed by electroporation with Neon System kit (Invitrogen) using the following parameters: 1,475 V, 20 ms, 2 pulses. After 48 h, cells were harvested for use in experiments and to check knockdown by immunoblot.

Adherent Cell Transfection

Human HEK293/NOD2 Cells were seeded 24 h prior to transfection in media without antibiotics. Transfection mixes were made, comprising Fugene (Promega) at a ratio of 3:1 to amount of DNA plasmid to be transfected, in the appropriate volume of Opti-MEM (Gibco, Thermo Fisher) (10% of volume of media in wells to be transfected). The transfection mixes were incubated at room temperature for 20 min and then added dropwise to the wells to be transfected. Cells were either cultured for a further 24 or 48 h before being used for downstream applications.

RNA Isolation

Typically 2–5 × 10^6 cells per condition were harvested and washed once with cold PBS. Pellets were resuspended in 350 μl RLT buffer (Qiagen) containing 1% (v/v) Mercaptoethanol (Sigma) and stored at −80°C. Samples were thawed on ice and homogenized by adding to Qiashredder columns (Qiagen) and centrifuged 2 min 13,000 rpm. RNA isolation was then peformed using RNeasy kits (Qiagen) according to manufacturer's instructions. The isolated RNA was eluted by added 25 μl nuclease free water (Ambion) to the RNeasy column membrane for 5 min, followed by centrifugation into fresh Eppendorfs 8,000 g 1 min. RNA concentration and purity were obtained using a Nanodrop 1000 spectrophotometer (Thermo Fisher) and samples were stored at −20°C until further analysis.

Reverse Transcription

RNA was reverse transcribed using a high capacity RNA to cDNA kit (Applied Biosystems). Five hundred ng to 2 μg of RNA was normalized to the same concentration for each sample using nuclease free water (Ambion) in polypropylene PCR tubes (Starlab). Then an RT mix containing 2 μl 10× RT buffer, 0.8 μl 25× dNTP mix, 2 μl RT random primers, 1 μl multiscribe RT, 1 μl RNase inhibitor (Applied Biosystems) and 3.2 μl nuclease free water was added to each PCR tube (10 μl total RT mix) (Starlab) for a total volume of RNA sample and RT mix of 20 μl. This was reverse transcribed using a Thermo Cycler (Applied Biosystems) with the program: 25°C 10 min, 37°C 120 min, 85°C 5 min. The cDNA was stored at −20°C.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

qPCR was performed using TaqMan chemistry (Applied Biosystems). cDNA was diluted 10-fold with nuclease free water. 4.5 μl of diluted cDNA was added in triplicate for each sample to wells of a white 0.2 ml 96 well PCR microplate (Starlab). 0.5 μl of TaqMan FAM-MGB labeled primer (Applied Biosystems) and 5 μl TaqMan Universal PCR Mastermix (Applied Biosystems) was added to each well, resulting in a 10 μl total reaction mix. The plate was covered with a polyolefin optical film (Starlab) and centrifuged at 400 g for 1 min. qPCR was then performed using the Bio-Rad C1000 Thermal cycler CFX Realtime system (Bio-Rad) using the manufacturer's recommended program: 50°C 2 min, 95°C 10 min, then 40 cycles of 95°C 15 s, 60°C 1 min. Mean cycle threshold (Ct) number was calculated from the triplicate values. Relative gene expression was calculated in comparison to the housekeeping RPLP0 control. The difference in gene expression between conditions was calculated using the $2^{-\Delta\Delta Ct}$. This is derived from:

$$\Delta CT = CT(\text{targetgene}) - CT(\text{control gene})$$
$$\Delta\Delta CT = \Delta CT(\text{targetcondition}) - CT(\text{control condition}).$$

Flow Cytometry

Typically 0.5 × 10^6 THP-1 cells per condition were plated in 1 ml of media in 12 well plates and differentiated for 16 h with 25 ng/ml PMA (Sigma). Following differentiation, the indicated treatments were applied. Cells were then harvested with gentle scraping and transferred to a FACS tube. The following staining protocols were then followed. Cytofluorometric evaluation was by the LSRII flow cytometer (BD Biosciences) with analysis of the data by FLOWJo.

MitoSOX Red Staining

The cells were pelleted by centrifugation and resuspended in room temperature HBSS (Thermo Fisher) to wash, then centrifuged. The cells were resuspended in 200 μl MitoSOX red solution (final concentration 5 μM MitoSOX red (Invitrogen) in HBSS) and incubated for 15 min in the 37°C cell culture incubator. Two hundred μl of HBSS was added, and the tube centrifuged. The cells were resuspended in 250 μl HBSS and cytofluorometric evaluation performed.

Dihydrorhodamine 123 (DHR) Staining

The cells were pelleted by centrifugation and resuspended in room temperature PBS to wash, then centrifuged. The cells were resuspended in 50 μl DHR solution (final concentration 2.5 μg/ml DHR 123 (Invitrogen) in PBS) and incubated for 30 min at 37°C in a water bath. As a positive control, 50 μl of PMA (final concentration 100 ng/ml (Sigma) in PBS) was added to control samples for the final 15 min. The cells were centrifuged and then washed once with PBS, before resuspension in 200 μl FACS staining buffer. Cells were fixed with the addition of 200 μl 1% PFA and cytofluorometric evaluation performed.

Seahorse Mitochondrial Stress Assay

The Seahorse XFe96 Extracellular Flux Analyser was used to measure mitochondrial respiration and glycolysis (Seahorse Bioscience). Seahorse 96 well assay plates (Seahorse Bioscience) were coated with Cell-Tak suspension (Corning). THP-1 cells were seeded at 1.5 × 10^5 cells/well. Next, the optimal working concentrations of the compounds used for the mitochondrial stress test (oligomycin, FCCP and antimycin/rotenone) and the glycolysis stress test (oligomycin and 2-DG) were determined for THP-1 cells. The aim was to maximize the response to each compound with the lowest concentration possible. An XFe96 sensor cartridge (Seahorse) was hydrated overnight prior to Seahorse assays by adding 200 μl of XF calibrant solution to each well and incubating in a CO_2 free incubator at 37°C. The sensor cartridge (Seahorse) was loaded with the test drug compounds immediately prior to the assay and loaded on the Seahorse Analyser. Twenty-four h before the assay was run, cells were harvested, counted and resuspended at 1.88 × 10^6 cells/ml. Two ml of cell suspension was plated in 6 well plates for each condition, with or without the appropriate ligand stimulation for the required duration. On the day of the assay, 1 ml of cell suspension from each condition was harvested into 1.5 ml Eppendorf tubes. For the mito stress test, cells were resuspended in mito stress test media with or without ligand(s) (XF base media (Seahorse Bioscience) supplemented with 1 mM sodium pyruvate (Sigma), 5 mM glucose (Life Technologies) and 2% FCS (Sigma) adjusted to pH 7.4 at 37°C and sterile filtered). Eighty μl of cell suspension (1.5 × 10^5 cells) was then seeded in quadruplicate for each condition and the plate was centrifuged at 200 g for 1 min. Following 30 min in a CO_2 free incubator at 37°C, 95 ul of fresh mito stress test media was

added to each well, and after a further 15 min in a CO_2 free incubator the assay was run. Final concentrations of injected drugs were 1 μM oligomycin, 1 μM FCCP, 0.3 μM rotenone and 0.3 μM antimycin.

STED Immunofluorescence Microscopy

Cells were plated in 8-well detachable tissue culture chambers on a PCA slide (Sarstedt) coated with 0.01% poly-l-lysine (Sigma)—cells were at a density of 1–2 × 10^5 cell per well in 250 ul of appropriate media. At the end of the experiment, cells were washed twice with PBS, fixed with 4% paraformaldehyde (Sigma) for 15 min and permeabilized with 0.5% (v/v) Triton X-100 (Sigma). Cells were blocked overnight at 4°C with 150 μl blocking solution per well (5% (v/v) human serum (Sigma), 5% (v/v) goat serum (Sigma), 5% (v/v) FCS (Sigma). The following day, cells were incubated with primary antibody diluted in 140 μl blocking solution at a pre-optimized or manufacturer recommended concentration for 1 h at room temperature. Following three washes with PBS (250 μl per well, 5 min gentle shaking), cells were incubated with the species appropriate fluorescently labeled secondary antibody for 1 h at room temperature. Cells were washed three further times with PBS, and the detached slide was then mounted with Vectashield mounting media containing DAPI (Vector Laboratories). Alternatively, cells were incubated with 200 μl PBS containing 1:100 DAPI (Thermo Fisher) for 15 min at room temperature and the detached slide was mounted with Vectashield mounting media without DAPI (Vector Laboratories). A Leica SP8 STED system was used for imaging. ImageJ was used for image processing and analysis.

Bacterial Killing Assay

1 × 10^6 THP-1 cells were seeded per condition in 1 ml R10 without antibiotics in a 24 well plate, and differentiated overnight with 25 ng/ml PMA. After 16 h, the media was changed for 500 μl fresh R10 media without antibiotics. Two h later, *Salmonella enterica* serovar Typhimurium strain LT2 (ATCC 700220) was added at a multiplicity of infection (MOI) of 20:1. Thirty min post-infection, wells were washed twice with PBS and 500 μl fresh R10 supplemented with Gentamicin 100 μg/ml was added. After a further thirty min, wells were washed once with PBS and 500 μl fresh R10 supplemented with Gentamicin 30 μg/ml was added. At the end of the designated post-infection period, the medium was removed (and stored at −80°C until later analysis by ELISA) and 500 μl of PBS with 1% (v/v) saponin was added to the wells, followed by incubation for 5 min at 37°C. Five hundred μl of PBS was added and serial dilutions plated on LB/agar plates, incubated overnight at 37°C, and colonies then counted. Alternatively, when cell viability post-infection was assessed, cells were detached by incubating with 500 μl of trypsin per well for 5 min at 37°C, then viability assessed by trypan blue staining (Invitrogen) and counting of live/dead cells.

Statistical Analysis

Prism (GraphPad) was used to determine the statistical significance. When making multiple comparisons on a data set, analysis was by one-way ANOVA with *post-hoc* Bonferroni analysis. For experiments with two sample groups (one condition, one control) and a single comparison, analysis was by paired, two-tailed Student's *t*-test. Error bars represent Standard Error of the Mean (SEM).

DATA AVAILABILITY

The MS proteomics data is available upon request from the authors.

ETHICS STATEMENT

Ethical approval for the study was obtained from National Research Ethics Service (NRES) Sheffield Research Ethics Committee (REC reference: 16/YH/0247) and from West Midlands - The Black Country Research Ethics Committee (REC reference: 09/H1204/30).

AUTHOR CONTRIBUTIONS

TC, DC, and AS designed experimental studies, interpreted the data, and wrote the manuscript. TC, DC, SS, SP, AA, SW, MdC, M-LT, RF, and BK performed experiments, acquired, and analyzed data. HP and BK provided critical reagents, helped interpret data. AS supervised and obtained funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01495/full#supplementary-material

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REVIEW
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NAD-Biosynthetic and Consuming Enzymes as Central Players of Metabolic Regulation of Innate and Adaptive Immune Responses in Cancer

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Cancer cells, particularly in solid tumors, are surrounded by non-neoplastic elements, including endothelial and stromal cells, as well as cells of immune origin, which can support tumor growth by providing the right conditions. On the other hand, local hypoxia, and lack of nutrients induce tumor cells to reprogram their metabolism in order to survive, proliferate, and disseminate: the same conditions are also responsible for building a tumor-suppressive microenvironment. In addition to tumor cells, it is now well-recognized that metabolic rewiring occurs in all cellular components of the tumor microenvironment, affecting epigenetic regulation of gene expression and influencing differentiation/proliferation decisions of these cells. Nicotinamide adenine dinucleotide (NAD) is an essential co-factor for energy transduction in metabolic processes. It is also a key component of signaling pathways, through the regulation of NAD-consuming enzymes, including sirtuins and PARPs, which can affect DNA plasticity and accessibility. In addition, both NAD-biosynthetic and NAD-consuming enzymes can be present in the extracellular environment, adding a new layer of complexity to the system. In this review we will discuss the role of the "NADome" in the metabolic cross-talk between cancer and infiltrating immune cells, contributing to cancer growth and immune evasion, with an eye to therapeutic implications.

Keywords: immunometabolism, metabolic reprogramming, immune cell regulation, NAD, tumor microenvironment

COMPOSITION OF THE TUMOR MICROENVIRONMENT: SUPPORTIVE AND IMMUNOREGULATORY CELLS

The solid tumor microenvironment (TME), as well as the lymphoid niche, is a dynamic and multicellular ecosystem with complex interactions (1, 2). Intercellular crosstalk within this niche is driven by multiple receptor-ligand systems, as well as by locally synthesized soluble proteins, including chemokines/cytokines, interleukins, interferons, growth, and angiogenic factors (3, 4). This unique environment is essential for tumor growth, metastatic dissemination, and

drug-resistance. Furthermore, the cellular and soluble components of the TME have an important role in shaping metabolic reprogramming of cancer cells, an established hallmark of cancer, and in creating an immunosuppressive environment (5–8), as showed in **Figure 1**.

The formation of the TME and the regulation of immune responses are orchestrated by different types of host cells, including endothelial cells (ECs), mesenchymal stem/stromal cells [MSCs, including cancer-associated fibroblasts (CAFs) and tumor-associated MSCs (TA-MSCs)], and tumor-infiltrating immune cells [i.e., tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and tumor-associated neutrophils (TANs)]. Their concerted action promotes tumor growth and spreading (1, 2, 9, 10) (**Figure 1**).

Endothelial Cells (ECs)

ECs support blood supply, nutrient transport, metabolic homeostasis, and immune cell trafficking, and are involved in inflammatory response (11).

To provide nutrients to the growing tumor, ECs form tumor-associated (angiogenic) vessels originating from locally pre-existing vessels or recruiting bone marrow-derived endothelial progenitors. They also represent the first interface between circulating blood cells, tumor cells, and the extracellular matrix, thereby playing a central role in regulating leukocyte recruitment, tumor cell features, and metastasis dissemination (12). Tumor-associated EC are dysfunctional, partly as a consequence of local hypoxia, which induces the production of soluble factors promoting neo-angiogenesis and contributing to tumor dissemination and chemoresistance (13, 14). Among these factors, vascular endothelial growth factor A (VEGF-A) can also play a critical role in the control of immune tolerance, linking immune suppression with angiogenesis (15).

Mesenchymal Stem/Stromal Cells (MSCs)

MSCs strongly affect the development and progression of various cancers (16). Stromal cells represent the main cell component with both supportive and immunoregulatory functions; they derived from multipotent cells of mesodermal origin which virtually reside in all tissues with an important role in tissue regeneration (16). MSCs have been found to migrate to tumors and to evolve into TA-MSCs and CAFs with an active role in tumor survival, proliferation, migration and drug resistance, and therefore, recently emerged as attractive targets or tools for anticancer approaches (17, 18).

CAFs are the most abundant resident cells of the TME. Numerous studies have demonstrated that CAFs have prominent roles in cancer pathogenesis (19, 20). Mechanistically, CAFs shape the extracellular matrix (ECM) structure, which supports the tumor cells (i) to invade and interact with stromal cells through the secretion of growth factors, cytokines and chemokines including interleukin-6 (IL-6), transforming growth factor-β (TGF-β) and CC-chemokine ligand 2 (CCL2); (ii) to amplify immune evasion recruiting immune cells, especially immunosuppressive cells into the tumor stroma; (iii) to promote the establishment of an intratumoral vascular network through proinflammatory and proangiogenic mediators (21). CAFs also activate epithelial-mesenchymal transition (EMT) in cancer cells, conferring their pro-invasive and stem-like features (22). In addition, CAFs are plastic cells that co-evolve with cancer cells and acquire a pro-tumor phenotype, contributing to tumor evolution (23). Due to the pro-tumor role of CAFs in support cancer development they become promising therapeutic targets for cancer therapy (21).

Tumor-Infiltrating Lymphocytes (TILs)

TILs are additional immune components, crucial in driving immune responses within the TME, adding more complexity in the composition of the TME (3). TILs are white blood cells, including T and B cells, that have left the bloodstream and migrated toward a tumor or tissue resident (1, 24). Their abundance varies according to tumor type and stage and in some cases relates to disease prognosis, tumor progression, and response to anticancer therapy (1, 25, 26). T cell differentiation status, survival, activation or "stemness properties" are determining factors of antitumor potency (27) and functions of TILs dynamically change within the TME (28). Sometimes TILs, specifically cytotoxic $CD8^+$ memory T cells and $CD4^+$ T helper 1 (Th1), which are normally antigen "experienced," kill tumor cells (29), and the presence of lymphocytes in tumors is often associated with a better prognosis during immunotherapy treatment, including the adoptive transfer of naturally- TIL or genetically-engineered T cells and the use of immune-checkpoint inhibitors (26, 30). However, very often, during cancer progression and chronic inflammation, T cells become exhausted due to the persistent antigen exposure. T cell exhaustion is a state of T cell dysfunction defined by poor effector function, sustained expression of inhibitory receptors, such as programmed cell death protein 1 (PD1) and cytotoxic T lymphocyte antigen 4 (CTLA4), and transcriptional programs altered compared with functional effector or memory T cells (31).

Regulatory T (Treg) cells are another TME cell type that has immunosuppressive functions in cancer, inhibiting recognition, and clearance of tumor cells by the immune system (30, 32, 33). Tregs are characterized by the expression of CD4, CD25, and forkhead box P3 (FOXP3) as their master regulator. $Foxp^{3p}$ Treg can originate in the thymus (naturally occurring Treg) or can be induced (iTreg) in the periphery by soluble cytokines and cell-cell contact (34) and are essential for maintaining peripheral tolerance and limiting auto-immune diseases. However, the proportions of Tregs are much higher in the circulation of patients with solid and hematologic malignancies and accumulation of Tregs in the tumor microenvironment is associated with disease progression and reduced survival (35, 36). From a functional point of view, Tregs inhibit both cellular and humoral immune responses by suppressing expansion and activation of conventional $CD4^+$ and cytotoxic $CD8^+$ T cells, and natural killer cells, mainly through the secretion of suppressive cytokines, such as TGF-β and IL-10. The development of agents that specifically inhibit Treg functions or remove them from the TME will permit new approaches for anticancer immunotherapy (37).

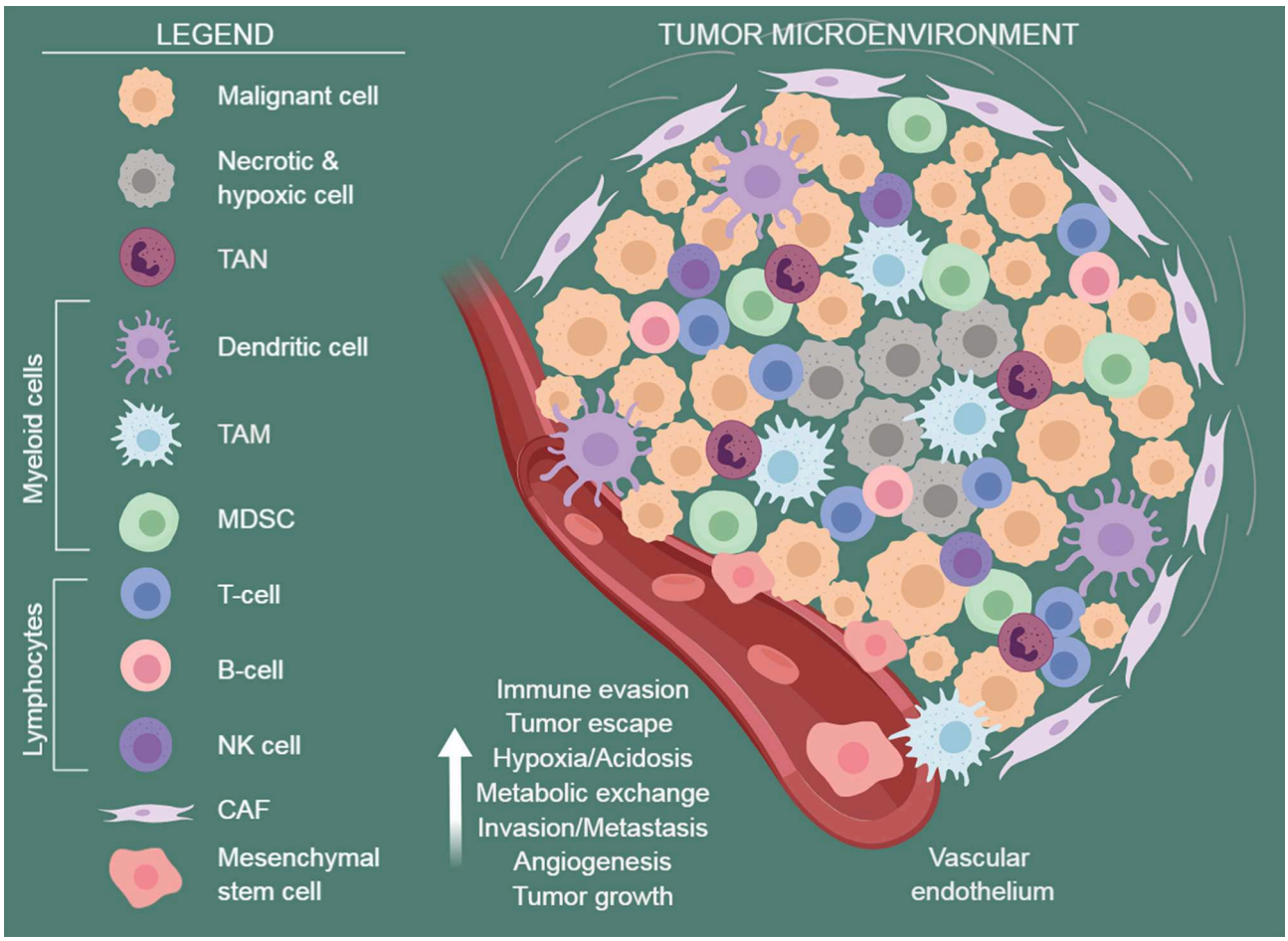

FIGURE 1 | The tumor microenvironment. A schematic view of the tumor microenvironment components. Established cancers are usually surrounded by a wide array of stromal cells and infiltrating immune cells of both innate and acquired immunity, such as MDSCs, macrophages, dendritic cells, neutrophils, NK cells, and lymphocytes. They form a complex regulatory network that supports tumor growth by creating a tolerogenic environment that enables cancers to evade immune surveillance and destruction. TAN, tumor-associated neutrophils; TAM, tumor-associated macrophages; MDSC, myeloid-derived suppressive cells; CAF, cancer-associated fibroblasts. Figure arrange using BioRender software https://biorender.com/.

Tumor-Associated Macrophages (TAMs)

TAMs are important mediators of tumorigenesis, resident in the tissue or deriving from peripheral reservoirs such as the bone marrow (BM) and spleen (2). Macrophages are functionally plastic and can be polarized into the immune stimulating and antitumor M1 subtype, or into "alternatively activated" M2 macrophages producing type II cytokines, promoting anti-inflammatory responses, and having pro-tumorigenic functions (38, 39). Macrophage polarization is finely tuned in response to different microenvironmental stimuli (40). For example, hypoxia may mediate this transition from tumor suppressing to tumor promoting macrophages (41). Furthermore, it has been shown a reciprocal regulation between CAFs and M2 macrophages: CAFs promote monocyte recruitment and polarization toward the M2 phenotype, leading to the enhancement of proangiogenic features, in parallel M2 macrophages are able to induce fibroblast activation (42). It is well-known that TAMs have a clear role in supporting multiple aspects of tumor progression (43). For example, TAMs promote tumor cell invasion through a paracrine loop that involves tumor-derived colony-stimulating factor 1 (CSF-1) and macrophage-derived epidermal growth factor (EGF) (43, 44). Moreover, TAMs induce immune suppression [reviewed in (45)] mediated by (i) expression of inhibitory receptors, including human leukocyte antigens (HLA)-E and HLA-G and T cell immune checkpoint ligands, such as PDL1, PDL2, CD80 and CD86, which directly inhibit T cell functions and NK cells; (ii) release of several cytokines, such as IL-10 and transforming growth factor-β (TGFβ), that contribute to feed a strong immunosuppressive microenvironment by inhibiting $CD4^+$ (Th1 and Th2 cells) and $CD8^+$ T cells and inducing Treg cell expansion and recruitment through CCL2, CCL3, and CCL20. Lastly, they induce depletion of essential amminoacids for cytotoxic activity of T cells including l-arginine and tryptophan, or production of kynurenine by indoleamine 2,3-dioxygenase (IDO) that inhibits T cell cytotoxicity.

Reversion of TAMs back to an M1 phenotype has also been reported (46), highlighting a potential therapeutic opportunity in which re-education of TME-resident macrophages might have beneficial anti-tumorigenic effects (45).

Myeloid-Derived Suppressor Cells (MDSCs)

Along with TAMs, MDSCs are considered major promoters of tumor immune evasion (47). This population of myeloid cells, functionally defined as immunosuppressive, arises as a consequence of aberrant myelopoiesis typical of cancer (48). During tumorigenesis, MDSCs are mobilized from BM, via CXCR4/CXCL12 axis (49) and infiltrate tumors, where they promote tumor neoangiogenesis, producing endothelial growth factors [e.g., VEGF, basic fibroblast growth factor (bFGF)] (47). At the same time, they disrupt the major mechanisms of immunosurveillance, including antigen presentation by dendritic cells (DCs), T cell activation, M1 macrophage polarization and NK cell cytotoxicity, as reviewed in Safari et al. (50) and Wang et al. (51). Pharmacological inhibitors of CXCR4, are now under clinical investigation for the mobilization of immune and hematopoietic stem cells (52). Noteworthy, depletion of MDSCs by chemotherapeutic agents (e.g., gemcitabine, cyclophosphamide) can efficiently contribute to their anticancer action (48, 50, 53).

Tumor-Associated Neutrophils (TANs)

More recently, a population of neutrophils, known as TANs, has been identified as tumor supporter promoting growth, invasion, and angiogenesis of cancer cells, although they have been classically considered to exhibit a defensive response against tumor cells. Like all other leukocytes, they migrate into tissues under the effect of specific chemokines, cytokines and cell adhesion molecules for example TGF-β and IL-8 induce the formation of a pro-tumorigenic (N2) phenotype capable of supporting tumor growth and suppressing the antitumor immune responses (54, 55). Accordingly, TGF-β blocking results in the recruitment and activation of TAN with an anti-tumor phenotype (54). The main tumor-promoting mechanisms of TANs include secretion of chemokines and/or cytokines, reactive oxygen species (ROS), and matrix-degrading proteinases, among others, conditioning tumor immune surveillance, metastasis, invasion, angiogenesis, and cellular proliferation (55, 56).

TUMOR-STROMA METABOLIC CROSS-TALK IN TME

It has been shown that the environment surrounding tumor cells is characterized by low oxygen tension (i.e., hypoxia) due to the abnormal blood vessel formation, defective blood perfusion, and unlimited cancer cell proliferation (14). The progression of hypoxia over time is a consequence of increased oxygen consumption and high glycolytic rate of aberrantly proliferating cancer cells (aerobic glycolysis or Warburg metabolism), leading to lactate dehydrogenase (LDH) activity, lactate excretion and TME acidosis, which alters the tumor-stroma "metabolic cross-talk" (**Figure 1**). *Vice versa*, hypoxia rapidly fosters energy production in tumor cells via glycolysis through hypoxia-inducible factor 1-alpha (HIF-1α)-mediated transcriptional control (57, 58). In addition, a hypoxic environment also modulates tumor-associated immune and stromal cells metabolism and fate. The rapid consumption of extracellular glucose and glutamine by tumor cells, especially in hypoxic conditions, leads to the accumulation of extracellular lactate, which was shown to affect several cell types within the TME (59). Increased lactate levels promote the insurance of an immune-permissive microenvironment by attenuating DCs and T cell activation, monocyte migration, and polarization of resident macrophages to TAMs (60–63). Furthermore, lactate accumulation promotes angiogenesis, stabilizes HIF-1α and activates NF-kB and PI-3 kinase signaling in endothelial cells, as well as inducing secretion of the proangiogenic factor VEGF from tumor-associated stromal cells (64–66). The secretion of lactate via the monocarboxylate transporter (MCT3) is coupled to the cotransport of H^+, which supports acidification of the cellular microenvironment (59). The surplus of CO_2 generated in mitochondrial decarboxylation reactions contributes to extracellular acidification as well (67). Then, a class of extracellular carbonic anhydrases (CA) can convert CO_2 to H^+ and $HCO3^-$. Accordingly, expression of CAIX isoforms is elevated during hypoxia and can be considered a proxy for HIF-1α signaling (68). A consequence of increased extracellular acidification is the stimulation of the proteolytic activity of MMPs that promotes the degradation of the extracellular matrix components enhancing tumor invasion (69).

Lactate in TME can be also recycled, as occurs in the Cori cycle in the liver. In this reciprocal metabolite changes between cancer cells and immune/stromal cells, lactate produced under hypoxic conditions by glycolytic cells can be re-uptaken by aerobic cells, via MCT1, and utilized for mitochondrial tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) (70, 71). This well characterized mechanism is known as the "reverse Warburg effect" (70, 72). In a model of epithelial cancer, tumor cells instruct the normal stroma to transform into a wound-healing stroma, providing the necessary energy-rich microenvironment for facilitating tumor growth and angiogenesis (72, 73). This metabolic cross-talk is evident in breast, prostate and ovarian cancer (74–76).

Both innate and adaptive immune cells increase their metabolic capacity upon stimulation, promoting energy generation, and biosynthesis supporting proliferation, effector molecule production, and differentiation (77). The impact of such altered metabolic state and levels of metabolites in TME on immune cell function is emerging. For example, a competition between tumor cells and T cells for the glucose pool in the aerobic microenvironment is linked to suppressed effector T-cell functions. In fact, activated T cells rely on glucose metabolism, up-regulating GLUT1 transporter via T cell receptor (TCR) and CD28-induced Akt activation (78, 79). Critical concentrations and/or lack of two amino acids, glutamine and arginine, necessary for T-cell activation, differentiation and proliferation, are therefore inhibitory to T cell functions (79).

The TME shows high levels of immunosuppressive metabolic byproducts, including a turnover in the TME release of adenosine triphosphate (ATP) and nicotinamide dinucleotide (NAD) which are metabolized by the ectoenzymes CD39, CD73, and the NADase CD38 to adenosine (80, 81). Adenosine binds to the T-cell adenosine A2R receptor inhibiting effector T-cell functions and stimulating Treg cells (82, 83). Furthermore, the adenosinergic axis is over-functional in hypoxic conditions, connecting adenosine-mediated immunesuppression to low oxygen tension (84, 85).

Overall, a better understanding of the critical players within the TME and their specific roles in immune regulation will help design of metabolism-targeted therapeutic strategies for improving immunotherapy regimens in cancer.

Recently, NAD pathway enzymes and metabolites were shown to affect immune-cell functions and fate and alter the cancer cell-TME crosstalk. The following paragraphs are focused on describing these molecular circuits and their therapeutic implications.

NAD HOMEOSTASIS: AN OVERVIEW

NAD is a vital molecule governing many metabolic processes. It is used as a redox coenzyme by several dehydrogenases, and as a co-substrate by various NAD-consuming enzymes (86, 87). Among them are (i) mono- or poly-ADP ribosyltransferases (including ARTs and PARPs), which transfer the ADP ribose moiety to acceptor proteins resulting in their modification and function regulation, (ii) sirtuins, which catalyze the NAD-dependent deacetylation of metabolic enzymes and transcription factors, thus controlling their activity; (iii) NAD glycohydrolase that generates different NAD metabolites, including ADP ribose (ADPR), cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), with calcium (Ca^{+2}) mobilizing activity. These enzymes are involved in the control of a wide range of biological processes, including transcription, DNA repair, cell adaptation to stress signals, and immune response (88). By catalyzing their reactions, they render NAD continuous re-synthesis an indispensable process. Various NAD biosynthetic routes guarantee the coenzyme regeneration, in different combination and with different efficiency depending on the cell-type and metabolic status (89, 90). A schematic overview of NAD homeostasis is shown in **Figure 2** and reviewed in Sharif et al. (87), Magni et al. (91), and Houtkooper et al. (92).

The route which recycles nicotinamide (Nam), produced by the breakage of the N-glyosidic bond in the various NAD-consuming reactions, back to NAD that is considered the major pathway ensuring NAD homeostasis. It involves the phosphoribosylation of Nam to nicotinamide mononucleotide (NMN) by the enzyme Nam phosphoribosyltransferase (NAMPT) and the subsequent adenylation of NMN to NAD by NMN adenylyltransferase (NMNATs). This same route also salvages extracellular Nam that can be of dietary origin or can be formed in the extracellular space by the NAD glycohydrolase activity of the CD38 ectoenzyme acting on extracellular NAD and/or NMN. NAD can also be synthetized from exogenous nicotinamide riboside (NR) and nicotinic acid (NA) through distinct routes that are initiated by NR kinase (NRK) and NA phosphoribosyltransferase (NAPRT), respectively. The former enzyme phosphorylates NR to NMN, whereas the latter enzyme phosphoribosylates NA to nicotinate mononucleotide (NAMN). NMNATs convert NMN to NAD, and NAMN to nicotinate adenine dinucleotide (NAAD). NAAD is finally amidated to NAD by the enzyme NAD synthetase. A *de novo* biosynthetic route, which starts from tryptophan and enters the amidated route from NA, is also operative in several tissues and cell-types. The first and rate- limiting step in this pathway is the conversion of tryptophan to N-formylkynurenine by either IDO or tryptophan 2,3 -dioxygenase (TDO). Four reactions are then required to transform N-formylkynurenine to an unstable intermediate, α-amino-β-carboxymuconate-ε-semialdehyde (ACMS), which undergoes either decarboxylation, directed toward oxidation, or spontaneous cyclization to quinolinic acid (QA) directed toward NAD formation. Indeed, QA is phosphoribosylated to NAMN by the enzyme QA phosphoribosyltransferase (QAPRT), and the formed NAMN enters the NA salvage pathway. Among the enzymes involved in NAD homeostasis, NAMPT, CD38, sirtuins, and IDO are overexpressed in different types of cancer (93) and have been shown to play a role in cancer immune tolerance (94, 95). In the following sections, we will review what is known about their expression and function in the TME.

NAMPT IN METABOLIC REGULATION AND ACTIVATION OF MYELOID CELLS

As the first and rate-limiting enzyme, NAMPT plays a pivotal role in the biosynthesis pathway of NAD from its nicotinamide precursor. It converts Nam and 5-phosphoribosyl-1-pyrophosphate (PRPP) into NMN in a complex reaction that can be significantly improved by a non-stoichiometric ATP hydrolysis (96). NAMPT is found both intracellularly and extracellularly (97, 98). Intracellular NAMPT (iNAMPT) is primarily located in the nucleus and cytosol. Previous studies reported NAMPT in mitochondria as well (99), but this remains a controversial finding (100, 101). As one of the main regulators of NAD intracellular level, NAMPT plays a crucial role in cellular metabolism (102). Conversely, the extracellular form of NAMPT (eNAMPT) has emerged as an important mediator of inflammatory programs (103). eNAMPT has been found in plasma and other extracellular fluids, including the supernatants of numerous cell types (103); however, while the mechanisms behind eNAMPT secretion remain unknown, they do not seem to rely on the classic pathway (104). Notably, the cytokine-like functions appear independent of the protein catalytic activity (105). In keeping with this view, NAMPT's substrates PRPP and ATP are apparently unavailable in the extracellular space to sustain the enzymatic activity (106).

eNAMPT was originally found to be secreted by activated lymphocytes and bone marrow stromal cells by Samal et al. (107) and called pre-B-cell colony enhancing factor [PBEF (107). In 2005, Fukuhara (108) identified eNAMPT

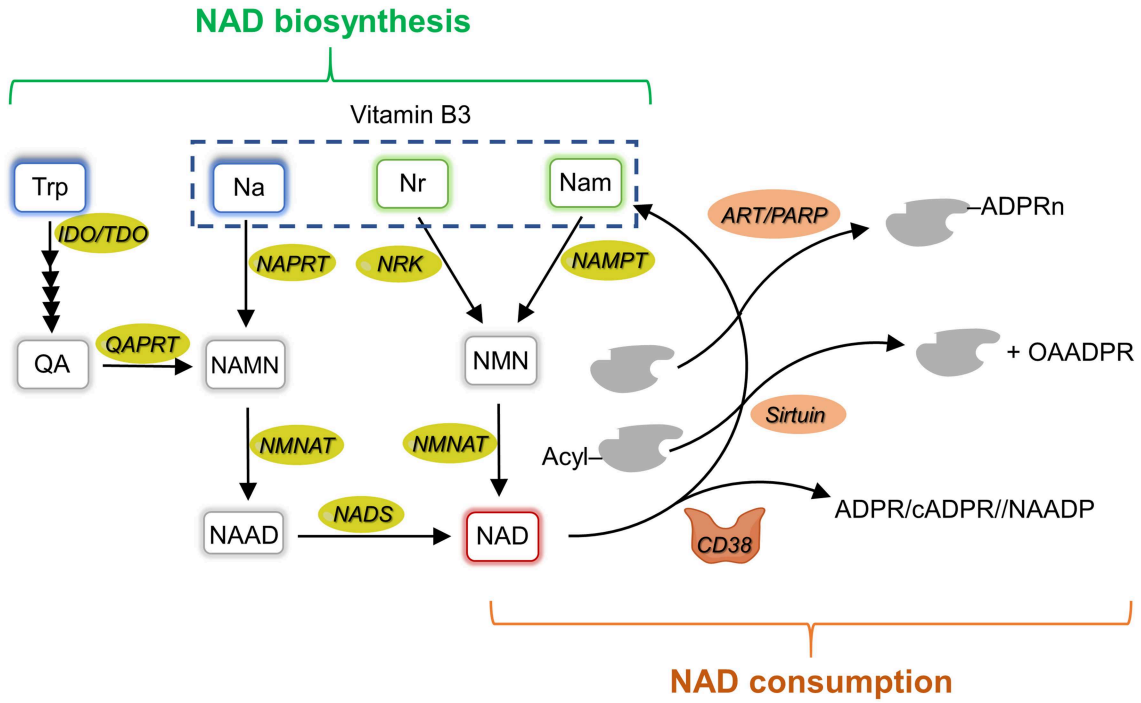

FIGURE 2 | NAD metabolism overview. Schematic representation of mammalian NAD metabolism including biosynthetic (left side, in green) and consuming (right side, in orange) pathways. Na, nicotinic acid; NAD, nicotinamide adenine dinucleotide; NAPRT, nicotinate phosphoribosyltransferase; NAMN, nicotinate mononucleotide; NAAD, nicotinate adenine dinucleotide; Nam, nicotinamide; NAMPT, nicotinamide phosphoribosyltransferase; NADS, NAD synthetase; NMN, nicotinamide mononucleotide; NMNAT, NMN adenylyltransferase; Nr, nicotinamide riboside; NRK, nicotinamide riboside kinase; QA, quinolinic acid; QAPRT, quinolinate phosphoribosyltransferase; IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; Trp, tryptophan; OAADPR, 2'-O-acetyl-ADP ribose; ART, ADP-ribosyltransferases; PARP, poly-ADP-ribose polymerase; ADPR, ADP-ribose; cADPR, cyclic ADPR; NAADP, nicotinic acid adenine dinucleotide phosphate.

as an adipokine and called it visfatin. These different names reflect its role in immune system and adipose tissue regulation.

Independent studies have conclusively shown that NAMPT expression and secretion can be induced by inflammatory signals in immune cells, in particular neutrophils, monocytes and macrophages (109). Both pathogen-derived lipopolysaccharide (LPS) and host-derived inflammatory stimuli, including tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and leptin, can up-regulate *NAMPT* transcription in macrophages and other several types of cells (110–113). Several studies showed stimulation of cytokine release after exposure of cells to exogenous NAMPT, highlighting a role of eNAMPT as an inflammatory mediator as reviewed in Garten et al. (103). Following NAMPT treatment, IL-1β, IL-6, TNF-α, and IL-10 are up-regulated in peripheral blood mononuclear cells (PBMCs) and $CD14^+$ monocytes (114). Co-stimulatory molecules such as CD54, CD40, and CD80 are also up-regulated in response to NAMPT treatment, an effect mediated through PI3-kinase and MAPKs p38, MEK1, and JNK (114). Furthermore, in macrophages NAMPT increases MMPs expression and activity (115). *In vitro*, eNAMPT promotes cell survival in macrophages subjected to endoplasmic reticulum (ER) stress, a frequent event in obesity and obesity-associated diseases. eNAMPT induces IL-6 secretion, followed by IL-6-mediated autocrine/paracrine activation of the prosurvival signal transducer STAT3, with a mechanism that is independent of the enzymatic activity (112).

Emerging evidence supports a role of NAMPT in regulating the differentiation program and the metabolic adaptation of myeloid cells. As described previously, activated macrophages can be divided in two subgroups *in vitro*: those with pro-inflammatory activity (M1) involved in first line of defense against bacterial infection, and those with anti-inflammatory activity (M2) that regulate tissue repair and wound healing (116), even if this is an oversimplification of the functional diversity occurring *in vivo*. Metabolic reprogramming of immune cells is required for both pro- and anti-inflammatory responses and a vast spectrum of metabolic statuses accompanies the complexity of phenotypes [reviewed in (117, 118)]. In general, an increase in glycolysis and in glucose uptake is typically associated to an M1 phenotype (119), while M2 macrophages rely on intact TCA cycle and OXPHOS as major source of ATP via electron transport chain and ATP synthase (120, 121). However, in addition to an augmented mitochondrial metabolism, alternatively activated macrophages can also use glycolysis when OXPHOS is disrupted (122). Another important pathway is the pentose phosphate pathway (PPP), which generates pentoses, 5-ribose phosphate and nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is essential in activated M1 macrophages because it fuels ROS production by NADPH oxidase (123), even if

other groups demonstrated that NADPH and NADPH oxidase play a role even in M2 differentiation (124). Concerning lipid metabolism, fatty acid synthesis is coupled to pro-inflammatory activity of macrophages, while beta-oxidation is typical of anti-inflammatory macrophages (117).

The increase of glycolysis associated with M1 activation of macrophages is orchestrated by the transcription factor HIF-1α. When cells experience low oxygen levels HIF-1α is stabilized and, upon binding of the HIF-1β subunit, initiates the transcription of genes such as glucose transporter and glycolytic enzymes (125, 126). NF-kB is required for transcriptional activation of HIF-1α (127); whereas, in M2 macrophages, genes involved in metabolic reprogramming are largely controlled by STAT6 and peroxisome proliferator-activated receptor gamma coactivator-1 beta (PGC-1β) (128).

Both iNAMPT and eNAMPT influence fundamental monocyte/macrophages processes such as differentiation, polarization and migration, even if the exact role of iNAMPT/eNAMPT in the process of myelopoiesis is incompletely elucidated so far (129–131) as summarized in **Figure 3**. For example, NAMPT has a role in the induction of an immunosuppressive and tumor-promoting microenvironment in chronic lymphocytic leukemia, where eNAMPT is important for the differentiation of monocytes toward tumor-supporting immunosuppresive M2 macrophage, promoting their differentiation, and polarization in tumor-supportive cells including TAMs (130). Recently, it was demonstrated that iNAMPT acts also on MDSCs, where NAMPT inhibits *CXCR4* transcription, via NAD/SIRT1/HIF-1α axis, and this, in turn, leads to a mobilization of MDSCs and enhances their production of suppressive nitric oxide (132).

Changes in NAD levels characterize different stage of macrophage polarization: in general, higher levels of NAD are typical of classically activated pro-inflammatory macrophages (M1), while NAD levels are lower in alternatively activated anti-inflammatory macrophages (M2). The NAMPT/NAD/SIRT1 axis seems to play a relevant role in myeloid cell functions as shown by the fact that efficient activation of M1 macrophages needs an increase of both NAMPT expression and cytosolic NAD (133). NAMPT-dependent generation of NAD is also crucial in the metabolic switch characterizing the transition from the early initiation phase of acute inflammation, which is anabolic and primarily requires glycolysis, to the later adaptation phase which is catabolic and relies on fatty acid oxidation (FAO) for energy (134). During these processes, also NAD-consuming deacetylases enzymes SIRT1 and SIRT6 have a role in regulating metabolism, increasing fatty oxidation and reducing glycolysis, respectively, coupling metabolic polarity with the inflammatory response, as described with more details later (135, 136). These data support the notion that NAD homeostasis has a crucial role in connecting bioenergetics and inflammation (134). A further feedback loop that links NAD to polarization of myeloid component has been suggested in monocytes, where NAMPT expression is induced by TNF-α via HIF-1α. In turn, NAMPT signaling involving NF-kB pathway activates activating protein 1 (AP1), inducing *IL6* and *TNFA* transcription modulating myeloid cell activation (137).

In congenital neutropenia, a disorder in which patients display accumulation of granulocytic progenitors and no mature neutrophils in bone marrow, it has been shown that granulocyte colony-stimulating factor (G-CSF) is effective as it up-regulates NAMPT, which in turn triggers NAD/SIRT1 dependent granulopoiesis via CCAAT/enhancer-binding protein α/β (C/EBPα/β) up-regulation (129). On the contrary, GM-CSF is not effective in congenital neutropenia because it is unable to activate iNAMPT upregulation and NAD/SIRT1 axis (138). Following the induction of myeloid differentiation with G-CSF, the NAD-consuming enzyme SIRT1 deacetylase C/EBPα at position Lys 161 (129, 138). NAMPT inhibition with FK866 led to the dramatic elevation of acetylated C/EBPα levels and reduced amounts of total C/EBPα protein, accompanied by diminished mRNA expression of C/EBPα target genes (G-CSF, G-CSFR, and ELANE). Moreover, treatment of acute myeloid leukemia cell line HL-60 with recombinant NAMPT or transduction of HL-60 cells with NAMPT-expressing lentiviral construct induced myeloid differentiation of these cells *per sé* (138).

An open question is whether the cytokine-like actions that eNAMPT exerts on myeloid cells are related to its enzymatic activity or are mediated by the binding to a cell surface receptor. The fact that treatment with low concentrations of recombinant eNAMPT is sufficient to activate specific intracellular signaling pathways suggests that eNAMPT has cytokine-like properties and binds to and activates a cell surface receptor. In 2015, Camp et al. identified eNAMPT as a new ligand of the Toll-like receptor 4 (TLR4) (105). The authors demonstrated that in human lung endothelial cells, eNAMPT activates an inflammatory response via activation of NF-kB signaling pathway by binding TLR4-MD2 (105). However, the fact that recombinant eNAMPT is often produced in *E. Coli* strains renders the interpretation of these results controversial for the possible contamination of LPS, the natural ligand of TLR4, and activator of inflammatory programs. New studies have to confirm the TLR4 engagement by eNAMPT and correlate this with myeloid differentiation and plasticity.

The evidence linking myeloid cell fate and NAD/NAMPT could open the way to pharmacological inhibition of either iNAMPT and/or eNAMPT for re-education of myeloid cells. This could be useful in the context of acute inflammation, but also in cancer to force a reversion of immunosuppressive microenvironment, in combination with immunotherapy, as summarized in **Figure 3**.

For iNAMPT specific small molecules inhibitors exist, most known FK866 (also known as APO866) and GMX1778 (also known as CHS-828), among others (**Table 1**) (139–143, 159–161). However, most of the data on these drugs describe their effect on the tumor itself, and not on cells of the microenvironment (141, 161). Whether these inhibitors could also affect also eNAMPT activity is unknown, even if, as mentioned before, the enzymatic activity of eNAMPT is controversial. On the other hand, for eNAMPT, the group of Garcia, in order to block only the cytokine-like activity of eNAMPT, has devised a polyclonal eNAMPT neutralizing antibody (130, 144), that could be useful in those condition in which only the extracellular form of eNAMPT is detrimental and intracellular enzymatic activity needs to be preserved.

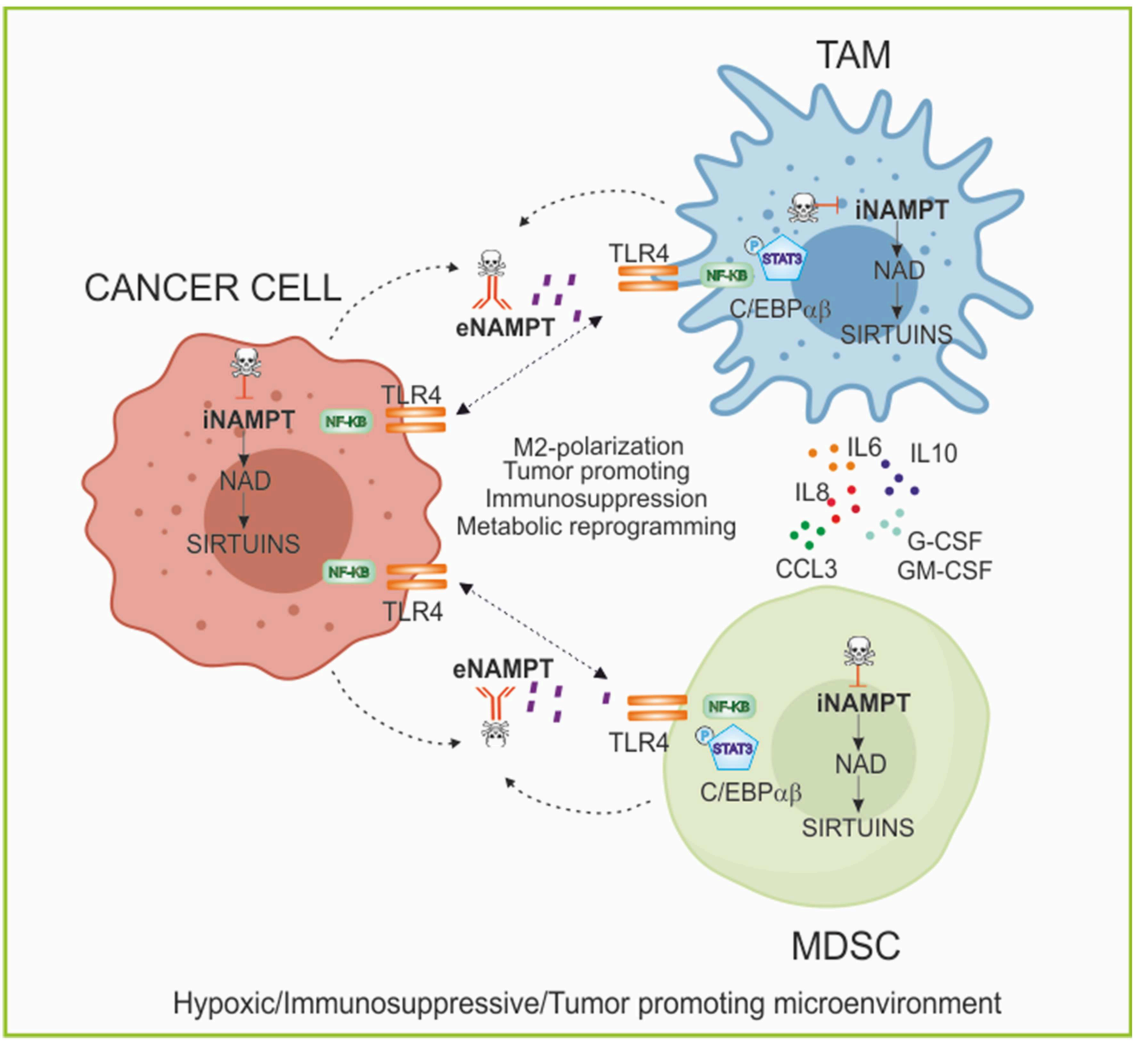

FIGURE 3 | NAMPT in regulating myeloid cell fate and immunometabolism. Role of iNAMPT/eNAMPT in skewing myeloid populations into tumor-supporting M2-like macrophages and myeloid suppressive cells. Specifically, the iNAMPT/sirtuins axis regulates the metabolic reprogramming of cancer and myeloid cells in condition of low oxygen tension; while eNAMPT/TLR4 axis activates intracellular signaling promoting differentiation of myeloid cells and secretion of anti-inflammatory and pro-tumor cytokines creating an immunosuppressive microenvironment. The block of NAMPT functions, using iNAMPT pharmacological inhibitors and/or neutralizing antibodies, can repolarize the myeloid populations and inhibit tumor growth. TLR4, Toll-like receptor 4; C/EBPα/β, CCAAT/enhancer-binding protein α/β; G-CSF, Granulocyte Colony-Stimulating Factor; GM-CSF, Granulocytes-Macrophage Colony-Stimulating Factor; TAM, tumor-associated macrophages; MDSC, myeloid-derived suppressive cells.

CD38 IN METABOLIC DYNAMICS OF T CELLS ACTIVATION

Cluster of differentiation (CD) protein CD38, first identified as a lymphocyte antigen, is a cell surface glycohydrolase that cleaves a glycosidic bond within NAD to yield Nam, ADP-ribose (ADPR), and cyclic ADPR (cADPR), and converts NAD phosphate (NADP) to NAADP, all calcium (Ca^{2+}) mobilizing molecules (162, 163). These molecules bind specific receptors, like the ryanodine receptor on endoplasmic reticulum, the lysosomal two-pore channel and the plasma membrane calcium channel transient receptor (TRPM2), activating calcium signaling, which in turn affects gene expression, cell cycle control, cell survival, energy metabolism, leukocyte trafficking, and inflammation (87).

CD38 is a transmembrane protein with four different forms, according to the cellular localization (164). The most common form of CD38 has a type II membrane orientation, i.e., with the catalytic domain facing the extracellular space. By contrast, the less abundant type III transmembrane form has its catalytic site facing the inside. Intriguingly, soluble intracellular and extracellular forms of CD38 have also been ascribed (165, 166). CD38 is widely expressed both in immune cell types (bone marrow progenitors, natural killer cells, monocytes, and activated T- and B-lymphocytes) and in non-hematopoietic cells (167).

TABLE 1 | Pharmacologic tools currently undergoing pre- or clinical evaluation to block NADome enzymes.

Agent	Mechanism of action	Indication	Trial Stage	References
NAMPT INHIBITORS				
APO866 (FK866)	NAMPTi	T/IC	Clinical phase I	(139)
CHS-828 (GMX 1778)	NAMPTi	T/IC	Clinical phase I	(140)
GNE-617, GNE-618	NAMPTi	T	Pre-clinical	(141)
KPT-9274	Dual NAMPTi/PAX4i	T	Clinical phase I	(142)
OT-82	NAMPTi	T	Clinical phase I	(143)
Blocking antibody	eNAMPT neutralization	T/IC	Pre-clinical	(144)
CD38 INHIBITORS				
Daratumumab	Blocking antibody	MM/ALL	Clinical phase III	(145)
Isatuximab	Blocking antibody	MM	Clinical phase II-III	(146)
MOR202	Blocking antibody	MM	Clinical phase II	(147)
Apigenin	CD38i	MD	Pre-clinical	(148)
SIRTUINS INHIBITORS				
Cambinol	SIRT1/2i	T/ND	Pre-clinical	(149)
Sirtinol	SIRT1/2i	T/ND	Pre-clinical	(150)
Selermide	SIRT1/2i	T/ND	Pre-clinical	(151)
Tenovins	SIRT1i	T/ND	Pre-clinical	(152)
EX-527	SIRT1i	T/ND	Pre-clinical	(153)
Nicotinamide	SIRTi/NAD precursor	T/ND	Pre-clinical, phase I-II	(154)
IDO INHIBITORS				
Indoximod	IDOi	T	Clinical phase I-II	(155)
Epacadostat (INCB024360)	IDOi	T	Clinical phase II-III	(156)
Navoximod	IDOi	T	Clinical phase I	(157)
BMS-986205	IDOi	T	Clinical phase I-II	(158)

I, inhibitor; T, solid and/or hematological tumors; IC, inflammatory conditions; MM, multiple myeloma; ALL, acute lymphoblastic leukemia; MD, metabolic diseases; ND, neurodegenerative diseases.

CD38 is also an unquestionable contributor to intracellular NAD homeostasis (168, 169) and this apparent "paradox" has been in part reconciled by recent reports demonstrating that CD38 can also degrade circulating NAD precursors such as NMN and NR, thus preventing their fueling of NAD biosynthesis (170, 171). Notably, CD38 enzymatic activity mediates many roles which include metabolism regulation and pathogenesis of heart disease, obesity, aging and inflammation, among the others. Nevertheless, it is well-established that CD38 overexpression is correlated to different hematological malignances including myelomas and leukemias (172). In this contest, a broad immune regulatory role for NAD and CD38 on T cell behavior has been reported (87, 145, 173) and summarized in **Figure 4**. In order to elucidate the impact of CD38 modulation of NAD homeostasis in T cell, a brief synthesis of T cell metabolism is necessary, as metabolism drives T cell life (36, 174, 175). One of the main challenges of the field in a translational perspective is to manipulate T cell metabolism in order to improve their immune response capacity. Defined metabolic pathways orchestrate T cell development, differentiation, function and persistence (176). TCA/OXPHOS-mediated ATP production is instrumental for the maturation of Naïve T (T_N) lymphocytes, a population of quiescent non-proliferative cells, in primary lymphoid organs (177). T cell activation is initiated after antigen recognition and TCR ligation. This step, requiring major histocompatibility complex, and co-stimulatory molecules, activates T lymphocytes inducing both a rapid proliferation rate and a differentiation program toward effector functions (176). To sustain both clonal expansion and active immune response, T cells shift to an anabolic metabolism which provides faster ATP production and nutrients supply. While cytolytic $CD8^+$ T (Tc) cells dominantly shift metabolism to glycolysis, activated $CD4^+$ T helper (Th) cells increase both glycolysis and FAO (178). FAO also supports metabolism of iTreg and long living memory T-cell (Tm) (178). All these T cell subsets, to achieve their metabolic profile, require a coordinated transcriptional program together with a specific system of nutrient uptake. T cells depend on the import of substrates such as glucose, amino-acids (especially glutamine), and glycerol. In T_N and Tm cells, increased expression of glucose and glutamine transporters is controlled by the transcription factor c-Myc (36) and regulated by a specific cytokine, IL-7 (175). AKT-mTOR and TLR signaling, as well as the transcription factors HIF-1α, c-Myc and FoxP3, have been shown to directly regulate Treg metabolic programming and development, while HIF-1α and mTOR control the glycolytic phenotype and activation (IFN-γ production) of effector T-cells, Th1, Th2, and Th17 lineages (36). Metabolism underpins T cell cycle through quiescence and activation states and T-cells failure to engage specific metabolic programs is a biological

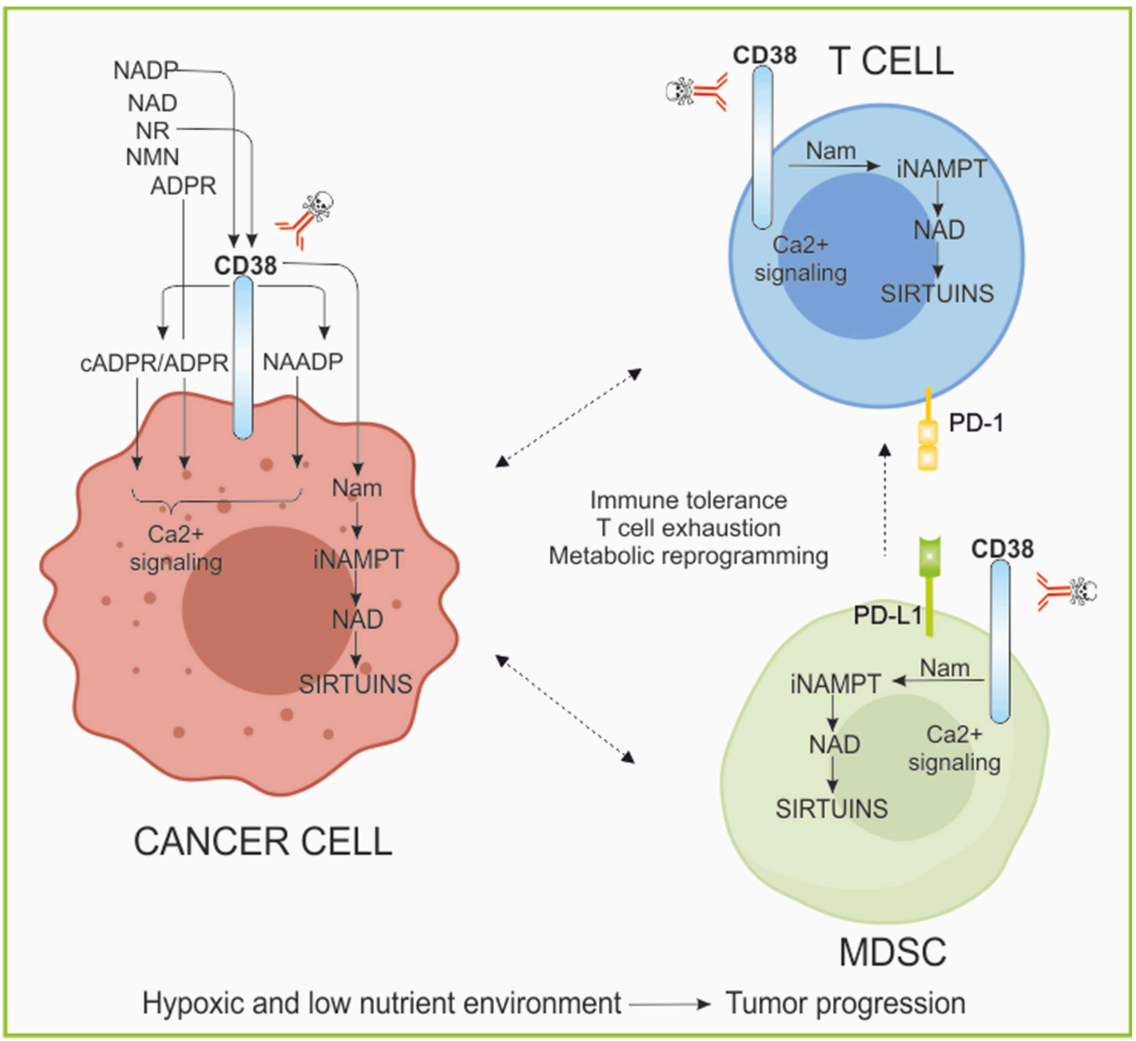

FIGURE 4 | CD38/NAD axis regulates T cell phenotype and responses. The ectoenzyme CD38, expressed by tumor cells and immune cells is involved in the activation of calcium (Ca^{2+}) signaling through the generated metabolite cADPR/ADPR/NAADP. Moreover, it metabolizes NAD, releasing Nam, rendering the substrate of NAMPT available for continuous NAD regeneration. These reactions occur also in immune cells modifying NAD concentrations and affecting sirtuins activities. The NAD/CD38/SIRTUINS axis regulates T cell immune cell fate, metabolism, and gene transcription. Evidence of high CD38 expressing immune suppressive cells have been reported in several tumors. CD38 inhibition was sufficient to re-establish T cell proliferation, antitumor cytokine secretion, and killing capability. ADPR, ADP-ribose; cADPR, cyclic ADPR; NADP, NAD phosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; Ca^{2+}, calcium; NR, nicotinamide riboside; NMN, nicotinamide mononucleotide; Nam, nicotinamide; MDSC, myeloid-derived suppressive cells; PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand.

phenomenon accompanying tumor aggressiveness and T cell exhaustions (176). The crosstalk between cancer cell and tumor TILs is played at different levels. As already mentioned, it has been shown that the establishments of nutrients competition between tumor cells and TILs has a primary role in influencing T cell fate and dysfunctions (34, 179–181). Malignant cells push their metabolism toward a Warburg phenotype. The consequent induction of a hypoxic and nutrient-deprived environment (low glucose, glutamine, glycine, and serine) shapes a tumor sustaining microenvironment and immune tolerance (179). Indeed, T cells migrating to tumors sites must adapt to both (i) nutrient-depleted environments (182) and contemporarily to (ii) the presence of hypoxic tumor-derived metabolites including lactate, adenosine, cyclic adenosine monophosphate (cAMP), IDO/kynurenine.

In this context, CD38-mediated Ca^{2+} mobilization can directly affect T cell metabolism. In the physiology of a T lymphocyte, Ca^{2+} controls T cell gene expression and consequently differentiation, development and cytotoxicity (183). Alteration of Ca^{2+} signaling affects immune deregulation and consequently tumor initiation and progression (183–186). A second level of T cell metabolic reprogramming control by the CD38/NAD axis also involves sirtuins (173). Indeed, a lot of literature has been produced on the role of SIRT1, as a key modulator of immune cell functions, as described in a dedicated section of this review (166, 187, 188). In this case, the inverse

correlation between expression of CD38 and intracellular NAD contents, act on SIRT1-mediated post-transcriptional control of key genes involved in T cell functions (173). Furthermore, it was recently shown that CD38 is highly expressed by specific subsets of immunosuppressive TILs (i.e., Treg and Th17) (34, 36, 173, 189) and by MDSC, another key immunosuppressive cellular component of tumor milieu represented (190). Both, $CD38^{high}$MDSC cells-mediated suppression of activated T-cells and the concomitant expression of CD38 with exhaustion markers on T cells, for example PD1, pointed to an active role of CD38 in modulating T cell metabolism and fate toward the generation of an immune tolerant landscape in tumor (173). Evidence of high CD38 expressing Treg have been reported for multiple myeloma and acute lymphoblastic leukemia, where the use of mAbs against CD38 (daratumumab, isatuximab, and MOR202, **Table 1**) is more than a promising therapeutic option to reestablish a functional immune surveillance (145–147, 189, 191, 192). In these tumor models, suppression of $CD38^+$ cancer cells associate with an increase in T-helper and cytotoxic T lymphocytes, T-cell functional response and TCR clonality (191, 192). A functional relationship between CD38 and Th17 has also been highlighted (173). Th17 is a $CD4^+$ T cell subpopulation secreting IL17, which gained interest in the field of immunotherapy due to their self-renewal, plasticity and hematopoietic stem-like phenotype (173, 193). Adoptive T cell transfer (ACT) therapy is a powerful strategy developed for controlling cancer (194, 195). The emerged staminal potential of Th17, together with their ability to persist for long times at tumor sites, made of this T cell subset an ideal candidate to improve ACT efficacy (173, 196, 197). Chatterjee et al. recently demonstrated that, SIRT1-dependent deacetylation of the transcription factor forkhead box O1 (FOXO1) drives the functional homing in different organs of a hybrid Th1/Th17 population 24 h after ACT. Most importantly, they reported that, the decrease of CD38 expression on Th17 cells leads to the increase of intracellular NAD concentration, reinforcing the SIRT1-dependent immune efficacy of this T cell population (173). For these reasons, the inhibition of CD38 has been proposed not only to specifically target $CD38^{high}$ immune suppressive cell populations (MDSCs, Treg), but also to improve tumor control via ACT therapy or using immunomodulatory drugs (173, 191, 192).

Lastly, very recently CD38 was considered as major acquired mechanism of resistance to PD-1/PD-L1 blockade, causing $CD8^+$ T cell suppression. Co-targeting of CD38 and PD-L1 improves anti-tumor immune response. CD38 manipulation was sufficient to regulate $CD8^+$ T cell proliferation, antitumor cytokine secretion, and killing capability (198).

SIRTUINS AND EPIGENETIC REGULATION OF IMMUNE RESPONSE

Sirtuins, initially described as transcriptional silencers in yeast (199), represent a class of NAD-dependent enzymes with deacetylase activity. So far, seven isoforms (SIRT1-7) constitute the family of mammalian sirtuins, which differ in subcellular compartmentation, enzymatic activity, and *in vivo* substrate selectivity (200). As a primary cellular location, SIRT1, SIRT6, and SIRT7 are found in the nucleus, SIRT2 in the cytoplasm, and SIRT3-SIRT5 in mitochondria (201). However, recent reports have shown that sirtuins are not anchored to precise subcellular compartments, and may shuttle between them, depending on cell type or physio-pathological conditions (202–205). The canonical reaction catalyzed by sirtuins is the transfer of an acetyl group from protein lysine residues to the ADPR moiety of NAD. As a result, the reaction produces Nam, first released, the deacetylated lysine, and 2'-O-acetyl-ADP ribose (206). Although lysine deacetylation is the primary activity of sirtuins, recent studies have shown that these enzymes can remove a variety of other acyl-lysine groups (207). Some sirtuins act as ADP-ribosyltransferases, although the biological relevance of such activity is incompletely understood. Mammalian sirtuins target different proteins in an isoform-specific fashion (207, 208), allowing their regulation of multiple processes like energy metabolism, epigenetic regulation of gene expression, DNA repair, inflammation, cellular stress resistance, healthy aging, tumorigenesis, autophagy, and apoptosis as reviewed in Haigis and Sinclair (208), Finkel et al. (209), and Houtkooper et al. (210).

Emerging evidence demonstrated that sirtuins are key regulators of inflammatory stress response in immune and non-immune cells (95, 211–213). Sirtuins are involved in epigenetic regulation, through deacetylation of histones and/or non-histone proteins, of metabolic, phenotypic, and bioenergetics reprogramming of immune cells (immuno-metabolism) (210, 212–214).

SIRT1 is the most extensively studied sirtuins, especially for its role in aging (210, 214). In addition, SIRT1 is involved in controlling stem cell development, cell differentiation and autophagy, metabolic reprogramming and inflammation (209, 215). SIRT1 is also the most studied among sirtuins involved in immune regulation and here we summarized some SIRT1 activities in epigenetic regulation of metabolism and immune response (**Figure 5**).

Epigenetic mechanisms are essential to the development and differentiation of the immune system, as well as in related pathologies (216–218). Epigenetic mechanisms include multilevel intracellular events that influence chromatin structure and gene expression such as histone methylation and acetylation, as well as DNA methylation, non-coding RNAs and chromatin remodeling (219). Further, numerous signals (i.e., TCR, TLRs, inhibitory receptors, and cytokines) drive changes in the epigenome that result in downstream modulation of immune responses (77). TLR signaling in macrophages regulates differentiation/polarization and activation in response to pathogens affecting gene expression and metabolic reprogramming (220, 221). In particular, TLR4 engagement by LPS in macrophages drives a shift toward a glycolytic metabolism impairing mitochondrial respiration (222), resulting in a marked shifts in NAD/NADH ratios, which influence the activities of SIRT1, potentially altering deacetylation of histone and non-histone substrates (134, 223). Liu et al. found in TLR4-stimulated THP-1 promonocytes that SIRT1 support a switch from increased glycolysis to increased FAO as early inflammation converts to late inflammation (134). The shift to late acute inflammation and elevated FAO required

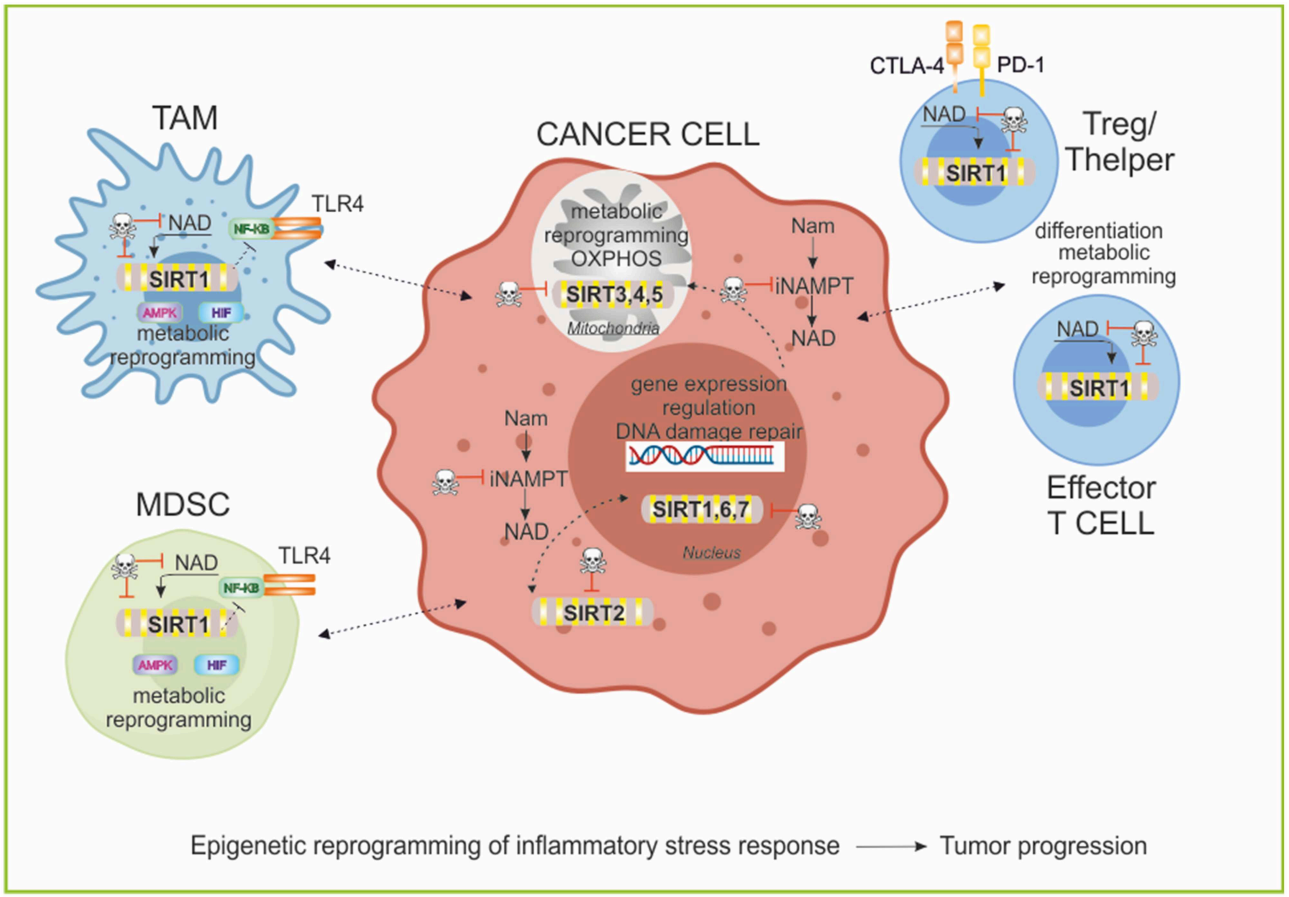

FIGURE 5 | Sirtuins and epigenetic regulation of immune cell functions. Sirtuins are a family of 7 members with different subcellular localization. These NAD-dependent deacetylases are involved in epigenetic regulation of metabolic reprogramming of cancer and immune cells and in promoting T cell differentiation and function. Particularly, in myeloid cells SIRT1 decreases inflammation negatively regulating NF-KB pathway, and inducing a metabolic rewiring mediated by its activity on AMPK/PGC-1α and HIF-1α stabilization. In T cell populations, SIRT1 modifies the phenotypic plasticity of Thelper and Treg and induces T cell tolerance. The manipulation of the Sirtuins/NAD axis is an important area of study for therapeutic implications in cancer research to repolarization of immune cell responses and to block tumor progression. TLR4, Toll-like receptor 4; AMPK, AMP-activated protein kinase; HIF, Hypoxia-inducible factor; PD-1, programmed cell death protein 1; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; TAM, tumor-associated macrophages; MDSC, myeloid-derived suppressive cells.

peroxisome proliferator-activated receptor gamma coactivator (PGC-1α),a known target of SIRT1 (187, 224–227). A circuit of AMP-activated protein kinase [(AMPK)/SIRT1/PGC-1α] results in the deacetylation and modulation of the activity of downstream SIRT1 targets that include the PGC-1α and the FOXO1 and FOXO3a transcription factors. The AMPK-induced SIRT1-mediated deacetylation of these targets explains many of the convergent biological effects of these two energy sensors, AMPK and SIRT1, on cellular metabolism (225, 226).

Recent studies have showed that the regulation of innate immunity and energy metabolism are connected through antagonistic crosstalk between NF-κB and SIRT1 signaling pathways (228). NF-κB signaling has a major role in innate immunity defense, while SIRT1 regulates the oxidative respiration and cellular survival (229). However, NF-κB activation can stimulate glycolysis during acute inflammation, whereas SIRT1 activation inhibits NF-κB signaling and enhances oxidative metabolism and the resolution of inflammation. SIRT1 inhibits NF-κB signaling directly by deacetylating the p65 subunit of NF-κB complex (230). SIRT1 stimulates oxidative energy production via the activation of AMPK, peroxisome proliferator activated receptor (PPARα) and PGC-1α and simultaneously, these factors inhibit NF-κB pathway and suppress inflammation (225, 226, 231). Using a myeloid cell-specific SIRT1 knockout (Mac-SIRT1 KO) mouse model, Schug et al. show that ablation of SIRT1 in macrophages renders NF-κB hyperacetylated, resulting in increased transcription of proinflammatory target genes. Consistent with increased proinflammatory gene expression, Mac-SIRT1 KO mice challenged with a high-fat diet display high levels of activated macrophages in liver and adipose tissue, predisposing the animals to development of systemic insulin resistance and metabolic derangement (232). In some cases, the effects of SIRT1 in regulating metabolism of immune cells are mediated by HIF-1α (233). SIRT1 can bind and deacetylate HIF-1α resulting in a stabilization or in an inhibition of the protein, depending on

the cells and context (234, 235). The SIRT1-HIF-1α axis bridges the innate immune signal to an adaptive immune response by directing affecting metabolism, cytokines production, and differentiation of immune cells (236). For example, (i) SIRT1 can limit the function and differentiation of MDSCs through HIF-1α-induced glycolytic metabolic reprogramming (237), (ii) SIRT1 can regulate T helper 9 (Th9) cell differentiation through the mTOR/HIF-1α-dependent glycolytic pathway (238). The interplay between HIFs and sirtuins may also extend to stress settings such as hypoxic tumors, in which cellular redox balance is perturbed (64, 239).

For adaptive immune cells, SIRT1 has a key role in mediating the differentiation of T cell subsets in a NAD-dependent manner. T cells exhibit remarkable phenotypic and functional plasticity during immune responses (240). SIRT1 is involved in (i) Th and Treg cell differentiation (238, 241); (ii) SIRT1 signals in DCs can repress PPARγ activity and promote T helper 2 (Th2) cell responses in airway allergy through metabolism-independent manners (242); (iii) SIRT1 interacts with c- Jun and inhibits CD4 T cells to mediate T cell tolerance (243); (iv) SIRT1 regulates CD8 T-cell differentiation interacting with basic leucine zipper transcription factor ATF-like (BATF) and regulating both epigenetic remodeling and energy metabolism of T cells (244). Furthermore, (v) SIRT1/FOXO1 axis regulates metabolic reprogramming of terminally differentiated memory T cells, as previously described (188).

Finally, SIRT1 has been shown to play also important roles in physiological processes affecting organismal longevity as well as stem cell function and self-renewal (245, 246). In macrophages, SIRT1 is emerging as critical positive modulator of self-renewal, regulating G1/S transition, cell cycle progression and a network of self-renewal genes (247).

Similar functions in regulating inflammation and metabolism are exerted also by SIRT2 and SIRT6 (134, 213, 248).

Interactions of cellular metabolic and epigenetic pathways and how these two key biological processes interplay to feedback modulate immune cell function is attracting in cancer therapy. Sirtuins/NAD axis has proven to be a crucial link between epigenetics and metabolism, and hence, it is an important area of study for therapeutic implications (215). While there are only specific activators or inhibitors for SIRT1 exist, drugs that affect NAD levels or NAD precursors offer the possibility to regulate all seven sirtuins coordinately (239). These compounds can be used alone or in combination with existing cancer therapies. The effects of SIRT1 inhibitors (e.g., cambinol, sirtinol, tenovins, Ex-527, **Table 1**) are currently studied mainly in the context of cancer (239). Very recent data show the impact of SIRT1 inhibition or genetic deletion on T cell responses, particularly on Treg differentiation. Genetic deletion or pharmacologic inhibition of SIRT1 through EX-527 improves $Foxp3^+$ Treg number and function through increased Foxp3 transcription its acetylation, leading to decreased Foxp3 turnover from ubiquitination and poly(ADP)ribosylation. As a result, targeting SIRT1 increases both central and inducible $Foxp3^+$ Tregs and promotes their suppressive functions, as summarized in Chadha et al. (249). SIRT1 inhibition is therefore useful in the context of graft-vs.-host disease (GVHD), to extend allograft survival (249–251). However, there are a number of studies in which SIRT1 deletion or inhibition led to proinflammatory conditions, indicating that regulation of the system is still incompletely understood (249, 252). Interestingly, the Nam generated in deacetylase reactions by SIRTs acts as a negative feedback regulator of SIRT activity (253, 254). This Nam is converted back to NAD by the action of NAMPT and NMNATs. Hence, NAD-biosynthetic enzymes, in particular NAMPT, also regulate sirtuins signaling (255) providing the rational to use NAMPT inhibitors to interfere with Sirtuins functions.

Overall these results indicate that sirtuins broadly coordinate innate and adaptive immune reprogramming and represent druggable immunometabolic enhancement targets, useful also to repolarize immune cells in TME.

IMMUNOSUPPRESSION VIA TRYPTOPHAN CATABOLISM: THE ROLE OF KYNURENINE PATHWAY ENZYMES

Amino acid catabolism is a key effector in driving immune tolerance. IDO is a cytosolic, heme-dependent enzyme responsible for the rate-limiting step of *de novo* NAD synthesis from tryptophan in extrahepatic tissues. The catalyzed-reaction yields N-formylkynurenine and commits the aminoacid toward its conversion to QA through the kynurenine pathway (90), which accounts for >90% of tryptophan catabolism (256). Tryptophan is an essential amino acid in protein metabolism, a precursor for the synthesis of the neurotransmitter serotonin and tryptamine, as well as for the synthesis of NAD and the hormone melatonin (257, 258).

In recent years, IDO has drawn enormous attention due to its immune regulatory functions (259–261) and summarized in **Figure 6**. IDO is not constitutively expressed in immune cells. Rather, various stimuli, and signaling pathways induce transcription and translation of metabolically-active IDO enzyme protein. Among them, TLRs, tumor necrosis factor superfamily members (TNFRs), interferon beta receptor (IFNBR), the interferon gamma receptor (IFNGR), transforming growth factor beta receptors (TGFBRs) and the aryl hydrocarbon receptor (AhR) all can activate signaling mechanisms that either induce or maintain IDO expression. NF-KB activation is a central downstream signal of these pathways regulating IDO expression (262).

By catalyzing the initial and rate-limiting step of tryptophan degradation, IDO reduces the local tryptophan concentration and produces immune modulatory tryptophan metabolites (263). In particular, cells expressing IDO and TDO produce the tryptophan catabolite kynurenine that, by interacting with the aryl hydrocarbon receptor expressed by T cells, Tregs and DCs, regulates immunity (264). Additionally, inhibition of $CD8^+$ T-cell-mediated cytotoxic function was found to be an important mechanism behind IDO's immune-modulating property (264). Due to the role in regulating T cell response and fate, IDO function is critical in organ and tissue graft survival, in viral infection, in tissue-specific autoimmunity and the promotion of cancer cell survival (265). The biologic function of the IDO pathway was originally described as both counter-regulatory

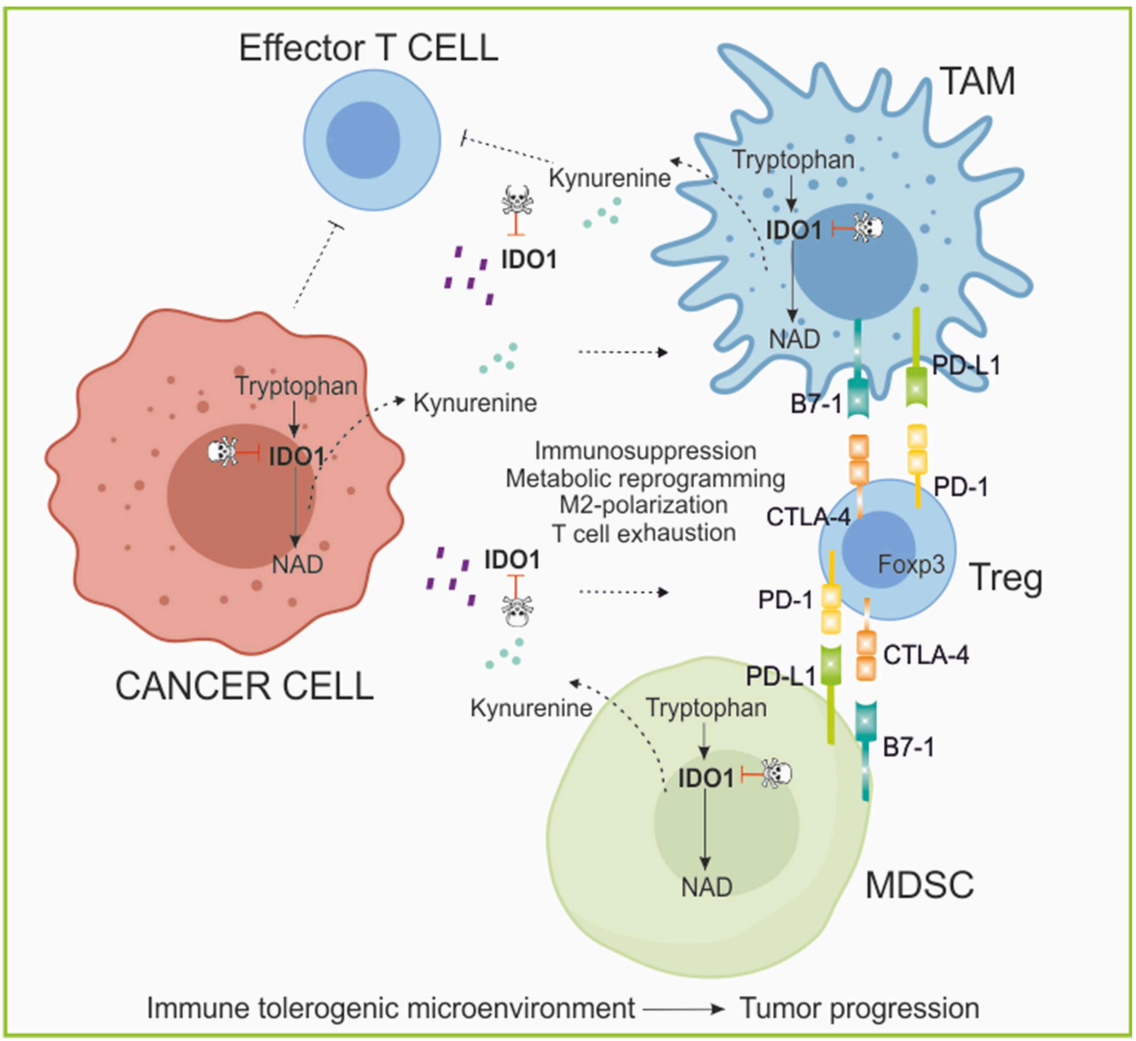

FIGURE 6 | The role of IDO/kynurenine in cancer immunoediting. IDO1 is an enzyme involved in the catabolism of tryptophan (kynurenine pathway). IDO and kynurenine can be secreted by tumor and tolerogenic immune cells in the microenvironment where exert an immunosuppressive function polarizing myeloid cell toward M2 phenotype (TAM; MDSC) and suppressing effector T cell functions, while stimulating expansion and activation of permissive Treg population, increasing immune escape mechanisms (PD-1/PD-L1; CTLA-4/B7-1 crosstalk). Moreover, in the extracellular space, IDO1 depletes the essential amino acid tryptophan from the tumor microenvironment, favoring tumor growth. IDO1 inhibitors in combination with Immunotherapy aim to reverse immunoediting (backward arrow) by inhibiting and activating local immunosuppressive and tumor eradication mechanisms, respectively. PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; Foxp3, forkhead box P3; TAM, tumor-associated macrophages; MDSC, myeloid-derived suppressive cells.

(controlling inflammation) and tolerogenic (creating acquired antigen-specific tolerance in T cells) (257).

Escape from the immune response is essential for cancer progression, however, mechanisms underlying this process remain unclear. Kynurenine in the tumor microenvironment was recently shown to favor immunosuppression (265, 266). Tryptophan catabolism was shown to create an immunosuppressive milieu in tumors and in tumor-draining lymph nodes through accumulation and secretion of immunosuppressive tryptophan catabolites that bind and activate AhR (267), leading to induction of T-cell anergy, apoptosis, increased conversion of naïve $CD4^+$ T cells into Tregs and polarization of DCs and macrophages toward an immunosuppressive phenotype (190, 261, 265, 268).

Clinically, studies of ovarian, lung, colorectal, breast cancer, brain tumors, melanoma, and others have shown that increased expression of IDO was associated with poor survival outcomes (258, 265, 269, 270). In most studies, the ratio of kynurenine to tryptophan was measured in patient plasma as a measure of IDO and TDO activity (156, 267). Moreover, not only tumor can express IDO, but also immune cells including both TAM and MDSC express high levels of IDO, in response to inflammatory cytokines, of which IFN-γ is the most potent inducer, amplifying the circuit of immunosuppression (190, 271, 272).

According to the role of IDO in driving immunosuppression, in the last years IDO became a valid target in cancer therapy (273, 274). Competitive inhibitors of IDO are currently

being tested in clinical trials in patients with solid cancer, with the aim of enhancing the efficacy of conventional chemotherapy, vaccine or checkpoint inhibitors (275). Agents currently account for the majority of the trials: indoximod (1-methyl-D-tryptophan), an inhibitor of the IDO pathway (155, 276), epacadostat (INCB024360) (156) and BMS-986205 (158) (**Table 1**), with encouraging results. Importantly, the use of IDO inhibitors can be also overcome the resistance to immunotherapies targeting immune checkpoints, strongly supporting the combination therapies with IDO inhibitors irrespective of IDO expression by the tumor cells (277). Additional IDO inhibitors are in the development pipeline, as well as agents that may target TDO, or a second isoform of IDO (IDO2) (275).

CONCLUDING REMARKS

Anticancer strategies targeting simultaneously oncogenic and metabolic pathways, de-regulated in cancer cells, seem to be ideal and have shown some promising results. Interestingly, local conditions in the tumor microenvironment affect also metabolic responses of immune cells, favoring immune-tolerance, and immune-escape mechanisms. One of the goals of immunotherapy could be to re-educate the immune system to kill tumors, by reprogramming their metabolism. The network of immunosuppressive mechanisms in the TME is complex, multifactorial, and mutually reinforcing. A better knowledge of the main players of this cross-talk can help in designing more effective combination therapies. In this picture, NAD-metabolizing enzymes are receiving increasing attention to due to their role in conditioning several aspects of immune cell fate and functions. It is foreseeable that modulators/inhibitors of the NADome (summarized in **Table 1**) will become useful alone or in combination with current anti-cancer therapeutic strategies to regulate both tumor growth and immune populations of TME.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Macrophage Rewiring by Nutrient Associated PI3K Dependent Pathways

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Class 1 Phosphoinositide-3-Kinases (PI3Ks) have been widely studied and mediate essential roles in cellular proliferation, chemotaxis, insulin sensitivity, and immunity. Here, we provide a comprehensive overview of how macrophage expressed PI3Ks and their downstream pathways orchestrate responses to metabolic stimuli and nutrients, polarizing macrophages, shaping their cellular identity and function. Particular emphasis will be given to adipose tissue macrophages, crucial players of insulin resistance and chronic metabolically triggered inflammation during obesity. An understanding of PI3K dependent wiring of macrophage responses is important as this is involved in various diseases ranging from obesity, type 2 diabetes to chronic inflammatory disease.

Keywords: macrophage, PI3K, nutrient sensing, adipose tissue macrophages, metainflammation, insulin

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INTRODUCTION

The PI3K family is a central metabolic regulator, responsible for phosphorylating inositol lipids at the 3′ position of the inositol ring. PI3K generated phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) triggers the recruitment and activation of several signaling proteins to the plasma membrane, thereby relaying various extracellular stimuli including Toll-like receptor (TLR) ligands, insulin and G-protein coupled receptor ligands (1, 2). Although there are three classes of PI3K enzymes (3), this mini-review will focus on class I PI3Ks and their function in macrophages in response to metabolic stimuli that are upregulated during obesity, including insulin, glucose, cholesterol and free fatty acids (FFAs). Indeed, macrophages that reside in adipose tissue (ATMs) are exposed to increased levels of these stimuli in the obese state and are significant players in metabolically triggered inflammation (herein referred to as meta-inflammation), which is crucial in the pathogenesis of type 2 diabetes (T2D) and atherosclerosis (4–7). Here, we present an overview of how the aforementioned stimuli regulate macrophage function and propose that PI3Ks are central integrators of these environmental cues.

THE PI3K PATHWAY AND ITS EFFECTS ON MACROPHAGE POLARIZATION

In mammals, class I PI3Ks are subdivided into class IA and class IB. Class IA consists of three catalytic (p110α/β/δ) and five regulatory subunits (p85α/β, p55α/p50α, and p55γ), in part generated through splicing or alternative transcription (p55α/p50α), associated to mainly receptor tyrosine kinases. Class IB only features one catalytic (p110γ) and two regulatory subunits (p84/p101) associated to G-protein-coupled receptors. The catalytic subunit of PI3K heterodimerizes with a

regulatory subunit that dictates localization and activity of the complex leading to recruitment of signaling molecules that bind PtdIns(3,4,5)P3 through their pleckstrin-homology (PH) domains including protein kinase B (PKB, also known as AKT), phosphoinositide-dependent kinase 1 (PDK-1), protein kinase C (PKC) and Bruton's tyrosine kinase (BTK). PI3K activation further blocks degradation and increases synthesis of proteins via mTOR signaling. AKT mediates effects involved in glucose transport, glycogen synthesis, and protein synthesis. Some of these metabolic effects are achieved through AKT mediated phosphorylation of Forkhead (FOXO) transcription factors (8–10). Given the crucial role of the PI3K pathway in cellular biology, mechanisms exist to limit its activation. PtdIns(3,4,5)P3 turnover is terminated by lipid phosphatases, such as phosphatase and tensin homolog (PTEN), a prominent tumor suppressor (11).

In macrophages, the PI3K pathway regulates the response to different metabolic and inflammatory signals and modulates macrophage polarization. Briefly, based on their microenvironment and the consequent functional programs elicited, macrophage phenotypes are defined as M1 and M2. Classically activated M1 macrophages adopt a pro-inflammatory phenotype in response to interferon gamma (IFN-γ) and lipopolysaccharide (LPS) and are critical for host defense against pathogens. Alternatively activated M2 macrophages play important roles in wound healing and resolving inflammation. M2 macrophages can further be subdivided into M2a (activated by interleukin (IL)-4 and IL-13), M2b (activated by immune complexes and TLR ligands), and M2c (activated by IL-10 and glucocorticoids). Importantly, these activation states are likely dynamic and influenced by the changing local milieu, therefore macrophages may not form clear cut activation subsets *in vivo* (12). Indeed, as discussed later, recent evidence indicates that ATMs adopt a unique metabolically activated state in response to their microenvironment.

Numerous studies have implicated PI3Ks in limiting pro-inflammatory responses in TLR stimulated macrophages, especially upon LPS mediated TLR4 activation. The mechanisms are diverse ranging from indirect effects such as suppression of TLR4 induced signaling cascades (e.g., MAP kinase signaling) to direct mechanisms, including AKT mediated modulation of FOXO transcription factors or the promotion of M2 responses. Indeed, LPS driven ERK, p38, and JNK pathways in monocytes and macrophages are enhanced upon pharmacological blockage of PI3K activity (13). Bone marrow macrophages (BMMs) deficient in p110γ or p85α exhibit augmented IL-6, IL-12, and TNF levels following LPS challenge, providing genetic evidence that PI3Ks attenuate LPS induced inflammation (14, 15). Further, PTEN deficient macrophages, which exhibit sustained PI3K activity, display decreased LPS driven pro-inflammatory cytokine expression and are skewed toward an M2 phenotype compared to controls (15, 16). In addition, downstream AKT signaling is required for the dampening effects of PI3Ks on TLR4 signaling and might involve phosphorylation and thereby termination of FOXO transcription factor activity. This is particularly important as FOXO1, which when active potentiates TLR4 expression (14, 17). Of note, three distinct isoforms of AKT exist: AKT1, 2 and 3 and studies utilizing AKT isoform-specific deficient mice suggest unique roles for the isoforms in mediating pro and anti-inflammatory signaling (18, 19). LPS stimulated $Akt1^{-/-}$ macrophages express augmented levels of iNOS (inducible nitric oxide synthase), NO (nitric oxide), TNFα, and IL-6, whereas LPS treated $Akt2^{-/-}$ macrophages produce low levels of these pro-inflammatory mediators suggesting deletion of *Akt1* promotes M1 while deletion of *Akt2* results in M2 responses (20). In line, $Akt2^{-/-}$ macrophages express increased levels of the M2 markers arginase 1 (Arg-1), FIZZ1, and exhibit more IL-10 upon LPS treatment compared to controls, while AKT1 deficiency results in enhanced bacterial clearance *in vivo* (20, 21). Interestingly, similar to $Akt2^{-/-}$ macrophages the M2 phenotype of $Pten^{-/-}$ macrophages is associated with elevated Arg-1 levels that are mediated by binding of the transcription factor CEBP-β to the Arg-1 promoter, suggesting sustained PI3K activity impinges particularly upon AKT1 in the context of macrophage polarization (16, 20). However, whether specific AKT isoforms are regulated by specific PI3K classes remains unknown.

ADIPOSE TISSUE MACROPHAGES

Although murine ATMs are a heterogeneous population of cells, ATMs in the lean state can generally be described as $F4/80^{+}CD11b^{+}CD206^{+}$ cells. Physiological adipose tissue growth is associated with minimal inflammation, while during pathological fat expansion, characteristic of obesity, limited angiogenesis of adipose tissue is associated with prevalent adipocyte hypertrophy, fibrosis and death (22). Here, ATM numbers dramatically increase due to local proliferation and recruitment of monocytes into adipose tissue that occurs partly through a monocyte chemoattractant protein 1/C-C chemokine receptor type 2 (MCP-1/CCR2) dependent axis and is influenced by adipose tissue lipolysis (23–26). Indeed, recruited ATMs express CCR2, but also CD11c, CD64, and CD9 (27). $CD11c^{+}$ ATMs overexpress pro-inflammatory genes and ablation of $CD11c^{+}$ cells in adipose tissue of obese mice leads to reduced inflammation and improved insulin sensitivity (28). Nonetheless, while in obesity, recruited ATMs overexpress several classic inflammatory (M1) markers e.g., *Il6* and *Nos2* (29), their phenotype is highly plastic and dependent on the microenvironment. Here, saturated FFAs (e.g., palmitate) or cholesterol, insulin and glucose that are prevalent in obese adipose tissue induce a state of metabolic activation (MMe) in ATMs, distinct from classic M1 activation. MMe activation is associated with elevated cell surface expression of lipid metabolism associated proteins including ATP binding cassette transporter (ABCA1), cluster of differentiation 36 (CD36), and perilipin 2 (PLIN2). This is related to augmented peroxisome proliferator activated receptor gamma (PPAR-γ) binding to the promoters of these genes. Further, autophagy and particularly sequestome-1 (p62) are important as opposed to controls attenuated levels of these lipid mediators occur in p62 null MMe macrophages (30). MMe activation correlates with lysosomal biogenesis as more active biogenesis occurs in newly recruited

$CD11c^+$ ATMs (27, 31). Recent work has corroborated that ATMs represent a heterogeneous population of cells and that irrespective of obesity, there are populations of lipid laden ATMs associated with the vasculature of adipose tissue exhibiting high endocytic capacity. This suggests active ATM reprograming in response to diverse macromolecules and nutrients present in the bloodstream (32). Together, ATMs respond to their environment by upregulating lipid/lysosomal programs, which is likely heighted during obesity, allowing them to fulfill their main function of clearing up dying adipocytes, buffering lipids, preventing ectopic lipid spill over, and ensuing insulin resistance (25, 26, 33, 34). But how does PI3K activity within macrophages, reconcile with the environmental cues that dictate ATM function and metabolic health? Although cytokines or adipokines secreted by adipose tissue can influence systemic inflammation as well as local macrophage responses (31), here we will focus exclusively on metabolic stimuli relevant to obesity and T2D and their effects on myeloid cells, particularly macrophages (**Figure 1**). We propose that during obesity, the metabolic milieu encountered by macrophages modulates PI3K signaling driving changes in macrophage function.

INSULIN STIMULATED PI3KS PROMOTE CELL SURVIVAL AND ATTENUATES LIPID LOADING IN MYELOID CELLS

Insulin represents an essential hormone for the maintenance of whole-body glucose disposal, regulating carbohydrate, protein and lipid metabolism in insulin-sensitive organs such as adipose tissue, muscle and liver (10). Upon insulin binding, the insulin receptor (IR) self-phosphorylates and activates insulin receptor substrates (IRS) which mediate downstream effects through engaging central signaling pathways including the PI3K/AKT, mTOR, and MAPK pathways (9). In this complex network, PI3Ks are a critical signaling node, mediating many of the metabolic and mitogenic effects of insulin. Importantly, the exact function of insulin signaling in immune cells remains largely unknown, although recently it was shown that T cell specific insulin signaling promotes a specific metabolic program, inducing nutrient uptake to support optimal T cell effector functions (35). Tissue resident macrophages, including ATMs, liver and peritoneal macrophages (pMOs) express the *INSR* gene with pMOs exhibiting highest expression. *INSR* upregulation in pMOs is linked with obesity and M1 macrophages exhibit more expression compared to unstimulated (M0) or M2 macrophages (36). Further, macrophages mainly express IRS-2 but not IRS-1 (37, 38). Although insulin stimulation of macrophages engages the PI3K/AKT signaling cascade (39, 40), it does not activate some important other nodes of the insulin signaling network such as the c-Jun N-terminal kinase (JNK) and p38 pathways (36). Macrophage glucose transport is facilitated mainly via glucose transporter 1 (GLUT1), which is rapidly induced by insulin, an effect that has been described to be more prominent in M1 vs. M0 or M2 macrophages, suggesting possible anti-inflammatory actions of insulin (**Figure 1**) (36, 41). In line, insulin promotes IL-10 expression dose dependently in pMOs and RAW264.7 macrophages and insulin priming attenuates TLR4 expression, LPS induced nuclear factor kappa B (NF-κB), p38 MAPK activation, and IL-1β production (42). Further, treatment of obese individuals with insulin reverses the pro-inflammatory phenotype of macrophages, eliciting anti-inflammatory effects (43). Concordant with a potential role in resolving macrophage mediated inflammation, insulin-stimulated macrophages exhibit increased expression of phagocytosis associated NAPDH oxidase activity and decreased apoptosis (44, 45). Nonetheless, insulin and PI3K signaling are unlikely to solely promote anti-inflammatory effects. Insulin is reported to increase TNF-α release in human monocytes (46). LPS-stimulation of IR deficient macrophages failed to induce IL-6 and IL-1β expression suggesting insulin signaling might be required for inflammation (47).

In obesity, surprisingly, mice deficient for the IR specifically in myeloid cells exhibit a protective phenotype associated with decreased ATM accumulation and improved insulin sensitivity (48). A recent report has reproduced these findings, additionally suggesting that there are less pro-inflammatory ($F4/80^+CD11c^+CD206^-$) and more anti-inflammatory ($F4/80^+CD11c^-CD206^+$) ATMs present in obese mice lacking the IR in myeloid cells, proposing myeloid cell specific IR signaling modulates ATM phenotypes (38). The authors of this study additionally demonstrated that in obesity, myeloid specific $Irs2^{-/-}$ mice exhibit impaired insulin sensitivity, associated with more pro-inflammatory ($F4/80^+CD11c^+CD206^-$) and less anti-inflammatory ($F4/80^+CD11c^-CD206^+$) ATMs. This suggests distinct differences between IRS2 and IR in regulating ATM phenotypes. These differences were explained by findings showing that IL-4 promotes M2 macrophage polarization through IRS-2 and post obesity, hyperinsulinemia through engagement of the IR, leads to macrophage IRS-2 downregulation (38). Further, several studies have identified myeloid dysfunctions associated with macrophage cell intrinsic insulin resistance. In this context, macrophages were rendered insulin resistant through pre-incubation with high-dose insulin, genetic deletion of the *INSR* or by pharmacologic inhibition of insulin signaling. Pre-treatment of macrophages with high-dose insulin leads to *INSR* downregulation and suppression of insulin signaling, which is also observed in freshly isolated macrophages from insulin-resistant mice, such as the leptin-deficient *ob/ob* mouse (49). In line, monocytes isolated from diabetic subjects show decreased surface expression and tyrosine kinase activity of the IR and diminished insulin-stimulated PI3K/AKT signaling (50). In response to free cholesterol (FC) loading, $Insr^{-/-}$ macrophages exhibit attenuated AKT phosphorylation and an augmented ER stress response, that is independent of the degree of FC loading. This suggests macrophage PI3K signaling through the IR is required to withstand stressful stimuli. The functional consequences of this are increased apoptosis, unconnected to obvious changes in pro/anti-apoptotic gene expression. Indeed, western diet-fed mice with IR deficiency on an *Ldlr* deficient background in hematopoietic cells develop larger, more complex lesions with increased necrotic cores and apoptotic cells (40, 51). Furthermore, insulin resistant macrophages, post-transcriptionally upregulate CD36 and

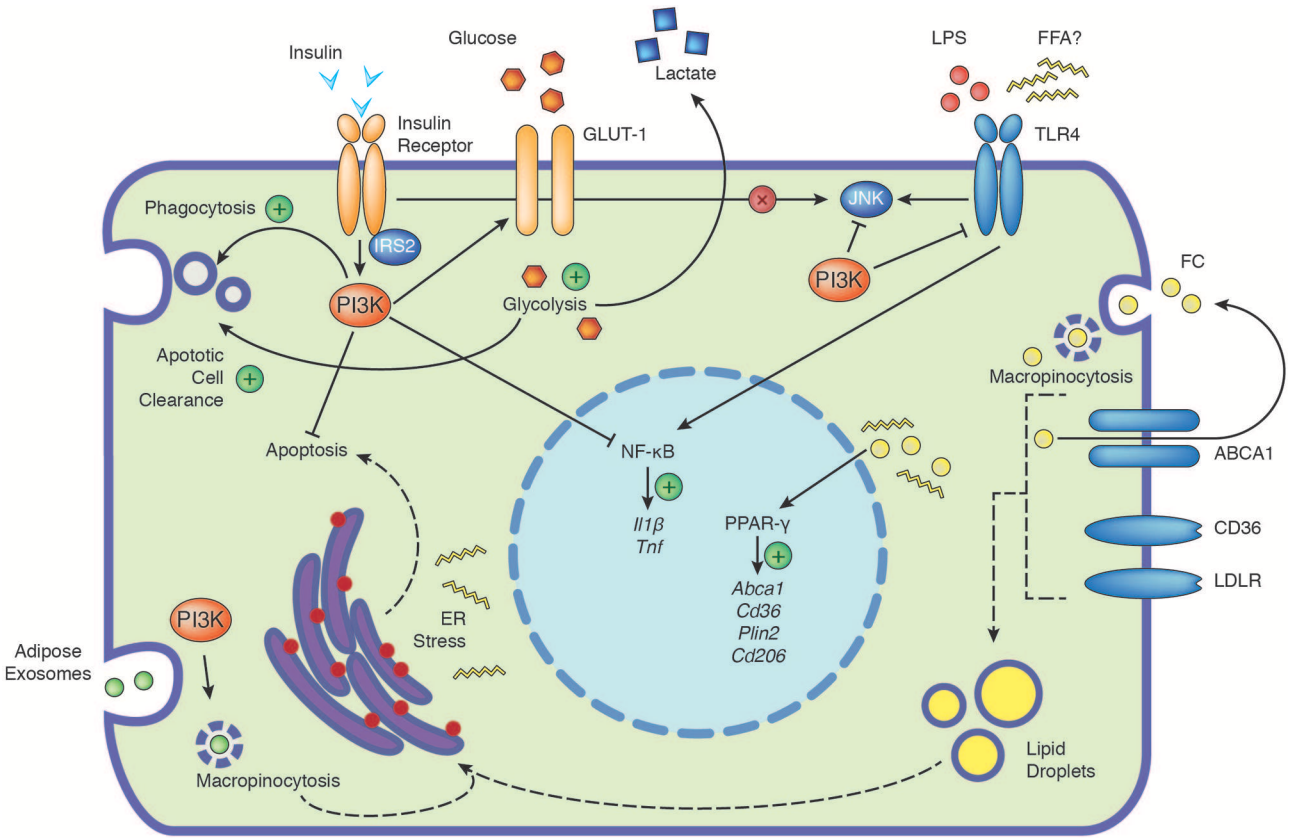

FIGURE 1 | The PI3K signaling cascade integrates signals from extracellular nutrients and influences cellular function. Depicted are the positive (plus) and negative influences of PI3K on the cellular responses to insulin, glucose, TLR4, free cholesterol and adipose exosomes. Insulin signaling in macrophages has no direct impact on JNK activation (cross). Dashed arrows indicate potential connections, see text for further details. ABCA1, ATP-binding cassette 1; CD36, cluster of differentiation 36; CD206, cluster of differentiation 206; ER, endoplasmic reticulum; FC, free cholesterol; FFA, free fatty acids; GLUT-1, glucose transporter 1; IL1β, interleukin 1β; IRS2, insulin receptor substrate 2; JNK, c-Jun N-terminal kinase; LDLR, low-density lipoprotein receptor; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphoinositide 3-kinase; Plin2, perilipin-2; PPAR-γ, peroxisome proliferator-activated receptors gamma; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

scavenger receptor A (SR-A), with increased CD36 protein levels dependent on defects in insulin stimulated PI3K signaling and proteasomal and lysosomal catabolism (40, 49). SR-A levels are coupled to increased ER stress as they are increased upon treatment with ER stress inducers (40). In agreement, primary *Insr*$^{-/-}$ macrophages exhibit enhanced binding and uptake of modified LDL. Conversely, *in vivo* treatment of *ob/ob* mice with rosiglitazone, an insulin sensitizing agent and PPAR-γ activator, reverses this phenotype resulting in improved insulin signaling and decreased modified LDL uptake (49). Interestingly, in human macrophages, both CD36 and SR-A basal levels are reported to depend on PI3K activity as selective pharmacological inhibition of Class IA p110β or δ and Class IB p110γ attenuates their expression and is associated with reduced macropinocytosis and foam cell formation upon modified LDL challenge (52). Thus, although there may be species-specific differences, intrinsic murine myeloid cell insulin stimulated PI3K dependent signaling promotes myeloid cell survival and modulates lipid metabolism, decreasing foam cell formation. Consequently, cell intrinsic macrophage insulin resistance and associated downregulation of PI3K signaling results in elevated macrophage lipid burden and death, impacting ectopic lipid spillover, further contributing to pathogenesis in obesity.

PI3KS PROMOTE GLUCOSE DEPENDENT ALTERNATIVE MACROPHAGE ACTIVATION

Hyperglycemia is a hallmark of T2D and glucose levels modulate intracellular macrophage metabolism through environmental glucose uptake and subsequent pyruvate and fatty acid generation and there are numerous excellent reviews on this topic (53, 54). Stable overexpression of GLUT1 in RAW264.7 macrophages promotes glucose uptake and metabolism (41). GLUT1, 3 and 5 expression increases as monocytes differentiate into macrophages and high expression is observed in foamy macrophages, which are typically found upon modified lipoprotein challenge and are reminiscent of ATMs (55–57). Enhanced glucose uptake

might promote macrophage survival as *Insr*$^{-/-}$ macrophages, exhibit increased cell death upon glucose deprivation (**Figure 1**) (40), an effect that could be particularly relevant in the context of modified lipoprotein presence (58). Interestingly, recent work indicates that GLUT1 and glucose transport is critical for the uptake of apoptotic cells (also known as efferocytosis), suggesting glycolysis may also promote anti-inflammatory phenotypes in macrophages in part through SLC16A1 mediated lactate release (**Figure 1**) (59). Other reports demonstrate that glucose promotes BMM proliferation and decreases LPS induced MHC-II expression, suggesting glucose levels might impact macrophage polarization (60). In line, high glucose levels have been described to induce the expression of Arg-1 and CD206 in macrophages in a PI3K dependent manner (61). Further, evidence of an importance for glucose in M2 responses is provided by studies demonstrating that PI3K-AKT dependent glucose utilization is critical for IL-4 responses (62, 63).

PI3KS CAN INFLUENCE FFA SIGNALING AND ATM ACCUMULATION

While physiologically FFA release through adipose lipolysis provides an important source of fuel, this process is dysregulated in the obese and insulin resistant state. The general dogma, particularly drawn from experimental murine studies, is that while unsaturated FFAs are anti-inflammatory, saturated FFAs are pro-inflammatory (64). Indeed, saturated FFAs such as palmitate promote skeletal muscle insulin resistance in part by blocking insulin mediated IRS-1 tyrosine phosphorylation and PI3K activity (65, 66). Given IRS-2 is predominantly expressed in macrophages (37), to our knowledge, no studies have addressed whether saturated FFAs decrease IRS-2 phosphorylation and render macrophages insulin resistant. Most studies utilizing macrophages in conjunction with palmitate have focused on its inflammation promoting effects. Indeed, palmitate triggered inflammation is JNK dependent, which is negatively regulated by PI3Ks (**Figure 1**) (67, 68). Although, palmitate has been suggested to mediate its effects via TLR4 (53), recent data indicates that JNK activation by palmitate is TLR4 independent (**Figure 1**). While LPS induced TLR4 signaling rapidly activates MAPK and NF-κB signaling and TLR4 endocytosis, palmitate activates these pathways much later and does not induce TLR4 endocytosis (54). The authors of this study demonstrated that LPS priming of macrophages altered cellular metabolism, gene expression and macrophage membrane lipid composition, which were necessary for palmitate induced inflammation (54). Notably, the effect of palmitate on inflammation might also depend on macrophage differentiation status. In fully differentiated macrophages, palmitate treatment elicits a pro-inflammatory phenotype, that is dependent on ER stress, as it is abrogated upon incubation with ER stress inhibitors (60). This is consistent with studies demonstrating that palmitate activates ER stress (64). However, during BMM differentiation chronic palmitate exposure been described to inhibit proliferation and promote an anti-inflammatory M2 phenotype, associated with increased PPAR-γ and CD206 expression (60).

Palmitate treatment of monocytes leads to macrophage inflammatory protein 1-alpha and beta upregulation (MIP-1α/β, also known as CCL3 and 4, respectively) and this occurs in a MAPK, NF-κB, and PI3K dependent manner indicating that PI3Ks can directly promote FFA mediated inflammation (69, 70). Interestingly, both chemokines are involved in neutrophil and monocyte recruitment, respectively (71), suggesting FFA mediated PI3K dependent signaling could promote increases in ATM number. Further evidence that palmitate mediated PI3K activation within myeloid cells regulates ATM content is provided by observations that palmitate treatment of macrophages induces netrin-1 and its receptor Unc5b, mediators that promote ATM retention and accumulation (72). Interestingly, in other cellular systems, netrin-1 acts in concert with its receptor in a PI3K dependent manner (73), although the functional relevance of PI3Ks to palmitate mediated ATM retention remains unexplored. These studies suggest that PI3Ks integrate signals derived from FFAs and thereby influences ATM accumulation and inflammatory status. However, many of the studies cited are limited by their exclusive use of *in vitro* models, disregarding the complexity of signals present *in vivo*.

CHOLESTEROL ACTIVATES THE PI3K PATHWAY

Cholesterol exists as free cholesterol (FC) or as cholesterol esters. During obesity, adipose tissue accumulates FC and this correlates with increased ATM content (74). Cholesterol and modified lipoproteins are taken up by macrophages through macropinocytosis, scavenger receptors (e.g., CD36, SRA-1) and the low density lipoprotein receptor (LDLR), leading to foam cell formation that impacts inflammation and viability (**Figure 1**) (75). FC is reported to impact macrophage inflammation in a concentration dependent manner, with lower and higher levels promoting anti and pro-inflammatory phenotypes, respectively (76). In macrophages, FC also induces AKT phosphorylation indicating it activates the PI3K pathway (40). Macrophages use cholesterol efflux pathways to maintain cellular lipid homeostasis with ABCA1 mediating the transport of cholesterol and phospholipids to lipid-free apolipoproteins such as apoA-I (75). ABCA1 upregulation in turn selectively attenuates FC, dampening inflammation by reducing TLR trafficking to lipid rafts, indicating the presence of feedback loops that resolve inflammation (77).

PI3K DEPENDENT UPTAKE OF ADIPOSE EXOSOMES

Exosomes are small (30–150 nm) endosomal derived membrane microvesicles secreted from cells that carry proteins, lipids, nucleic acids, and can reprogram recipient cells (78). Recent work demonstrates that the uptake of adipose exosomes (AdExo), promotes BMM differentiation into ATM like

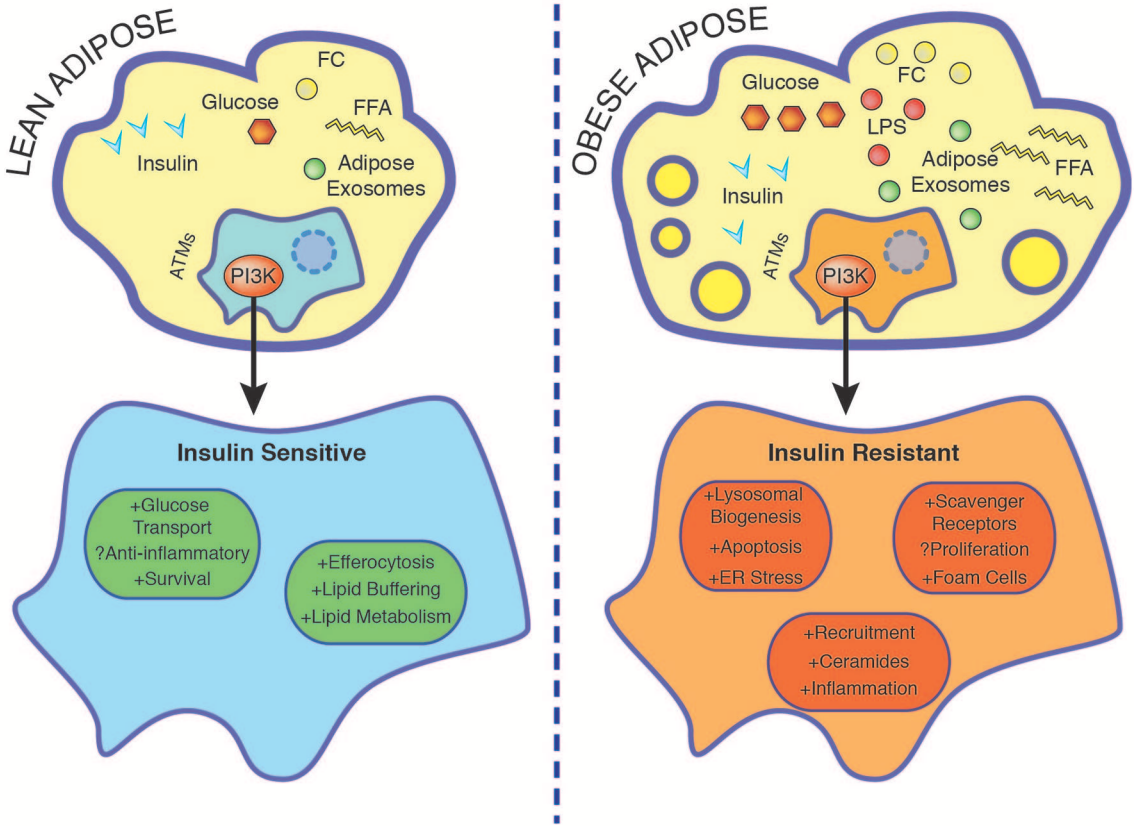

FIGURE 2 | Nutrient accumulation during obesity is associated with rendering macrophage insulin resistant. In the lean state ATMs buffer lipids and insulin promotes survival through PI3K. Insulin resistant ATMs display enhanced foam cell formation associated with increased lysosomal biogenesis, ER stress, apoptosis, and exacerbated inflammation. See text for further details. ER, endoplasmic reticulum; FC, free cholesterol; FFA, free fatty acids; LPS, lipopolysaccharide.

cells by inducing lysosomal biogenesis (79). Interestingly, AdExo do not carry FFAs but are particularly rich in FC and triglycerides and are taken up by macrophages through macropinocytosis, PI3K dependently, suggesting PI3Ks might modulate macrophage lipid loading in response to AdExos (**Figure 1**). They thus represent a novel intercellular communication route for the transfer of these lipids to macrophages (79).

PI3KS INTEGRATE THE ENVIRONMENTAL CUES THAT DICTATE MACROPHAGE PHENOTYPES IN OBESITY

Within adipose tissue during obesity, the metabolic stimuli outlined above, although elevated, likely exist at differing levels within the microenvironment and synergize their signaling with other stimuli, notably, LPS. This poses the question of how do myeloid cells respond to the combined actions of these stimuli and where do PI3Ks fit into this context during obesity? We propose a model where attenuated PI3K signaling within myeloid cells is central to meta-inflammation.

HOW MIGHT PI3KS AFFECT THE SYNERGY BETWEEN METABOLIC STIMULI IN MACROPHAGES IN OBESITY?

Obesity alters the gut microbiome and is associated with increased circulating LPS, which initiates adipose tissue inflammation and macrophage activation in a manner dependent on intact TLR4 signaling, a phenomenon coined "metabolic endotoxemia" (80, 81). Interestingly, TLR4 ligation and palmitate presence synergistically augment macrophage ceramide production through *de novo* synthesis in the ER and this is implicated in augmenting IL-1β synthesis (82). This might be especially relevant given TLR4-dependent priming of macrophages is reported to be necessary for FFA induced inflammation and thus might act as a initiating stimulus promoting FFA mediated inflammation (**Figure 2**) (67, 83). Numerous studies demonstrate that ceramides alter PI3K signaling by promoting insulin resistance through either dephosphorylating AKT or through blocking AKT translocation to the plasma membrane (84–86). Together a potential synergy between LPS and FFAs might impact macrophage intrinsic insulin sensitivity through PI3Ks.

As TLR4 dependent signaling is increased in obesity, as outlined earlier, PI3Ks would presumably limit pro-inflammatory responses through various mechanisms including the promotion of M2 responses (13–16). Generation of alternatively activated macrophages would also be favored by prevalent hyperglycemia in obesity in a PI3K-AKT dependent manner (61–63). However, high doses of insulin render macrophages insulin resistant, decreasing PI3K signaling and thus inhibiting insulin stimulated glucose uptake through GLUT-1 (36). Together, decreased myeloid cell PI3K signaling in the insulin resistant state would shift macrophage phenotypes toward pro-inflammation through a synergistic effect of high insulin, glucose, and LPS.

High glucose/insulin/palmitate stimulation of macrophages leads to upregulation of lipid metabolism genes (ABCA1, CD36, and PLIN2) and cellular programs associated with lysosomal biogenesis and autophagy, mimicking the effects of FC (30, 75). FC activates the PI3K pathway and elevated AdExos in obesity are taken up by macrophages through macropinocytosis, PI3K dependently (40, 79). Decreased PI3K signaling in insulin resistant macrophages would therefore contribute to enhanced systemic levels of these metabolic stressors. Furthermore, attenuated PI3K signaling in insulin resistant macrophages leads to upregulation of scavenger receptors and compensatory proteasomal and lysosomal catabolism (40, 49). This in turn induces a vicious cycle of modified lipid uptake, further promoting ER stress and apoptosis, which is aggravated in insulin resistant macrophages (40, 58). Consequently, we propose in obesity, cell intrinsic macrophage PI3K signaling would be downregulated and result in elevated lipid burden and death. This would impact the lipid buffering capacity of ATMs, further promoting ectopic lipid spillover and meta-inflammation (**Figure 2**). To sum up, the different metabolic inputs outlined in this review affect the degree/strength of PI3K signaling and together synergistically determine macrophage cell survival, lipid metabolism, and inflammatory phenotype.

CONCLUDING REMARKS

Slightly over 25 years ago, the concept of meta-inflammation was born by the discovery that adipose expressed TNF reduced adipose GLUT4 levels and neutralization of TNF in obese rats improved insulin sensitivity (5). Supportive of the key role of peripheral inflammation in obesity, obese myeloid-specific IKKβ or JNK deficient mice exhibit improved systemic insulin sensitivity (6, 7). Peripheral NF-κB activation is critical, as inhibiting this pathway in hepatocytes prevents IL-1β and insulin induced IR tyrosine phosphorylation and p85 association with IRS-1 (7). Since then, the contribution of ATMs to systemic inflammation has received much attention with the dogma that inflammatory pathways attenuate downstream PI3K signaling and initiate and exacerbate inflammatory responses, particularly in peripheral metabolic tissues such as the liver. However, only recently the importance of how environment re-programs and wires tissue resident macrophages has been appreciated (87).

We present an emerging paradigm where environmental stimuli encountered by ATMs during obesity reprogram them in a manner that is associated with macrophage intrinsic insulin resistance and drastic changes in intracellular lipids leading to oxidative and ER stress and upregulation of lysosomal and proteasomal programs. We propose myeloid cell PI3K activation integrates these environmental cues through its influences on saturated FFA responses, ATM accumulation, cell survival and the degree of lipid loading. This would presumably have consequences on ectopic lipid spill over and peripheral insulin sensitivity. While mice possessing global deletions of p85α/β and p55α/p50α exhibit improved insulin sensitivity (88–90) and mice with global deletions in p110α and p110β display impaired insulin sensitivity (91, 92), given that these subunits are deleted in all insulin sensitive tissues the exact function of myeloid cell specific PI3Ks during obesity and insulin resistance remains an enigma. To our knowledge, there is only one study that conditionally deleted a PI3K subunit in myeloid cells. By crossing floxed p110γ mice with mice expressing the Cre recombinase under the control of the *Tie*2 promoter, Breasson and colleagues demonstrated efficient deletion of p110γ in endothelial cells and adipose associated immune cells. These animals exhibited improved insulin sensitivity associated with increased CD206 expression in adipose tissue, independent of differences in ATM content, suggesting p110γ is dispensable for ATM recruitment but promotes M1 responses in obesity (93). Undoubtedly, myeloid cell specific deletions of class 1 PI3Ks in the context of obesity coupled to isolating primary macrophages from these mice and challenging them with the metabolic stimuli outlined in this review, will yield fruitful insights into the contribution of class I PI3Ks to obesity and ATM function.

AUTHOR CONTRIBUTIONS

OS, JB, AV, and GS has conceived and written this manuscript. JB designed the figures as presented.

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REVIEW
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Metabolic Control of Innate Lymphoid Cell Migration

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Innate lymphoid cells (ILCs) are specialized immune cells that rapidly respond to environmental challenges, such as infection and tissue damage. ILCs play an important role in organ homeostasis, tissue repair, and host defense in the mucosal tissues intestine and lung. ILCs are sentinels of healthy tissue function, yet it is poorly understood how ILCs are recruited, strategically positioned, and maintained within tissues. Accordingly, ILC migration is an area that has recently come into focus and it is important to define the signals that control ILC migration to and within tissues. In this context, signals from the local tissue microenvironment are relevant. For example, ILCs in the intestine are exposed to an environment that is rich in dietary, microbial, and endogenous metabolites. It has been shown that the Vitamin A metabolite retinoic acid promotes ILC1 and ILC3 homing to the intestine. In addition, recent studies have discovered cholesterol metabolites (oxysterols) as a novel class of molecules that regulate ILC migration through the receptor GPR183. ILCs are considered to be largely tissue-resident cells, yet recent data indicate that ILCs actively migrate during inflammation. Furthermore, the discovery of circulating ILC precursors in humans and their presence within tissues has fueled the concept of local ILC-poiesis. However, it is unclear how circulating ILCs enter tissue during embryogenesis and inflammation and how they are directed to local tissue niches. In this review, I will discuss the metabolic signals that regulate ILC homing and their strategic positioning in healthy and inflamed tissues. It is becoming increasingly clear that ILC function is closely linked to their tissue localization. Therefore, understanding the tissue signals that control ILC migration could open new avenues for the treatment of chronic inflammatory diseases and cancer.

Keywords: innate lymphoid cells, migration, metabolism, oxysterol, inflammation, cancer

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BACKGROUND

Innate lymphoid cells (ILCs) are immune cells of lymphoid origin that quickly respond to perturbations of tissue homeostasis. Apart from their role in barrier immunity and host defense, ILCs are also essential for organ homeostasis, recovery from tissue injury, and metabolism (1–5). In addition to cytotoxic natural killer (NK) cells, three different ILC types can be distinguished based on signature transcription factors and effector cytokines, similar to $CD4^+$ T helper lymphocytes: (1) T-BET^+ ILC1s produce interferon-gamma (IFNγ); (2) $GATA3^{high}$ ILC2s produce interleukin-5 (IL-5) and IL-13; (3) RORγt^+ ILC3s produce IL-17 and/or IL-22. RORγt^+ ILC3s include fetal lymphoid tissue-inducer (LTi) cells and adult LTi-like cells that have a similar phenotype ($CCR6^+NKp46^-$) and mainly reside in lymphoid tissues (6, 7). LTi cells are now considered a separate ILC lineage due to their unique ontogeny (5, 8). α4β7^+CXCR6^+ ILC3 precursors (ILC3Ps)

develop into LTi cells in the fetal liver (9). In contrast, adult LTi-like ILC3s can derive from bone marrow precursors that upregulate RORγt in peripheral tissues, such as the intestine, in a Notch-dependent manner (9). Perinatal RORγt$^+$ ILCs give rise to long-lived ILC3s in the small intestine (10), yet it is unclear whether and to what extent embryonic LTi cells persist in the adult. Therefore, the developmental relationship between fetal LTi cells and adult LTi-like ILC3s remains to be defined. Adult mice also have T-BET-expressing CCR6$^-$NKp46$^+$ ILC3s that are derived from CCR6$^-$NKp46$^-$ ILC3s (11). Dietary phytochemicals acting through the aryl hydrocarbon receptor (AHR) are required for the post-natal expansion of these CCR6$^-$ adult ILC3s (12–14). Both fetal LTi cells (15) and adult ILC3s in the intestine (16, 17) are dependent on the Vitamin A metabolite retinoic acid.

Many features of ILCs are shared with T cells, but ILCs also have unique, non-redundant, functions, such as the ability to orchestrate the formation of lymphoid tissues, which is carried out by ILC3s with LTi function (12, 13, 18–21). In mice, the prenatal formation of lymphoid tissues (lymph nodes and Peyer's Patches) is carried out by CD4$^+$ fetal LTi cells, whereas adult ILC3s mediate the development of intestinal cryptopatches and isolated lymphoid follicles that develop after birth (22, 23). Besides their beneficial effects, ILCs have been implicated in chronic inflammatory responses that underlie human disease (24–26).

The main focus in the research on ILCs has been on cell lineage relationships, transcription factors, and effector function—mostly based on analogies with T lymphocytes. ILC migration has only recently become an active area of investigation. Their strategic position within tissues allows ILCs to fulfill their role as sentinels of healthy tissue function. Furthermore, local ILC clustering and rapid migration in response to inflammatory signals may explain why ILCs exert such powerful effects on tissue immunity (**Figures 1**–**3**). However, much remains to be learned about the pathways that regulate the migration and tissue localization of ILCs. In this review, I mainly discuss the migration of ILCs other than NK cells.

TISSUE DISTRIBUTION OF ILCs

ILCs are found in many organs, but are enriched in mucosal tissues (intestine, lung) that are most exposed to the environment (10, 16, 27–31). Furthermore, the relative abundance of ILC subsets differs between tissues in mice (10, 16, 28) and humans (30, 31), with ILCs perhaps less compartmentalized in humans than in mice (31). For example, ILC3s are abundant in the small intestine and ILC2s in the skin as well as in adipose tissue, whereas NK cells predominate in bone marrow, spleen, liver, and lung (32). In addition, there are regional differences in ILC distribution within the same organ. For example, NKp46$^+$ ILC3s are enriched in the small intestine, whereas in the colon adult LTi-like ILC3s are more prevalent. In addition, ILC2s are more abundant in the colon than in the small intestine (32). Moreover, ILC abundance differs between steady-state and inflamed tissue (33–35). Finally, developmental age of the organism influences ILC tissue distribution. For example, LTi-like ILC3s are present in the fetal gut, whereas NKp46$^+$ ILC3s are largely absent (10), only expanding after birth in response to diet-derived AHR ligands (12–14) and signals from the maternal microbiota (36). Similarly, ILC2s seed the mouse lungs within the first 2 weeks of life (37). The differential tissue distribution of ILCs is likely related to their migratory behavior, e.g., due to temporal seeding of tissues during embryogenesis (10, 38) and due to organ-specific expression of integrins and chemokine receptors on ILCs (**Figures 1**–**3**).

Parabiosis studies in mice established the concept that, in contrast to NK cells, ILCs in both lymphoid and non-lymphoid tissues are largely tissue-resident cells (39). This implies that, similar to tissue macrophages, ILCs are maintained within tissues by local self-renewal. However, recent studies have challenged this concept with the discovery of circulating CD117$^+$ ILC precursors (ILCPs) in humans (40) and the observation that inflammatory ILC2s in the mouse can migrate from the intestine to the lung during helminth infection (41). Circulating ILCs might therefore constitute mobile a pool of cells that can be activated and recruited to inflamed tissue on demand in order to support host defense carried out by tissue-resident ILCs (**Figures 1**, **3**). Apart from ILCPs, human blood also contains ILC2s (27), but no mature ILC1s and ILC3s (40). In addition to more abundant NK cells, circulating putative ILCPs and mature ILCs, mainly ILC1s, are also found in mice (42, 43).

Interestingly, ILCs occupying vascular vs. tissue compartments seem to have distinct functions. A recent study demonstrated that NK cells circulating between blood and peripheral tissues have effector function, whereas NK cells trafficking to lymph nodes are long-lived and proliferative (44). This different migratory and functional behavior has been associated with the differential expression of transcription factors (44).

TISSUE NICHES AND ILC FUNCTION

Like other immune cells, ILCs occupy distinct niches within tissue, which is important for their function and likely regulates their homeostasis. For example, in the intestine, ILCs reside in three main anatomical compartments: (i) LTi-like ILC3s are clustered in lymphoid tissues, such as cryptopatches and isolated lymphoid follicles (45, 46); (ii) NK cells/ILC1s, ILC2s, NKp46$^+$ ILC3s are dispersed in the lamina propria (47–49); (iii) Intraepithelial ILC1s are located within the epithelium (30, 50). This anatomical compartmentalization corresponds to the diversity of ILC function in the intestine (**Figure 2**). For example, LTi-like ILC3s in Peyer's Patches and isolated lymphoid follicles interact with B cells to stimulate IgA production (51, 52), which promotes host commensalism with the local microbiota (53). Furthermore, lymphoid tissue-resident commensal bacteria are contained by IL-22-producing ILC3s (54). In addition, ILC3s in cryptopatches are in close proximity to the cypts, where intestinal stem cells reside. Accordingly, IL-22 production by ILC3s has been shown to maintain crypt stem cells after tissue damage (55–57). In contrast, NKp46$^+$ ILC3s are mostly resident in the small

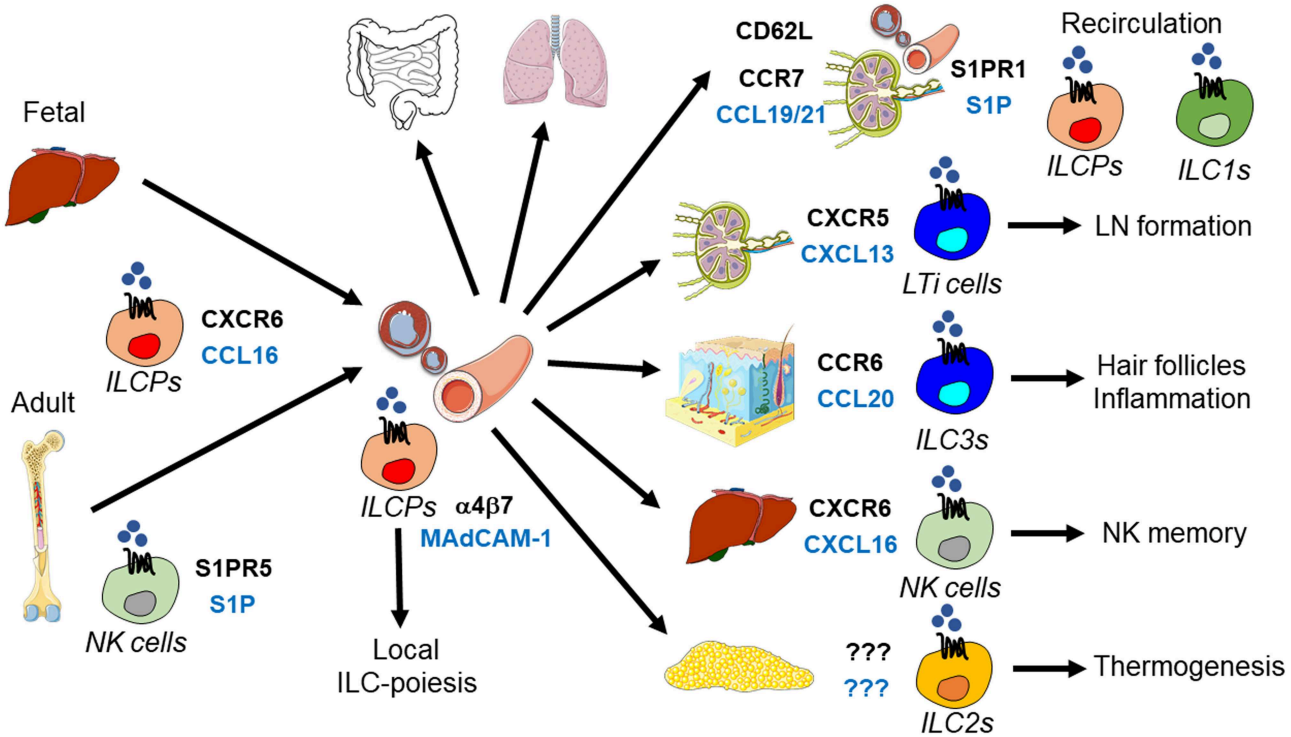

FIGURE 1 | Tissue homing and positioning of ILCPs and mature ILCs. ILCPs derived from fetal (liver) or adult (bone marrow) hematopoiesis enter the blood to home to lymphoid and non-lymphoid organs. This process requires CXCR16 and α4β7 integrin, which bind to CXCL16 and MAdCAM-1, respectively. NK cells egress from the bone marrow is mediated by S1PR5-S1P. Recent data support the concept that circulating ILCPs are a source of local ILC-poiesis after their migration into tissues. ILCPs and ILC1s recirculate between lymph nodes and blood through the use of CD62L, CCR7, and S1P receptors. During embryogenesis, LTi cells are essential for lymph node (LN) formation, which is initiated by their CXCR5-CXCL13-dependent clustering at lymph node anlagen. CCR6-CCL20 positions ILC3s to hair follicles and recruits ILC3s during skin inflammation. In the liver, CXCR6 and its ligand CXCL16 are essential for NK cell memory responses. ILC2s promote thermogenesis, but the signals regulating their migration in adipose tissue are unknown.

intestinal villi, located close to the epithelium, where they mediate host defense against pathogens (58). Interestingly, NKp46$^+$ ILC3s seem to produce IL-22 mainly in response to pathogen-induced IL-23 secretion by myeloid cells, whereas lymphoid tissue-resident LTi-like ILC3s produce IL-22 constitutively in a microbiota-dependent manner (59). Finally, IFNγ-secreting ILC1s within the intraepithelial compartment are involved in colitis (50).

Although intestinal ILC3s are tissue-resident (39), they are not completely sessile cells. For example, in the steady state, there is a constant influx and egress of ILC3s to and from cryptopatches and there is increased ILC3 mobilization from cryptopatches during inflammation (21, 60). The significance of steady-state ILC3 migration in and out of cryptopatches is unknown, but could potentially serve the purpose of sampling or sensing cues from the environment (such as crypt material) to respond to perturbations of the intestinal stem cell compartment. Accordingly, it has been suggested that cryptopatches act as a platform to rapidly amplify ILC-mediated immune responses, not only through cytokine production, but also through ILC movement into surrounding tissue (60).

Interestingly, occupancy of tissues niches by ILCs is regulated by quorum sensing-like mechanisms. Thus, it has recently been shown that receptor activator of nuclear factor kappa B (RANK)-RANK ligand (RANKL) interactions adjust the numbers of mouse CCR6$^+$ LTi-like ILC3s to the size of the niche, likely within cryptopatches (61). Therefore, regardless of their outer environment, clustering of CCR6$^+$ ILC3s allows them to keep one another in check. This mechanism likely operates also in human tonsil, where CCR6$^+$ ILC3s express both RANK and RANKL (61).

In many tissue niches, ILCs have an intimate relationship with non-hematopoietic cells, such as stromal cells. For example, an ILC3-stromal cell niche in secondary lymphoid organs has been reported in both mice and humans (62). Moreover, ILC2s occupy a distinct perivascular localization close to stromal cells in the lung (63, 64). In this specific niche, adventitial stromal cells promote ILC2 homeostasis in steady-state and in response to helminth infection through the production of IL-33 and thymic stromal lymphopoietin (TSLP) (64). In turn, ILC2-derived IL-13 supports the expansion and IL-33 production by adventitial stromal cells (64). The close proximity of lung ILC2s to blood vessels has been proposed to allow efficient recruitment of eosinophils from the blood (63), further underscoring the importance of ILC intra-tissue localization (**Figure 3**). Furthermore, ILC2s are strategically positioned within the airways, near airway branch points (65), where inhaled particles are thought to accumulate. This puts ILC2s in close

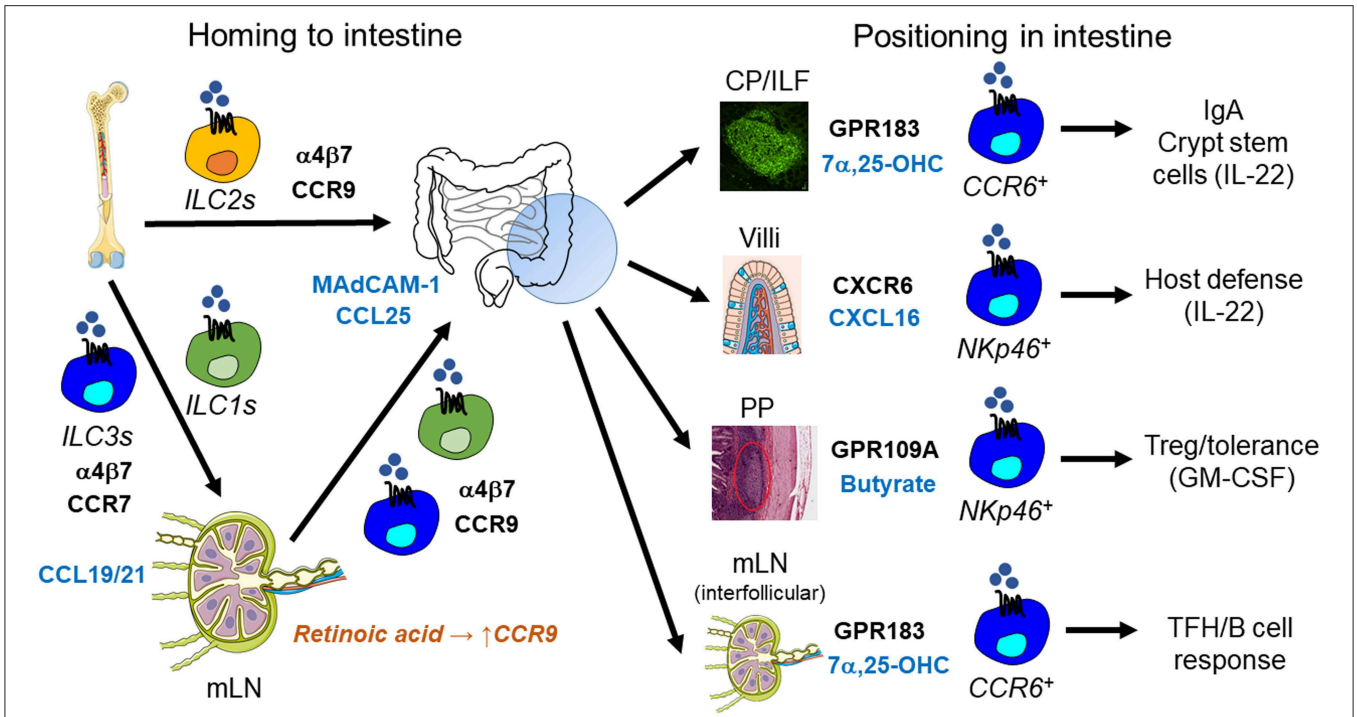

FIGURE 2 | ILC trafficking and positioning in the intestine. ILC homing to the small intestine is mediated by α4β7 integrin and the chemokine receptor CCR9 and their respective ligands MAdCAM-1 and CCL25. ILC2s in the bone marrow already express CCR9 and are therefore capable of homing to the small intestine directly. In contrast, retinoic acid is required to induce CCR9 expression in ILC1s and ILC3s after their CCR7-dependent trafficking to mesenteric lymph nodes (mLN). After homing to the intestine, ILC3s are positioned within different tissue niches by distinct signals: (i) $CCR6^+$ LTi-like ILC3s localize to cryptopatches (CP) and isolated lymphoid follicles (ILF) through the GPR183-mediated sensing of 7α,25-dihydroxycholesterol (7α,25-OHC). CP/ILF-resident ILC3s stimulate IgA production by interacting with B cells and support crypt stem cells through the constitutive production of IL-22. (ii) $NKp46^+$ ILC3s are positioned to intestinal villi through CXCR6-CXCL16, which promotes host defense against intestinal pathogens via IL-22. (iii) Microbiota-produced butyrate regulates the regional residence of $NKp46^+$ ILC3s in Peyer's Patches (PP), which controls intestinal tolerance through GM-CSF secretion and regulatory T cells (Treg). (iv) GPR183 and its ligand 7α,25-OHC are also essential for the localization of $CCR6^+$ LTi-like ILC3s to the interfollicular region within mLNs, where they interact with T follicular helper cells (TFH) and B cells to regulate IgA production.

contact with neuroendocrine cells that activate ILC2s through the release of calcitonin gene-related peptide (65). ILC-neuron interactions also occur in intestinal cryptopatches (66) and accordingly neuronal circuits have been shown to regulate ILC function in different contexts (66–71).

Furthermore, there is the emerging concept that different tissue microenvironments specify ILC function, as has been shown for ILC2s (72). Local tissue niches might also regulate ILC function through stimulating ILC plasticity. *In vitro*, ILC plasticity occurs through the exposure to polarizing cytokines, such as IL-1β and IL-12, which induces the conversion of ILC2s and ILC3s into ILC1s (35, 73–76). However, it is unclear where these factors are produced *in vivo* and where ILC trans-differentiation occurs within tissue. One possibility is that migratory signals induced by inflammation guide ILCs to specific niches, where they are exposed to polarizing cytokines. Alternatively, the polarizing cytokines might be produced within the same niche in response to inflammatory stimuli.

Overall, the signals and migratory receptors regulating the co-localization and interaction of ILCs with stromal cells and other immune cells, such as T cells, are largely unknown. Subsets of ILCs interact with T cells through the expression of major histocompatibility complex (MHC) class II, CD1d, OX40 ligand (OX40L), and CD30 ligand (CD30L) (77). In the intestine, MHC class II^+ ILC3s suppress CD4 T cell responses against the local microbiota (78, 79), whereas the interaction of MHC class II^+ ILC2s with CD4 T cells promotes type 2 immunity in the lung (80). Moreover, OX40L-expressing ILC2s stimulate Th2 and regulatory T cell (Treg) responses in lung and adipose tissue (81). Interestingly, adult LTi-like ILC3s constitutively express co-stimulatory ligands (OX40L, CD30L), whereas fetal LTi cells do not (82).

Another interesting area for future investigation is the occupation of tissue niches by ILCs and their adaptive counterparts, i.e., T cells. This is particularly relevant since both ILCs and T cells largely dependent on the same factors (γ_c cytokines) for their homeostasis and expansion, therefore likely competing with each other. Accordingly, intestinal ILC2s and ILC3s expand in T cell-deficient mice, e.g., in mice lacking *Rag* genes (16), most likely due to increased availability of IL-2 and IL-7. This notion is supported by the finding that intestinal ILC3s outcompete T cells for IL-2 (79) and that IL-7 consumption by

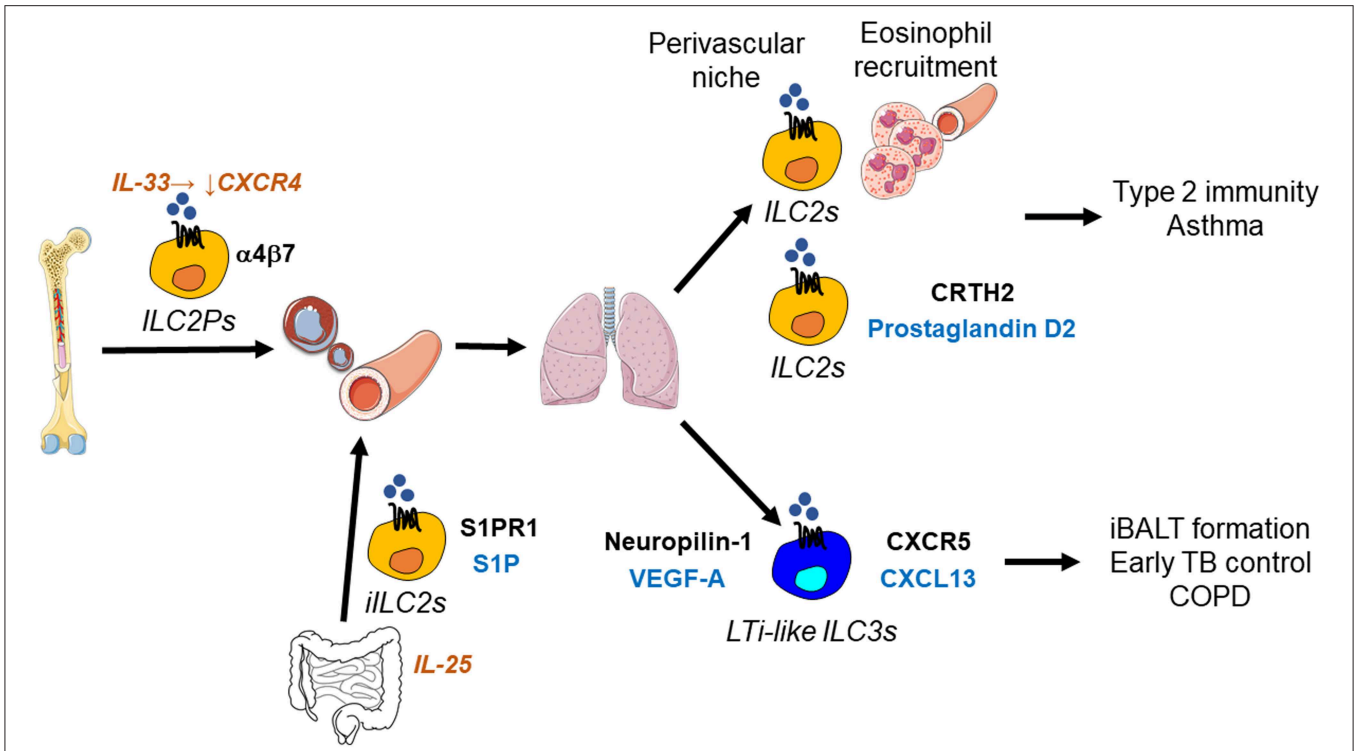

FIGURE 3 | ILC migration in the lung. Suppression of CXCR4 by IL-33 enables ILC2Ps to egress from the bone marrow and home to the lung, where they occupy a perivascular niche. This strategic localization likely allows ILC2-mediated recruitment of eosinophils from the circulation into the lung. In addition, prostaglandin D2 stimulates ILC2 migration in the lung through the interaction with its receptor CRTH2 on ILC2s. ILC2s thereby promote type 2 barrier immunity, but also allergic inflammation as in asthma. During helminth infection, IL-25-responsive inflammatory ILC2s (iILC2s) are capable of trafficking from the intestine to the lung, where they support anti-helminth immunity. This inter-organ trafficking requires S1PR1-S1P. LTi-like ILC3s migrate in the lung using CXCR5-CXCL13 and likely neuropilin-1-VEGF-A. LTi-like ILC3s mediate the formation of inducible bronchus-associated lymphoid tissue (iBALT), which is essential for the early control of tuberculosis (TB) in the lung, but may also be involved in inflammatory responses occurring in chronic obstructive pulmonary disease (COPD).

ILCs regulates the amount of IL-7 that is available to T cells (83). Finally, it has been suggested that IL-2 produced by proliferating T cells maintains LTi-like ILC3s in lymphoid structures (77), in accordance with the observation that mesenteric lymph node-resident ILC3s are reduced in T cell-deficient mice (84). However, in many tissues it has not been thoroughly investigated whether ILCs and T cells occupy distinct or overlapping niches.

ILC TRAFFICKING TO TISSUES

Trafficking Receptors on ILCs

Mature ILCs are largely tissue-resident cells (39), yet the signals that control the migration of ILCPs and mature ILCs into tissues during embryogenesis, adult life, and inflammation are still incompletely understood. Similar to T lymphocytes, ILC trafficking to tissues is regulated by integrins and chemokine receptors (**Table 1**) that are often expressed in an ILC subset-specific manner with similar chemokine receptor expression as the corresponding T helper subsets (32).

For example, LTi-like ILC3s express CCR6 and CXCR5 (10, 11), both transcriptional targets of RORγt, which are also preferentially expressed on Th17 cells (CCR6) and T follicular helper cells (CXCR5). CCR6 and CXCR5 are already expressed on ILC3Ps that migrate to peripheral tissues from the fetal liver and adult bone marrow (9). In contrast, NKp46$^+$ ILC3s express CXCR6 (29, 92), the receptor for CXCL16, which mediates their localization to the lamina propria (58). ILC1s also express CXCR6 (92). Furthermore, CXCR6 promotes the homing of NK cells to the liver, which is important for NK cell memory (93). Lymphoid tissue-resident human ILC3s with LTi activity, as well as murine fetal CD4$^+$ LTi cells, not only express CCR6 and CXCR5, but also Neuropilin-1, which mediates their migration toward vascular endothelial growth factor-A (VEGF-A) (99). Finally, distinct subsets of intestinal ILC3s express CD49a (integrin α1) (86). Moreover, similar to Th2 cells, both mouse and human ILC2s express CCR4 (27, 29, 100) and other skin-homing receptors, such as cutaneous leukocyte-associated antigen (CLA) and CCR10 that bind to endothelial cell-leukocyte adhesion molecule 1 (ELAM-1) and CCL27/CCL28, respectively (32). It has been reported that ILC2s in broncho-alveolar lavage fluid highly express CCR4 (and CCR7) after IL-33 administration (101), suggesting a role for CCR4 and its ligands CCL17 and CCL22 in ILC2 migration following activation, although this prediction requires experimental validation. CCR8 is another chemokine receptor that shows shared expression in ILC2s and Th2 cells (29), which may mediate ILC homing to the skin

TABLE 1 | Receptors involved in ILC migration.

Receptor	Ligand	Source	Function	References
INTEGRINS/SELECTINS				
α4β7 integrin	MAdCAM-1	Endothelial cells	LTi cell entry into embryonic LNs; ILC trafficking to small intestine and colon	(18, 85)
CD49a	Collagen	Tissue matrix	ILC tissue retention?	(30, 86)
αEβ7 integrin	E-cadherin	Epithelial cells	ILC1 interaction with intestinal epithelium?	(50)
CD69	NA	NA	Inhibition of ILC tissue egress by antagonizing S1P receptors	(41)
CD62L (L-selectin)	MAdCAM-1 GlyCAM-1 CD34	Endothelial cells	LN entry and recirculation of ILCPs, ILC1s, NK cells	(42, 43)
CHEMOKINE RECEPTORS				
CCR6	CCL20	Epithelial cells	ILC3 positioning to hair follicles; recruitment into inflamed skin	(43, 87)
CCR7	CCL19/CCL21	Stromal cells	LTi cell clustering at LN anlagen; ILC1/ILC3 migration from BM to mLNs; ILC LN entry and recirculation; ILC3 trafficking from intestine to mLNs; ILC3 recruitment to tumors	(43, 84, 85, 88–90)
CCR9	CCL25	Epithelial cells	ILC trafficking to small intestine	(85)
CXCR4	CXCL12	Reticular cells?	Inhibition of ILC2P egress from BM	(37)
CXCR5	CXCL13	Stromal cells	LTi cell clustering at LN anlagen; ILC3 recruitment to infected lung in tuberculosis; ILC3 clustering with stromal cells in tumors	(88, 90, 91)
CXCR6	CXCL16	CX3CR1$^+$ DCs Other cells?	Positioning of NKp46$^+$ ILC3s to small intestinal villi; NK cell homing to liver; ILC3P migration from fetal liver to periphery; ILCP egress from BM	(58, 92, 93)
OTHER RECEPTORS				
GPR109A	Butyrate	Microbiota	Regional ILC3 distribution in PPs	(94)
GPR183	7α,25-OHC	Stromal cells	ILC3 migration to cryptopatches; ILC3 recruitment to small intestine; ILC3 positioning within mLNs	(21, 95, 96)
S1P receptors	S1P	Red blood cells? Endothelial cells?	NK cell egress from BM and LNs; ILC egress from LNs; ILC2 inter-organ trafficking in helminth infection	(41, 42, 97)
CRTH2	Prostaglandin D2	ILC2s?	ILC2 recruitment to inflamed lung	(98)
Neuropilin-1	VEGF-A	Unknown	LTi cell recruitment to iBALT?	(99)

BM, bone marrow; iBALT, inducible bronchus-associated tissue; LN, lymph node; mLN, mesenteric lymph node; PP, Peyer's Patch.

in response to CCL1. In contrast, ILC1s and NK cells share preferential expression of CXCR3, the receptor for CXCL9, CXCL10, and CXCL11, with Th1 cells (102). Furthermore, expression of CD49a and CD49d (integrin α4) can be used to distinguish subpopulations of intestinal ILC1s (86). NK cells also express integrins, such as CD49a and CD49b (integrin α2) (32). In addition to subset-specific expression, migratory receptors are also expressed in a tissue-specific manner within the same ILC subset. For example, ILC2s in adipose tissue have higher expression of *Itgae*, *Ccr6*, and *Cxcr4* than ILC2s from other tissues (72).

Seeding of Tissues With ILCs During Development

In mice, ILCs derived from fetal liver hematopoiesis are among the first lymphocytes to seed barrier tissues, such as the intestine before birth (10, 12, 38) (**Figure 1**). This tissue seeding prepares the host for the colonization of the intestine with the microbiota and the intake of food-derived antigens. Moreover, LTi cells populate organs early to promote the formation of lymphoid tissues (22). ILCPs express α4β7 integrin, whose ligand MAdCAM-1 is widely expressed in the fetus, thereby allowing ILCP migration to a variety of tissues (32). The entry of LTi cells into embryonic lymph nodes is also dependent on α4β7 integrin (103). Furthermore, the interaction of CXCL13, induced by retinoic acid in mesenchymal organizer cells, with CXCR5 on LTi cells is required for the clustering of LTi cells at embryonic lymph node anlagen and lymph node development, with a minor contribution of CCL21 and its receptor CCR7 (88). Further work showed that the amount of maternal retinoic acid regulates the number of LTi cells and therefore the size of lymph nodes, which determines anti-viral immunity later in life (15). Before birth, LTi cells also cluster at embryonic anlagen to promote the formation of Peyer's Patches, which is dependent on expression of RET, a tyrosine kinase receptor for neurotrophic factors, on LTi cells (104). In addition, arginase 1-expressing ILCPs accumulate at Peyer's Patch anlagen, where they can give rise to ILC1s, ILC2s, and ILC3s in the fetal intestine (38). This ILCP clustering occurs in a CXCR5- and CCR7-independent manner, since, in contrast to LTi cells, these Arginase 1$^+$ ILCPs do not express CXCR5 and CCR7 (38). In contrast to LTi cells, Arginase 1$^+$ ILCPs also lack lymphotoxin expression and are therefore dispensable for Peyer's Patch organogenesis (38). Finally, fetal α4β7$^+$CXCR6$^+$CCR6$^+$CXCR5$^+$ ILC3Ps migrate from fetal liver to lymphoid organs and intestine (9, 105) in a CXCR6-dependent manner (92). ILCs are also found in human fetal tissues (27), suggesting that early colonization of tissues with ILCs is conserved between mice and humans.

Overall, embryonic tissue seeding of ILCPs is reminiscent of the colonization of tissues with embryonically-derived macrophages (106, 107). In contrast to organs that are seeded before birth (e.g., the intestine), other organs, such as the lung and spleen, are colonized with ILCs after birth. Later, during adult life, there is likely a second wave of ILCPs from bone marrow (or other tissues) that enter the circulation and gain access to tissues to contribute to the ILC pool found in peripheral organs. Again, this might be in analogy to macrophages, where, in the adult organism, circulating monocytes enter tissues and, under specific conditions, can differentiate into tissue-resident macrophages.

ILC Trafficking to Lymph Nodes

In adult mice, ILCs use similar mechanisms as naïve T cells for lymph node entry (**Figure 1**). For example, like NK cells, ILCs (especially ILCPs and ILC1s) enter peripheral lymph nodes using CD62L (L-selectin) and CCR7 (42, 43). In addition, LTi-like ILC3s are capable of trafficking from the intestine to draining mesenteric lymph nodes in a CCR7-dependent manner (84). In contrast, ILC1s, ILC2s, and NKp46$^+$ ILC3s do not migrate along this route. Accordingly, LTi-like ILC3s migrate toward the CCR7 ligand CCL21 *in vitro*, whereas ILC2s are unable to do so (84). Finally, the trafficking of LTi-like ILC3s to the spleen is not critically dependent on CCR7 (84).

ILC Trafficking to the Intestine

Tissue-specific signals from the local microenvironment likely play an important role in the trafficking of ILCs to the intestine, including cues from the microbiota, which might be particularly important for intestinal ILC3s (36, 86). In addition, metabolic cues are essential as has been demonstrated in a few studies so far (**Figure 2**). For example, it has been shown that the preferential homing of ILCs to the small intestine is controlled by diet-derived nutrients. ILCPs and mature ILC subsets express α4β7 integrin, CCR7, and CCR9 to varying degrees (85, 92) and, similar to T lymphocytes, ILC1, and ILC3 trafficking to the small and large intestine requires α4β7 integrin (85) that binds to MAdCAM-1, abundantly expressed on endothelial cells in the intestine. Furthermore, the Vitamin A metabolite retinoic acid is essential for the homing of ILC1s and ILC3s, but not ILC2s, to the small intestine (85). Specifically, it was found that ILC1s and ILC3s migrate from the bone marrow to mesenteric lymph nodes in a CCR7-dependent manner, where retinoic acid induces expression of α4β7 integrin and CCR9 (85), whose ligand CCL25 is abundant in the small intestine. In contrast, CCR9 expression is acquired by α4β7$^+$ILC2Ps already in the bone marrow and therefore retinoic acid-independent (85, 100). This feature likely links the nutrient status of the host to the type of local immune response through the preferential migration of specific ILC subsets to the small intestine. This concept is consistent with the observation that lack of Vitamin A, as it occurs in malnutrition, causes a reduction of ILC3s and impaired protection against bacterial pathogens in the intestine, whereas ILC2s and anti-helminth responses are increased (16). This switch to type 2 barrier immunity likely ensures continued commensalism with evolutionary partners (helminths, commensal bacteria) in the small intestine during nutrient deficiency. In contrast, the homing of ILC3s to the colon requires α4β7 integrin, but not CCR9, and is therefore independent of retinoic acid (85). It has not been explored whether other chemotactic receptors used by T cells, such as GPR15 (108), enable ILC homing to the colon.

Circulating ILCPs

ILCPs are present within tissues, such as the intestine and other organs, including blood, in mice and humans (9, 38, 40, 42, 109, 110). Despite the presence of ILCPs in both peripheral blood and tissues, parabiosis studies in mice indicate that ILCs other than NK cells in both lymphoid and non-lymphoid tissues are mainly tissue-resident (39). Subsequently, this concept has been challenged by the finding that human CD34$^-$CD117$^+$ ILCPs are present not only in blood, but also in a variety of lymphoid and non-lymphoid tissues (40), demonstrating that these ILCPs can leave the circulation and migrate into tissues. Furthermore, these circulating human CD117$^+$ ILCPs can be considered the equivalent of naïve T cells since they lack immediate effector function, but have the ability to differentiate into mature ILC1s, ILC2s, and ILC3s *in vitro* and upon adoptive transfer into mice. ILCPs were therefore proposed to serve as cellular substrates for local "on-demand" ILC-poiesis within tissue (111). Further studies are needed to clarify potential species-specific differences in ILC migration/tissue residency between humans and mice and to establish to what extent mature ILCs in tissues are replenished by circulating ILCPs.

Progenitors upstream of human CD34$^-$CD117$^+$ ILCPs express the adhesion/homing receptor CD34 and are found in a variety of tissues, but not in blood (111). It is plausible that loss of CD34 expression on CD34$^+$ ILCPs triggers the entry of CD34$^-$CD117$^+$ ILCPs into the circulation (111). Furthermore, IL-1β (in combination with IL-2 and IL-7) acts as a growth factor for CD117$^+$ ILCPs *in vitro* (40) and it has been suggested that production of IL-1β induced by disruption of tissue homeostasis promotes the migration of ILCPs from blood into tissue (111). However, as a cytokine, IL-1β lacks direct chemotactic activity and therefore other, yet unknown, chemotactic guidance cues and their corresponding receptors must be involved.

ILCP Egress From Bone Marrow

In mice, ILCPs and ILC2Ps, unlike common lymphoid progenitors, express the chemokine receptor CXCR6 and their egress from the bone marrow is partially dependent on CXCR6, thereby regulating ILCP entry into the circulation (92). In contrast, adult ILC3Ps migrate from the bone marrow to the periphery in a CXCR6-independent manner (9). Furthermore, a recent study demonstrated that IL-33 signaling is required for the egress of ILC2Ps from the bone marrow during the perinatal period by downregulating CXCR4 expression (37). Finally, the bioactive lipid sphingosine-1 phosphate (S1P) promotes lymphocyte egress from several organs (112) and S1P receptor 5 (S1PR5) is essential for the bone marrow egress of NK cells (97). However, it has not been investigated whether S1P receptors also regulate the egress of ILCPs from bone marrow.

ILC Recirculation

Both mouse and human ILCPs in the blood express CD62L, which promotes lymph node entry of ILCPs, whereas lymph node exit requires S1P receptors (42). The later possibility is further

supported by the finding that treatment with the S1P agonist FTY720, which disrupts S1P gradients and results in S1P receptor internalization from the cell surface (112), causes ILC-penia, while increasing the number of ILCs in lymph nodes (113). These studies are consistent with the concept that, similar to naïve T cells, ILCPs and some mature ILCs have the ability to re-circulate between blood and lymphoid organs (**Figure 1**). This notion is further supported by a recent study in mice, demonstrating that ILC1s (similar to NK cells) recirculate between blood and lymph nodes in a CD62L- and CCR7-dependent manner, whereas ILC3s in lymph nodes are mainly tissue-resident (43). Furthermore, among human ILCs, NKp44$^-$ ILC3s, likely representing ILCPs (40), have higher expression of genes encoding surface receptors involved in lymphocyte recirculation (CD62L, CCR7, S1PR1) than NKp44$^+$ ILC3s (114). Finally, compared to their circulating counterparts, human lymphoid tissue-resident ILC3s express CXCR5 and CCR7, known to regulate positioning within lymphoid organs (114).

Tissue Retention of ILCs

Finally, less is known about the factors that retain ILCs within tissues once they are recruited. This likely involves the same receptors that are required for the tissue retention of T lymphocytes (115) (**Table 1**). For example, intestinal and skin ILCs express CD69 (41, 48, 92), which antagonizes the egress receptor S1PR1 (116). In addition, ILC2s from human tissues express the collagen-binding integrin CD49a (30) that has been shown to promote T cell retention in tissues. Populations of mouse ILC1s and ILC3s also express CD49a (86) as do human intraepithelial ILC1s in the intestine (50). In addition, the latter subset expresses CD103 (αE integrin) (50), which together with β7 integrin binds to E-cadherin on epithelial cells. CD103$^+$ ILC2s have also been identified in mouse skin (117). Further studies (such as genetic ablation in mice) are required to demonstrate a direct role for specific receptors in the tissue retention of ILCs.

ILC POSITIONING WITHIN TISSUE

ILCs occupy strategic positions within tissues to perform their organ-specific functions. Proper ILC positioning within tissue is also critical for the spatial compartmentalization of tissue immunity. For example, as discussed above, ILCs inhabit tissue-specific niches, which facilitates the interaction with other immune cells as well as with non-hematopoietic cells. However, there is very limited knowledge regarding the signals and receptors that direct ILCs to local tissue niches. Recent work elucidated how metabolic signals ensure that ILCs are properly positioned in the intestine to carry out their function. We found that intestinal ILC3s lacking the G protein-coupled receptor GPR183 (also known as EBI2) exhibit aberrant localization (21). GPR183 recognizes hydroxylated metabolites of cholesterol, so-called oxysterols, with 7α,25-dihydroxycholesterol as the main GPR183 ligand (118, 119). We demonstrated that oxysterols sensed through the receptor GPR183 function as guidance cues to position ILC3s within intestinal cryptopatches, which is critical for lymphoid tissue formation in the colon (21) (**Figure 4**). Similar findings have been subsequently reported by two other

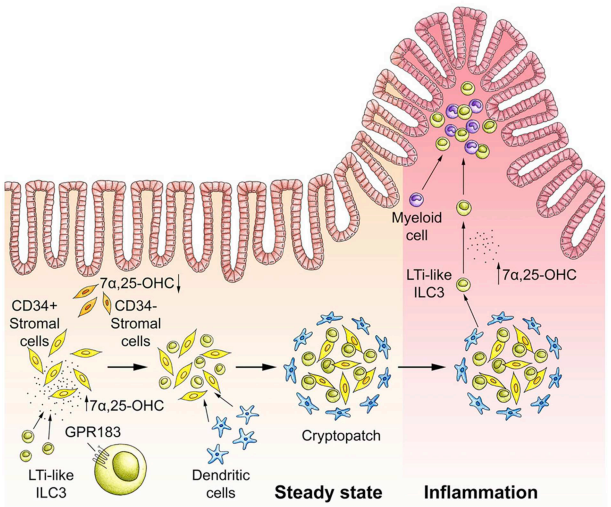

FIGURE 4 | The GPR183-oxysterol pathway positions ILC3s in the colon. In steady-state, LTi-like ILC3s are recruited to cryptopatches by the receptor GPR183 that senses locally produced cholesterol metabolites (oxysterols). The GPR183 ligand 7α,25-dihydroxycholesterol (7α,25-OHC) is produced by CD34$^+$ fibroblastic stromal cells. The interaction of GPR183$^+$ LTi-like ILC3s with stromal cells leads to cryptopatch formation in steady-state. Inflammation increases 7α,25-OHC production and promotes the migration of GPR183$^+$ ILC3s and myeloid cells to inflammatory foci within the colon. Adapted from reference (21).

labs (95, 120). Chu et al. found that GPR183 also regulates ILC3 recruitment to the small intestine (but not to the colon), possibly through promoting α4β7 integrin surface expression on ILC3s (95). We further showed that oxysterols are produced by specialized stromal cells located within cryptopatches/isolated lymphoid follicles (21). The intriguing possibility that dietary cholesterol in breast milk is a source of oxysterols required for post-natal lymphoid organogenesis in the colon remains to be explored (119).

This complements previous work showing that CXCL16 produced by CX3CR1$^+$ dendritic cells guides the positioning of CXCR6$^+$ NKp46$^+$ ILC3s to the villi of the small intestine, where they contribute to epithelial defense through the production of IL-22 (58). Notably, the chemokine receptors CCR6 and CXCR5 (and their respective ligands CCL20 and CXCL13), although specifically expressed by LTi-like ILC3s, are not required for ILC3 migration to cryptopatches, neither in the small nor large intestine (121, 122). However, it has recently been reported that skin ILC3s are positioned within hair follicles in a CCR6-dependent manner (87). In mesenteric lymph nodes, ILC3s are found in a specific anatomical location, the interfollicular region (84), and GPR183 also promotes the proper positioning of ILC3s to this region, whereas CCR6 and CXCR5 are dispensable (95, 96).

Apart from NK cells that largely lack GPR183, ILC subsets other than LTi-like ILC3s also express GPR183 to varying degrees (21). However, the specific functions of GPR183 in other ILC subsets is unknown. Overall, our recent work and that of others

indicates an important role for local cholesterol metabolites in directing ILC migration.

Other lipids, such as leukotrienes and prostaglandins, are likely relevant for the intra-tissue localization of ILCs (**Figure 3**). For example, human ILC2s are phenotypically defined by the expression of CRTH2 (27), the prostaglandin D2 receptor, and prostaglandin D2 induces the chemotaxis of ILC2s *in vitro* (123). Accordingly, CRTH2 mediates accumulation of mouse ILC2s in the inflamed lung (98). Moreover, human ILC2s found in asthma are responsive to the lipid mediators prostaglandin D2 and lipoxin A4 (124). As mentioned above, amino acid derivatives, such as a tryptophan metabolites, can regulate ILC homeostasis through AHR. However, it is currently unknown whether amino acid-derived molecules can also act as chemotactic cues, guiding ILC positioning.

Apart from host-derived signals, the gut microbiota also likely regulates ILC migration and positioning through the local production of metabolites. For example, lymphoid-tissue resident commensal bacteria promote ILC3 localization to mesenteric lymph nodes and Peyer's Patches (125). Furthermore, the short-chain fatty acid butyrate controls the compartmentalization of ILC3s in Peyer's Patches (94). Specifically, it has been shown that butyrate, sensed through the receptor GPR109A on ILC3s, is more abundant in ileal than jejunal Peyer's Patches, thereby inhibiting the residence of $CCR6^{-}NKp46^{+}$ (and $CCR6^{+}NKp46^{-}$) ILC3s in ileal Peyer's Patches (94).

Overall, more metabolic signals remain to be identified that promote proper ILC localization in healthy and inflamed tissue. The use of lipid metabolites, rather than the exclusive use of genome-encoded proteins (chemokines), as guidance cues for ILCs within tissue might be advantageous for the host. Lipid metabolites can be rapidly produced and inactivated through enzymatic conversion, as exemplified by the GPR183 ligand 7α,25-dihydroxycholesterol since its synthesis from cholesterol is controlled by two enzymes, cholesterol 25-hydroxylase (CH25H) and 7α-hydroxylase (CYP7B1); and it can be further metabolized into bile acid precursors that lack chemotactic activity by the enzyme 3β-hydroxysteroid dehydrogenase type 7 (HSD3B7) (118). This allows tight regulation of 7α,25-dihydroxycholesterol abundance within tissue. Furthermore, lipid metabolites likely easily diffuse within tissue, thereby facilitating the generation of precise local chemotactic gradients. Finally, from the same precursor molecule, two bioactive metabolites with distinct functions can be generated: 25-hydroxycholesterol generated by CH25H from cholesterol has anti-viral and anti-inflammatory activity, whereas 7α,25-dihydroxycholesterol synthesized from 25-hydroxycholesterol by CYP7B1 regulates immune cell migration through GPR183 (118). This feature likely allows coordinated regulation of tissue-resident immune function by lipid metabolites.

ILC MIGRATION DURING INFLAMMATION

During infection and other tissue insults, ILCs must migrate to local sites of inflammation within tissue. For example, Neuropilin-1^{+} human LTi cells are present in inducible bronchus-associated lymphoid tissue (iBALT) in the inflamed lung in chronic obstructive pulmonary disease (COPD) (99). A recent study showed that, in Mycobacterium tuberculosis infection, ILC3s are recruited via the CXCL13-CXCR5 axis to the lung, thereby mediating the formation of iBALT associated with granulomas, which contributes to early control of infection together with the production of IL-17 and IL-22 (91). In addition, IL-17-producing ILC3s are present in the alveolar space in asthma patients (126). Similarly, ILC2s are increased in the broncho-alveolar lavage fluid of humans with idiopathic pulmonary fibrosis (127). Furthermore, it has recently been shown that ILCs are recruited into the inflamed skin in a CCR6-dependent manner (43). Finally, the accumulation of LTi-like ILC3s in mesenteric lymph nodes after helminth infection is dependent on CCR7-mediated trafficking (84).

The GPR183-oxysterol pathway also plays an important role in controlling ILC migration in inflamed tissue (119). Mobilization of ILC3s from cryptopatches into the surrounding tissue occurs during intestinal inflammation (60). We showed that the recruitment of ILC3s (and myeloid cells) to inflammatory foci in the colon is dependent on GPR183 (21) (**Figure 4**). It is reasonable to assume that increased oxysterol synthesis induced by tissue injury conveys perturbation of tissue homeostasis to the immune system, initiating ILC movement and the inflammatory response (119). It is currently unknown whether other metabolites produced in inflamed tissue regulate ILC migration and localization.

An important feature of ILCs is their ability to contribute to the repair of tissues damaged by infection, inflammation, and irradiation, which is likely dependent on their local migration and accumulation within damaged tissues. For example, LTi-like ILC3s restore lymphoid tissue architecture after viral infection (128), promote thymic regeneration after irradiation (129), and protect the intestine from graft vs. host disease-induced damage after hematopoietic stem cell transplantation (55). Similarly, ILC2s alleviate virus-induced damage to the lung (33).

Moreover, a recent study established the new concept of ILC inter-organ trafficking during inflammation. Specifically, it was shown that inflammatory IL-25-responsive ILC2s can migrate from the intestine to the lung during helminth infection to support host defense (41). The exit of ILC2s from the intestine into the blood via the lymphatic system was mediated by S1P (41), the critical factor regulating lymphocyte egress from tissues (112). CD69, expressed on tissue-resident ILCs, antagonizes S1PR1 through downmodulation of S1PR1 from the cell surface (116). In contrast, inflammatory ILC2s are $CD69^{-}$, allowing S1PR1-dependent egress into the circulation (41). A previous study in mice had found that intestinal NK cells, ILC1s, and ILC3s are $CD69^{hi}$, whereas ILC2s are $CD69^{lo}$, supporting the concept that intestinal ILC2s might be "less tissue-resident" than other intestinal ILC subsets (92). Finally, it has been suggested that signals from the local microbiota promote S1P receptor expression on ILC2s, thereby allowing them to exit the intestine (130).

It remains to be tested whether inter-organ trafficking of ILCs also occurs between other organs. In this context, it is

relevant that in various inflammatory conditions activated ILCs are found in peripheral blood. For example, human NKp44$^+$ ILC3s expressing homing receptors for skin and intestine appear in the circulation after conditioning for hematopoietic stem cell transplantation (131). Similarly, circulating ILC2s are increased in humans with asthma (132). These observations suggests that inter-organ trafficking might also occur in humans after mobilization of ILCs into the blood in response to inflammatory stimuli.

As outlined above, the recruitment of blood-borne ILCPs during infection may contribute to ILC heterogeneity within tissue. However, the signals activating blood-resident ILCs within tissue and recruiting them into the inflamed tissue from the circulation are unknown. This could involve tissue-derived signals sensed within the local vasculature and/or intra-tissue signals. Furthermore, the relative contribution of local expansion of resident ILCs vs. the recruitment of ILCPs to inflamed tissue is still unclear and may vary between tissues and the type of insult. For example, inflammatory signals could disrupt RANKL-RANK interactions, thereby allowing the local proliferation of CCR6$^+$ ILC3s (61).

ILC MIGRATION IN CANCER

Another largely unexplored area that warrants further investigation is ILC migration in cancer. The tumor microenvironment constitutes a unique metabolic milieu, resembling inflamed tissue. Among ILCs, NK cells are often the predominant population found in the tumor microenvironment, e.g., in human lung and colon cancer (30). Due to their cytotoxic activity, NK cells are a promising target for anti-cancer immunotherapy (133). However, in many human cancers, e.g., lung adenocarcinoma, NK cells are under-represented within the tumor compared to healthy tissue (134), especially the cytotoxic CD56^{dim} subset (135). This suggests that NK cell recruitment to tumors is suboptimal and targeting NK cell migration could be a valuable strategy in cancer immunotherapy. Several chemokines and their respective receptors mediating NK cell migration to tumors have been identified, such as CXCL8-CXCR1/CXCR2, CXCL10-CXCR3, CXCL12-CXCR4, and they are being explored as clinical targets (136).

Whereas, a role for NK cells in controlling cancer growth and metastasis has been well-established, the function of other ILC subsets in cancer, especially in regards to migration, is poorly understood (133). In several hematological malignancies, human ILC1s, ILC2s, and ILC3s are increased in the blood compared to healthy individuals (133), supporting the notion that ILCs can be mobilized into the circulation during malignancy. There is also some evidence that circulating human ILC2s could contribute to immunosuppression in gastric cancer (137).

Furthermore, ILC3s with likely LTi function are enriched in solid tumors both in mice and humans. For example, NKp46$^+$ ILC3s invade B16 mouse melanoma expressing IL-12 (138). It was shown in the same model that lymphoid tissue-resident (splenic) Rorc$^{fate-map+}$ ILCs have superior anti-tumor activity than intestinal or hepatic Rorc$^{fate-map+}$ ILCs and Rorc$^{fate-map-}$ ILC1s/NK cells (139). NKp46$^+$CCR6$^+$CXCR5$^+$ ILC3s with LTi properties are also enriched in tumor-associated tertiary lymphoid structures in human non-small cell lung cancer (140). Both studies showed that tumor-associated ILC3s upregulate adhesion molecules on the tumor vasculature, which likely promotes anti-tumor immunity through the recruitment of T cells. These tertiary lymphoid structures are of interest because of their importance for T cell-mediated anti-tumor immunity and their general positive prognostic value for cancer outcome, e.g., in lung cancer (141). However, tumor-associated lymphoid structures may also promote cancer growth. For example, one study reported that high amounts of CCL21 recruit CD4$^+$ LTi cells to tumors in a CCR7-dependent manner, which is associated with the formation of tumor-promoting lymphoid-like stroma in melanoma (89). In a mouse model of breast cancer, it was also shown that CCL21 recruits CCR7-expressing ILC3s with an LTi phenotype (CD4$^+$CCR6$^+$) to the tumor environment (90). Furthermore, CXCL13 was required for the clustering of CXCR5$^+$ ILC3s with mesenchymal stromal cells in the tumor microenvironment, which supported lymph node metastasis (90).

Interestingly, complementary to our findings in the intestine, it has recently been shown that oxysterol recognition through GPR183 is required for the development of iBALT (142), a common feature of active lung inflammation. However, this study did not determine the role of GPR183-expressing ILCs in this process. Overall, it seems plausible that the oxysterol-GPR183 pathway could also be involved in the formation of tertiary lymphoid structures in cancer.

CONCLUDING REMARKS

ILCs maintain healthy organ function and it is increasingly recognized that ILC function is critically dependent on their trafficking to and localization within tissues. Accordingly, ILC migration and the mechanisms of ILC tissue recruitment are areas that are beginning to be explored in more depth. It is important to comprehensively identify the guidance cues and receptors that control ILC localization and motility in tissues. In the long-term, cell surface receptors regulating ILC migration to inflamed or malignant tissues could serve as new therapeutic targets for human diseases.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Immunometabolic Checkpoints of Treg Dynamics: Adaptation to Microenvironmental Opportunities and Challenges

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In the last decades, immunologists have started to consider intracellular metabolism in relation with the dynamics and functions of immune cells, especially when it became clear that microenvironmental alterations were associated with immune dysfunctions. Regulatory T cells (Tregs) are equipped with a variety of immunological and metabolic sensors, and encompass circulating as well as tissue-resident cells, being therefore particularly susceptible to microenvironmental cues. Moreover, Tregs undergo metabolic reprogramming over the course of an immune response, allowing the use of alternate substrates and engaging different metabolic pathways for energetic demands. The study of metabolic mechanisms supporting Treg dynamics has led to puzzling results, due to several limitations, including the heterogeneity of population in the same tissues and between different tissues, the difficulty in considering all the interconnected metabolic pathways during a cellular process, and the differences between *in vitro* and *in vivo* conditions. Therefore, Treg reliance on different metabolic routes (oxidation rather than glycolysis) has been a matter of controversy in recent years. Metabolic reprogramming and altered bioenergetics are now identified as hallmarks in cancer, and are employed by cancer cells to determine the availability of metabolites and molecules, thus affecting the fate of tumor-infiltrating immune cells. In particular, the tumor microenvironment forces a metabolic restriction and a plethora of synergistic intrinsic and extrinsic stresses, leading to an impaired anti-tumor immunity and favoring Treg generation, expansion, and suppressive function. This leads to the understanding that Tregs and conventional T cells have different capability to adapt to metabolic hurdles. Considering the role of Tregs in dictating the outcome of tumor-specific responses, it would be important to understand the specific Treg metabolic profile that provides an advantage at the tumor site, to finally identify new targets for therapy. In this review, we will report and discuss the major recent findings about the metabolic pathways required for Treg development, expansion,

migration and functions, in relation to tissue-derived signals. We will focus on the adipose tissue and the liver, where Tregs are exposed to a variety of metabolites, and on the tumor microenvironment as the context where Tregs develop the ability to adapt to perturbations in nutrient accessibility.

Keywords: Treg, glycolysis, cancer, proliferation, mitochondria, oxydation

INTRODUCTION

Regulatory T cells (Tregs) are now recognized as a specialized CD4 T cell subset essential for immune homeostasis, as well as for protection from autoimmunity and excessive inflammation. In several mouse models, transient Treg depletion early in life, or congenital Treg deficiency, leads to the spontaneous development of lethal multiorgan autoimmune disorders (1, 2). In humans, a wide array of Treg defects, ranging from frequency to function to proliferative potential, have been reported in several autoimmune diseases, and therapies aiming at recovering physiological Treg activities (such as adoptive Treg cell therapy) are under development for these conditions (3). Conversely, increased Treg proportions can be found in the tumor microenvironment in many tumor types in both mouse models and human patients, correlating with a poor prognosis [with a few exceptions like colorectal cancer (4)]. Therefore, Treg depletion or blockade is now considered as a necessary step to elicit effective anti-tumor immunity (5). Recent data have revealed that CTLA-4, the first "immune checkpoint" to enter the clinic as cancer immunotherapeutic agent, is more expressed by Tregs than effector T cells in peripheral lymphoid organs and in blood and even more at the tumor site, and that anti-CTLA-4 antibodies may work through antibody-dependent cell-mediated cytotoxicity and Treg depletion (6–9); this finding proves the key role of Tregs as a non-redundant and even dominant immune checkpoint in the tumor microenvironment. Therefore, a deeper understanding of the most important pathways and molecules involved in Treg expansion, survival and contraction is urgently needed in order to design better therapies aiming at Treg manipulation *in vivo*.

We have recently started to appreciate the complexity of Treg dynamics, from their development to their rapid adaptation to microenvironmental and systemic changes. More importantly, in the last decades, we have started to take into consideration intracellular metabolism of T cells in relation to their dynamics and functions during immune responses, a concept known as cellular "immunometabolism" (10). Tregs can be considered a very peculiar CD4 T cell subset, since they physiologically reside in virtually all tissues and organs, and constitutively express a wide array of immune as well as metabolic sensors. Therefore, Tregs are equipped to promptly respond to any immune and metabolic signal in an "innate-like" fashion, even though we have not completely elucidated the consequences of immunometabolic signals in Treg activities. Based on their high sensitivity to external cues and on their fundamental role in switching between tolerance and immunity, Tregs can be considered as one of the key links between nutrient sensing and immune response, a mechanism selected by evolution to optimize energetic resources (11).

TREGS SWITCH BETWEEN QUIESCENCE AND PROLIFERATION IN MANY PHASES OF THEIR DEVELOPMENT AND FUNCTIONS

When CD25+ Tregs were discovered, they were originally described as anergic cells, based on their inability to proliferate *in vitro* in response to T cell receptor (TCR) stimulation and in the absence of exogenous IL-2 (12). What is more, anergy appeared as a prerequisite for suppressive function, since Tregs seemed to lose their suppression in condition of anergy reversal (12). Not only was anergy thought to be required for Treg function, but it seemed also involved in Treg differentiation. Indeed, since the very first experiments *in vitro*, the conversion of conventional T cells (Tconvs) into Tregs was favored in conditions of tolerogenic or sub-immunogenic stimulation that induced suboptimal levels of proliferation (13, 14). All these data contributed to consolidate the idea of anergy as a key component of the Treg identity. However, the observations that *de novo* induced Tregs can massively proliferate and that Tregs could preserve their suppressive function while proliferating have challenged this notion (14, 15). It is now recognized that, in many contexts, proliferation is not only involved but even required to ensure a full suppressive function by Tregs.

In the last decades, a large amount of data have clarified the requirement of active proliferation throughout the stages of Treg development and activation in mice, whilst the knowledge of these events remains still elusive in humans. A subset of Tregs, probably accounting for the majority of Tregs in lymphoid organs of naïve animals, develop in the thymus upon encountering self-antigens, and are called thymic Tregs (tTregs). A certain proportion of Tregs can develop in peripheral organs in response to non-self-molecules such as commensal and food antigens, are thus highly represented in the intestine, and are called peripheral Tregs (pTregs) (16). To date, no reliable markers are available to dissect the actual contribution of thymic vs. peripheral developmental routes to the Treg pool; however, several protocols have been developed to induce Treg differentiation *in vitro* (of so-called iTregs) from Tconvs, which recapitulate some features of Treg induction *in vivo*.

In the neonatal life, early after development, tTregs undergo a massive wave of proliferation that is probably their first proliferative burst. In mice, a distinct pool of Tregs largely expands in the perinatal life, persists longtime, and plays a vital

role in suppressing autoimmunity (17) through the induction of T cell anergy (18). These perinatal Tregs presented very high proportions of Ki67-positive and EdU-incorporating cells, and DNA replication was one of the top pathways emerging from their transcriptomic profile. A similar expansion of Tregs has been detected in the human peripheral blood during early neonatal life, probably in response to the immediate exposure to commensals (19). Tregs display a phenotype that is compatible with recent activation in healthy human neonates, while during neonatal sepsis they may play a role in controlling the clinical manifestations of the disease (20).

Following development, Tregs recirculate throughout the blood and populate lymphoid as well as non-lymphoid organs; in the latter, tissue-resident Tregs acquire a tissue-specific molecular profile and specialized functions (21). This polarization from a central (cTreg) into an effector (eTreg) status occurs in response to antigen stimulation as well as local inflammatory stimuli, and follows the activation of specific molecular programs driven by transcription factors like NFkB, Blimp and IRF4 (22). Tissue-resident Tregs are thought to play not only the well-known immunosuppressive functions, but also to exert some non-immune activities such as maintenance of tissue homeostasis and promotion of tissue regeneration upon injury (23). Whether Tregs undergo further rounds of division within tissues, and whether this event is required to become specialized resident cells or to ensure their long-term persistence, is still unclear. Some tissues are characterized by relatively high frequencies and numbers of Tregs in physiological conditions, such as the bone marrow (24) and the visceral adipose tissue (VAT) of lean adult mice; at least in the latter case, it has been clearly demonstrated that active proliferation, coupled with enhanced survival, sustains the physiological Treg expansion in this tissue (25, 26). Several data confirm the idea that human circulating Tregs contain a very high proportion of cycling cells (27, 28) even though the role of proliferation in the physiological homeostasis of human Tregs remains to be clarified.

In pathological conditions, for instance following acute tissue injury, the Treg population may expand thanks to the concomitant recruitment of cTregs that are locally differentiated into eTregs, to the local development of pTregs, and to the proliferation of resident and recruited Tregs (29). Thanks to their proliferative response and effector polarization, Tregs can promote the resolution of the injury through the suppression of inflammatory processes and the release of tissue-repairing molecules. However, when the source of damage is not eradicated, such as in the case of viruses establishing chronic infections or in the case of cancer, the Treg pool may continue to expand in a chronic fashion, until it even subverts the protective anti-viral or anti-tumor immunity (30).

All these pieces of evidence demonstrate that, during their life time, Tregs may continuously switch between quiescent survival and active replication and back; this switch may occur when Tregs are in a naïve status without compromising their "naiveness" as in the case of perinatal Treg expansion, or may occur during the physiological specialization of tissue-resident Tregs or the pathological polarization of effector Tregs in damaged tissues (**Figure 1**). Such highly dynamic behavior implies that Tregs may be particularly able to switch their metabolism depending on the immune signals that they receive and the nutrient availability in different contexts.

THE METABOLISM OF CONVENTIONAL T CELLS: HOW DO TREGS FIT WITH THIS MODEL?

Thanks to advances in the field in recent years, we have now recognized that T cells, probably the cells in the body capable of the largest clonal proliferation, strictly rely on metabolic switches to sustain their immune functions. Three main metabolic profiles can be generally assigned to the main stages of T cell activation: first, mature naïve T cells are thought to survive in a quiescent state thanks to a low level of oxidative phosphorylation and mitochondrial respiration; second, T cell stimulation through the TCR and costimulatory receptors primes a strong switch from an oxidative to a glycolytic-lipogenic metabolism, characterized by increased uptake of glucose (and other nutrients) from the extracellular environment, generation of ATP from substrate-level phosphorylation during glycolysis, conversion of pyruvate into lactate, and biosynthesis of macromolecules for cell growth and division; third, memory T cells are mostly oxidative cells, yet they are "metabolically primed" for a glycolytic switch, a combination that ensures both long-term survival and prompt response to an antigen recall (31, 32).

The glycolytic-lipogenic and the lipolytic-oxidative pathways are generally viewed as mutually exclusive, thanks to the reciprocal regulation operated at several checkpoints along the metabolic flux. As an example, in poor nutrient supply conditions and other stress conditions, the AMP-activated protein kinase (AMPK) is activated and negatively regulates the mammalian target of rapamycin (mTOR), thus inhibiting a variety of biosynthetic pathways such as fatty acid synthesis (mediated by acetyl-CoA carboxylase or ACC) and cholesterol synthesis. A reciprocal regulation also exists between fatty acid synthesis and oxidation, since malonyl-coA (the product of ACC reaction) is a major inhibitor of carnitine palmitoyltransferase 1a (or CPT1a, the key enzyme of fatty acid oxidation). Therefore, cells are thought to oscillate between an mTOR-driven anabolic metabolism and an AMPK-driven catabolic metabolism. However, the two programs have been found to coexist in selected conditions. Memory T cells, indeed, maintain the ability to uptake and convert glucose into fatty acids, and then perform cell-intrinsic lipolysis to fuel mitochondrial oxidation, in an apparently "futile" cycle whose implications have not been completely understood (33).

A correspondence between the immune status and the intracellular metabolism can be achieved thanks to many immune-metabolic links in signaling pathways in T cells: to mention just a few examples, TCR and CD28 stimulation directly activates mTOR, a major orchestrator of the glycolytic switch (34); CD28 primes mitochondrial fatty acid oxidation, thus driving proper memory development (35); the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), when not

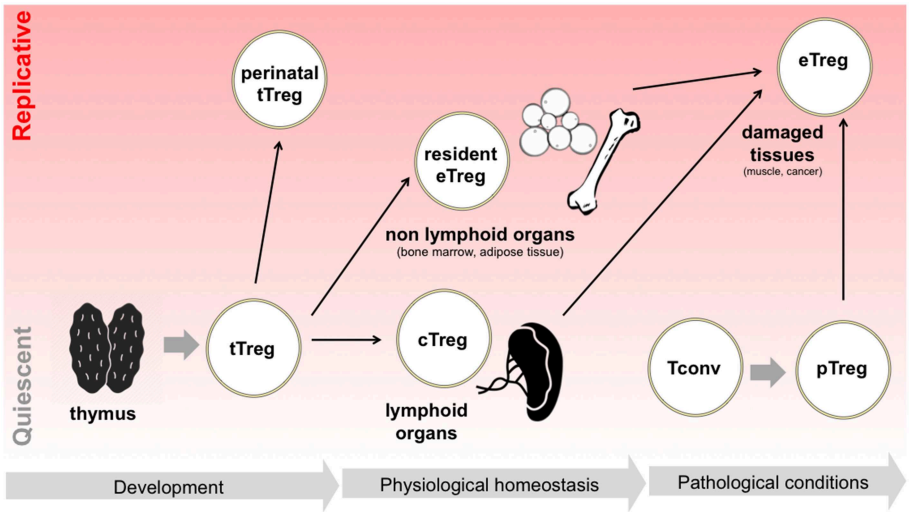

FIGURE 1 | Tregs switch between quiescence and replication in different developmental and activation phases. Following tTreg development, a perinatal wave of Treg replication occurs, which is thought to suppress T cell responses in neonatal life. Later in life, in physiological conditions, Tregs colonize both lymphoid organs, becoming cTregs, and non-lymphoid organs, becoming resident Tregs and acquiring eTreg phenotype: in the latter case, extensive Treg proliferation has been reported, for instance in adipose tissue. Finally, in pathological conditions such as in the case of acute tissue damage, Tregs are locally expanded, thanks to the proliferation of resident eTregs, to the differentiation of cTregs, and to the conversion of pTregs.

engaged in glycolysis, represses post-transcriptionally IFN-γ expression (36); and the tumor necrosis factor receptor-associated factor 6 (TRAF6), an adaptor that can be activated by some receptors of the tumor necrosis factor receptor (TNFR) superfamily, fosters memory development through mitochondrial fatty acid oxidation (37).

How can Tregs be categorized according to this scheme? The answer is still unclear: indeed, some data describe Tregs as mostly oxidative cells, while other data demonstrate the need of glycolysis for some Treg activities. One of the main reasons explaining these conflicting results may be the type of Tregs (tTregs vs. pTregs) that is analyzed in different studies: on the one side, pTregs seem to differentiate when effector T cell activation, which relies on the glycolysis-lipogenesis pathway, is blocked. Conversely, tTregs seem to depend on this pathway for their proliferation and fitness, resembling effectors (38); however, both tTregs and pTregs can either remain quiescent or enter cell cycle depending on the surrounding signals, and this can profoundly affect their metabolism. Therefore, probably the most important reason dictating their metabolic requirements is the stage of development and/or activity at which Tregs are analyzed; indeed, opposite results can be obtained when analyzing iTregs/pTregs during their differentiation from Tconvs, or when analyzing already established Tregs (irrespective of their peripheral or thymic origin) during their active proliferation. In turn, Tregs can be studied directly *ex vivo* from many different tissues and sources, or after many different types of stimulation and culture *in vitro*; this experimental heterogeneity may profoundly affect the outcome of metabolic analyses. Finally, the CD25high or Forkhead Box P3 (Foxp3) + compartment is actually a heterogeneous mixture of different subsets, which include cycling as well as resting Tregs, and also cells with an unstable regulatory phenotype.

Here we will report and discuss the main findings regarding Treg metabolism, classified according to whether they refer to *de novo* Treg induction, to the proliferation of previously established Tregs, or to different Treg activities.

TREG INDUCTION, PROLIFERATION AND FUNCTION RELY ON DISTINCT METABOLIC PATHWAYS

Metabolic Pathways Involved in Treg Induction

The first evidence connecting Treg induction to an oxidative metabolism came from the study of Michalek et al., where iTregs were differentiated through the classical protocol based on transforming growth factor β (TGFβ) exposure, or through mTOR inhibition with rapamycin; in both settings, etomoxir (at 200 μM), known as an inhibitor of fatty acid oxidation, could suppress iTreg polarization (39). Compared to T helper subsets polarized *in vitro* with specific cytokine cocktails, TGFβ-induced iTregs expressed lower levels of the glucose transporter 1 (GLUT1), performed less glycolysis and more fatty acid oxidation, and were not affected by supplementation of exogenous fatty acids (39). Both iTregs and so-called "natural" Tregs contained higher levels of phosphorylated AMPK, and metformin administration increased Treg frequency *in vivo* (39).

Subsequently, several studies have contributed to generate the hypothesis that Treg differentiation relies on a switch from glycolytic-lipogenic to oxidative metabolism. A large amount of data derive from the analysis of iTreg polarization *in vitro*, induced through standard protocols of anti-CD3/anti-CD28 stimulation in the presence of TGFβ. In this setting, iTregs display lower glycolytic rates and higher oxygen consumption

compared to T helper (Th) 1 and Th17, and their metabolic and transcriptional profile is suggestive of higher fatty acid oxidation (40–44). In these cultures (actually containing Foxp3+ and Foxp3- cells in some studies), the differentiation of iTregs was reduced in the presence of the electron transport inhibitor rotenone (irrespective of their proliferation) (43); conversely, the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) or the mTOR inhibitor rapamycin (45), or the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (46, 47), enhanced iTreg differentiation. On the side of lipogenesis, the genetic or pharmacological blockade of fatty acid synthesis enhanced iTreg polarization at the expense of Th17 cells (42).

Key metabolic "checkpoints" have been identified that affect iTreg differentiation by tipping the balance between glycolytic and oxidative metabolism. The first step of this axis is represented by glucose uptake, operated by several glucose transporters and in particular by GLUT1. When its expression was ablated in the T cell lineage, effector T cells displayed defective growth, proliferation and survival; however, Treg numbers were not affected, either in naïve mice or under inflammatory conditions (48). Downstream glucose capture, mTOR is a major sensor of environmental cues including immune signals and nutrient availability, and is also a major orchestrator of the glycolytic-lipogenic switch required for cell growth and proliferation; not only mTOR inhibition enhances iTreg differentiation (49, 50), but also mice carrying T cell-restricted mTOR deficiency show impaired T helper cell expansion, with enhanced Treg induction, *in vitro* and *in vivo*, in a model of viral infection (51). Downstream mTOR, the transcription factor HIF1α is activated and initiates a glycolytic program in T cells that is required for Th17 polarization at the expense of iTreg induction (45, 52). Pyruvate dehydrogenase (PDH), whose activity is negatively regulated by the pyruvate dehydrogenase kinase 1 (PDK1), dictates the fate of pyruvate between conversion into lactate or into acetyl-CoA; this axis was identified as a key regulatory node for Th17 and iTreg alternative polarization *in vitro* (43). Finally, the acetyl-coA carboxylase 1 (ACC1), a key enzyme for fatty acid synthesis, was shown to switch the polarization fate between iTregs and Th17 (42).

These findings strengthened the notion that iTreg/pTreg differentiation was favored in conditions of oxidative metabolism and was antagonized when the glycolytic-lipogenic pathway was fueled. However, some considerations should be made when interpreting these results, especially the studies performed in mouse models. First, most data come from mice carrying genetic ablation of key genes in the entire T cell lineage, thus effector T cell development, homeostasis and activation may be suboptimal or defective. In these settings, some of the Treg alterations, especially those observed *in vivo*, may be a consequence of impaired effector T cell homeostasis or activation, rather than regarding selectively iTreg/pTreg induction. Second, most data have been obtained in contexts of antigen immunization, autoimmune or inflammatory diseases: for instance, Foxp3+ cells (probably a mixture of tTregs and pTregs) did not upregulate proteins related to glycolysis, contrary to Th17 cells, in lymphoid organs of mice with experimental autoimmune encephalomyelitis (EAE) (43). Other examples are that administration of 2-DG and metformin increased the frequency of Foxp3+ cells in splenocytes of mice immunized with a model antigen (53), and that mice lacking HIF1α or ACC1 in the CD4 T cell lineage showed higher Treg frequencies and were more resistant to EAE (42, 52). These inflammatory conditions generally do not favor massive induction of expansion of Tregs, and thus do not allow studying Treg metabolism in a dynamic context. Third, discriminating the metabolic requirements of newly induced pTregs, compared to preexisting tTregs undergoing activation during disease, may be quite difficult *in vivo*.

Regarding the *in vitro* studies, most conclusions have been drawn from experiments based on the classical protocol of TGFβ culture. In this setting, iTreg induction appears as a "backup plan" when other differentiation programs are not allowed. Since T helper polarization usually requires T cell growth supported by a glycolytic-lipogenic switch, T cells may sense metabolic restriction as a signal of functional anergy, which consequently leads to Foxp3 induction and initiates a regulatory program. Supporting this idea, the increase of iTreg polarization induced by some metabolic inhibitors like the ACC1 inhibitor soraphen A, the mitochondrial blocker rotenone (41–43), or appearing in mTOR-deficient cells (51), was coupled to a suppression of T cell proliferation; conversely, higher T cell proliferation was induced by the AMPK agonist AICAR along with lower iTreg induction (46). Therefore, these results suggest the existence of a link between metabolic and immune anergy, of which Foxp3 induction may be an early consequence (54). At later stages, Foxp3 may stabilize this profile; indeed, ectopic Foxp3 expression is necessary and sufficient to induce a switch from glycolysis to oxidation (40, 43, 44) (**Figure 2**).

Recent data have clearly demonstrated that TGFβ has the intrinsic property of suppressing glycolytic metabolism in Tconvs undergoing transition into iTregs, as well as in tTregs, which would be otherwise highly glycolytic cells (56). Therefore, TGFβ-based protocols for iTreg polarization may provide limited information on the physiological mechanisms of iTreg differentiation. Indeed, strikingly different results have been obtained when human iTregs have been differentiated *in vitro* in a TGFβ-independent fashion, under conditions of suboptimal (weak and short) CD3/CD28 stimulation (55). In this setting, a population of CD25high cells developed, enriched in Ki67+ and FOXP3+ cells, in which the induction of high levels of FOXP3 was suppressed by 2-DG and enhanced by etomoxir. Two splicing isoforms of the human FOXP3 protein exist differing for the presence of the exon 2 and conferring different suppressive properties and lineage stability: the glycolytic enzyme enolase-1 was found recruited to the FOXP3 locus to repress FOXP3 expression, especially when 2-DG treatment inhibited glycolysis thus disengaging glycolytic enzymes. Impaired glycolysis and diminished expression of the FOXP3 isoform containing exon 2 were observed in Tconvs obtained from multiple sclerosis patients in correlation with their lower rates of conversion into iTregs (55). This study challenged the idea that iTreg differentiation was antagonized in conditions of high glycolytic rates; rather, it demonstrated that a proper metabolic activation of Tconvs, which included competence for

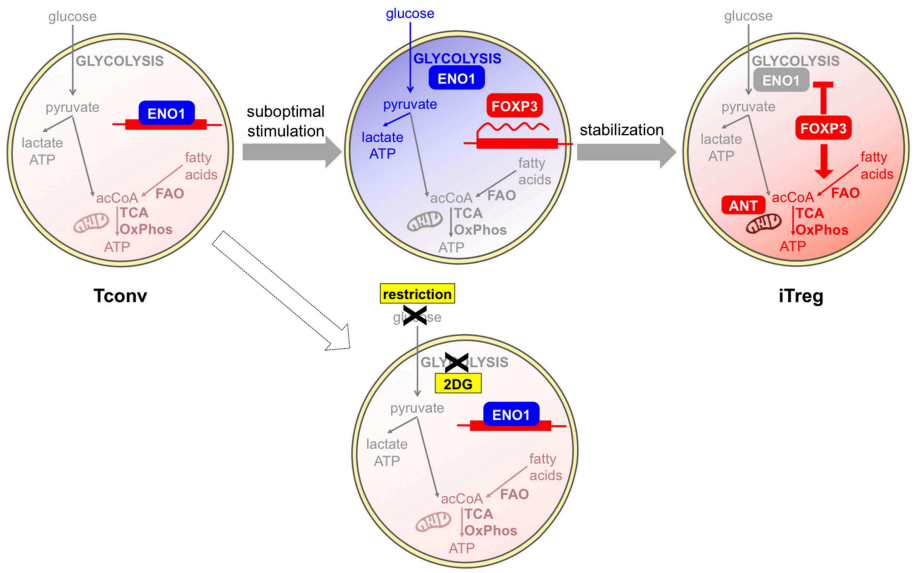

FIGURE 2 | Glycolysis and oxidation may be involved in different phases of iTreg development. Tconvs show a basal metabolism that is based on fatty acid oxidation (FAO) and the usage of acetyl CoA (acCoA) to fuel the TCA cycle and oxidative phosphorylation (OxPhos). Human Tregs can be differentiated *in vitro* from Tconvs following suboptimal antigen stimulation. This challenge induces a shift from the basal oxidative (light red) to the glycolytic (blue) metabolism: as a consequence, the glycolytic enzyme enolase-1 (ENO1) is engaged in the glycolytic cascade and thus displaced from the FOXP3 locus, where the FOXP3 gene can be transcribed. If glycolysis is blocked by a pharmacological inhibitor, or is impeded in conditions of glucose restriction, enolase-1 continues to repress FOXP3 transcription and iTreg development cannot occur (55). At later stages, FOXP3 itself can induce a stabilization of the iTreg program and a rescue of a quiescent state, through the enforcement of mitochondrial metabolism (red) that is mediated by an upregulation of mitochondrial proteins (44). A proper mitochondrial function is under the control of the ANT transporter and is required for iTreg induction (47).

glycolysis, was a prerequisite for proper iTreg development and that the metabolic requirements for iTreg differentiation were not antagonistic but rather parallel to the metabolic requirements for effector T cell activation. Based on this view, it could be hypothesized that regulation is allowed to develop along with immunity in conditions of optimal nutrient availability and competence for nutrient usage (**Figure 2**). Also in mouse models, weak TCR signal strength has been proven to preferentially promote Treg induction and/or expansion (13, 14, 57); however, one study has shown that low-dose antigen stimulation was not associated with strong mTOR activation (57). Therefore, differences in mTOR involvement and metabolic requirements may exist between human and mouse Treg induction under weak antigen stimulation.

Some compounds that have been used in many immunometabolism studies, such as 2-DG and etomoxir, have displayed some "off-target" effects that were not recognized before. In macrophages, 2-DG has been shown to block not only glycolysis but also oxidative phosphorylation and ATP production (58). Etomoxir, when used at low doses, blocks the oxidation of long-chain fatty acids through the inhibition of CPT1a, the rate-limiting enzyme for this process. However, when used at high doses, it can block mitochondrial respiration directly, irrespective of the nutrient (glucose, glutamine or fatty acids) fueling oxidative phosphorylation in T cells (47). Also in macrophages, high concentrations of etomoxir can display CPT1a-independent effects that are mediated by the depletion of intracellular free coenzyme A (59). Therefore, results obtained with the use of these pharmacological inhibitors *in vitro* should be interpreted with caution, while approaches based on the genetic ablation of key metabolic enzymes may probably shed light on the exact metabolic requirement for Treg differentiation (60). Accordingly, when CPT1a was genetically abolished selectively in the T cell lineage, TGFβ-driven iTreg polarization *in vitro* was not affected, and Treg frequency was found to be normal *in vivo* in these mice in physiological conditions. Instead, etomoxir at high doses suppressed iTreg polarization through the inhibition of adenine nuclear translocator (ANT), a transporter that affects ATP concentration in the mitochondrial matrix, the mitochondrial membrane potential, and the activity of the electron transport chain (47). These data questioned the assumption that fatty acid oxidation was a driving force for iTreg/pTreg differentiation. Together with data from De Rosa et al. (55), these results support the idea that Treg development from converting T cells may require the optimal activation of multiple routes, which may not involve long chain fatty acid oxidation but does include mitochondrial respiration.

Metabolic Pathways Involved in Treg Homeostasis and Proliferation

As summarized above, several findings indicate that Tregs actively proliferate during their lifetime and that the Treg population may comprise a high proportion of cycling cells, from many districts, in physiological and pathological conditions.

Their hyporesponsiveness *in vitro*, which has been interpreted as a functional and metabolic "anergy," may rather derive from a functional and metabolic activated status, making them refractory to strong stimulation in culture (11). Thus, it is reasonable to suppose that Tregs rely on a glycolytic-lipogenic metabolism for their fitness and their proliferative bursts (**Figure 3**).

Several data regarding freshly isolated human and mouse Tregs (likely comprising a mixture of tTregs and pTregs) confirm this hypothesis. Procaccini et al. have shown that, contrary to conventional T cells, Tregs show constitutively active mTOR directly *ex vivo*: a transient mTOR inhibition, achieved through rapamycin administration or through nutrient starvation and interruption of the leptin/leptin receptor signal, turns these cells from anergic into highly proliferative cells both *in vitro* and *in vivo*. Thus, it has been proposed that an "oscillatory" mode of mTOR activity is responsible for the exit of Tregs from a hyporesponsive into a proliferative status (61). These findings have been confirmed also by Gerriets at al. who showed that Ki67 high Tregs from the spleen of naïve mice express higher levels of GLUT1 and mTOR activity (62). A proteomic and biochemical profile of freshly extracted human Tregs has revealed that these cells are highly glycolytic *ex vivo* and utilize both glycolysis and fatty acid oxidation for their proliferation *in vitro* (as assessed with the use of 2-DG or etomoxir). In contrast, effector T cells are mostly oxidative *ex vivo* but rely on glycolysis when cultured *in vitro* (72). In line with these findings, others have found that freshly extracted human Tregs express high levels of genes related to glycolysis and lipid metabolism, and capture glucose at high efficiency (73, 74).

The pivotal role played by mTOR in Treg expansion has been demonstrated in several models. Mice carrying the Treg-intrinsic ablation of mTORC1 or mTOR spontaneously developed a severe scurfy-like autoimmune and inflammatory systemic disease (63, 67). Even if Treg numbers appear normal in mice with Treg-restricted mTORC1 deficiency, these cells displayed a severely impaired competitive fitness *in vivo*, which was accompanied by a decreased glycolytic rate, and which explained their defective suppression at the systemic level (63). Notably, the cholesterol synthesis was the most affected pathway by mTORC1 deficiency, and mTORC1-deficient Tregs failed to incorporate efficiently glucose-derived carbons into lipids; these findings demonstrate that Tregs utilize an mTOR-dependent glycolysis-lipogenesis for their expansion (63).

Much effort has been made to elucidate the role of fatty acid oxidation and mitochondrial metabolism in Treg expansion and homeostasis. Liver kinase B 1 (LKB1) is a bioenergetic sensor that phosphorylates AMPK and thus triggers oxidative catabolism, thus allowing cell survival under stress conditions. Mice lacking LKB1 specifically in Tregs display spontaneous type-2 inflammatory disease caused by defective Treg development and survival (64, 65, 71). Notably, LKB1-deficient Tregs display transcriptomic and metabolomic profiles compatible with impairment in tricarboxylic acid cycle, mitochondrial function and fatty acid oxidation (64, 65). The activity of LKB1 was not mediated by AMPK, since the Treg-restricted deficiency of AMPKα1 and AMPKα2 did not affect Treg numbers and did not induce any spontaneous inflammatory disorder (64, 65). Fatty acid oxidation was also not required for the physiological Treg development; according to data obtained in mice bearing a genetic CPT1a deficiency specifically in Tregs, CPT1a was dispensable for Treg development, Foxp3 expression, and suppressive function (47). Therefore, the AMPK-driven fatty acid oxidation does not seem to be involved in Treg homeostasis in physiological conditions.

When the mitochondrial complex III was genetically ablated in Tregs, a scurfy-like disease was observed, characterized by apparently normal Treg numbers but defective Treg competitive fitness and Treg suppression at the systemic level (66). Similar findings have been obtained in mice bearing a Treg-specific deletion of mitochondrial transcription factor A (TFAM), which is essential for electron transport chain activity: here a specific defect in eTreg development was identified (67). In complex III-deficient mice, the Treg defect was accompanied by diminished oxygen consumption, concomitantly increased glycolysis, and lower NAD+/NADH ratio (an index of the electron transport chain activity). However, the metabolic alterations observed in these mice might affect Tregs indirectly, through a novel metabolic-epigenetic circuitry; loss of mitochondrial complex III results in higher content of two metabolites, 2-hydroxyglutarate and succinate, known to inhibit DNA demethylases, and indeed complex III-deficient Tregs show DNA hypermethylation and altered gene expression (66).

Metabolic Pathways Involved in Treg Activities: Migration, Suppression, and Stability

In both physiological and pathological conditions, circulating or lymphoid Tregs can undergo further functional and metabolic reprogramming while accomplishing their functions: indeed, Tregs can be attracted into specific tissues where they can experience further rounds of proliferation and/or functional specialization, can exert specific activities such as immune regulation and tissue repair, and can corroborate or instead destabilize their regulatory program, depending on the microenvironmental signals (29, 75, 76). Whether Tregs need to activate specific metabolic pathways to accomplish each of these tasks has not been completely elucidated. However, many studies (mentioned below) suggest that some "division of labor" may exist between different metabolic pathways in Tregs: on the one side, glucose usage and glycolysis are required for migration and rather antagonize Treg stability and suppressive function; on the other side, oxidative phosphorylation may be needed for Treg immune suppressive function (**Figure 3**).

The role of glycolysis in Treg migration has been demonstrated in several settings. Compared to wild-type Tregs, HIF1α-deficient Tregs show lower glycolysis and higher oxidative phosphorylation of both fatty acids and glucose, when cultured in normoxic or hypoxic conditions; functionally, HIF1α-KO Tregs suppress CD8 T cell proliferation *in vitro* with higher potency under hypoxia but display impaired migratory ability *in vitro* and *in vivo*. In mice with a Treg-restricted HIF1α deficiency, the reduced Treg recruitment to the site of

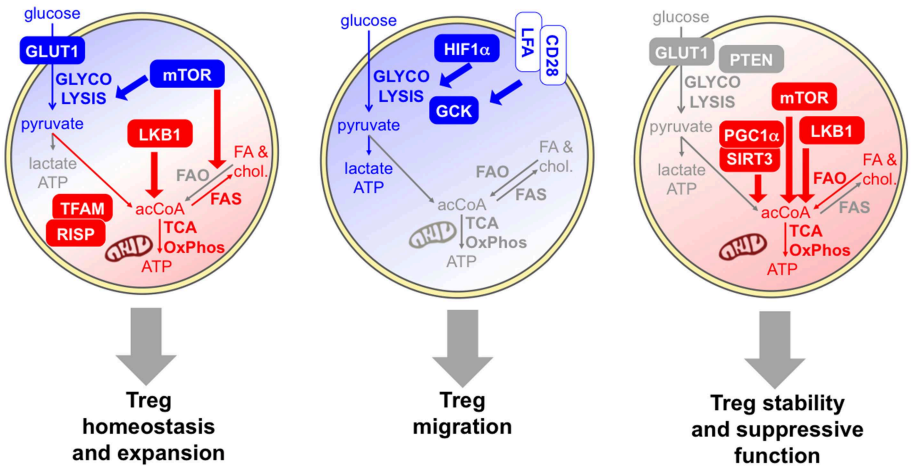

FIGURE 3 | Specific metabolic pathways and key molecules are involved in distinct Treg activities. Several data indicate that Tregs rely on a combination of glycolytic (blue) and mitochondrial (red) metabolism for their homeostasis and expansion. Proliferative Tregs express GLUT1 and constitutive mTOR (61, 62), and Tregs require an mTOR-dependent glycolysis-lipogenesis for their expansion (63). Indeed, fatty acid synthesis (FAS) and cholesterol synthesis occur. LKB1 is required for Treg development and survival through the support of mitochondrial function (64, 65). Evidence that mitochondrial activities are required for Treg fitness comes from experimental models of deficiency of mitochondrial complex III (RISP) (66) or mitochondrial transcription factor A (TFAM) (67). However, FAO may not be required for Treg development (47). The key role of glycolysis (blue) in Treg migration is demonstrated by the evidence that HIF1α-deficient Tregs show lower glycolysis and display impaired migratory ability *in vitro* and *in vivo* (68), and by the observation that pro-migratory stimuli, like LFA1 or CD28, enhanced glucose uptake and glycolytic rate while promoting migration, through the induction of the glycolytic enzyme GCK (69). Regarding Treg suppressive function and stability, some data indicate a glycolytic boost may compromise Treg suppressive function and stability *in vivo*: this event occurs in mice lacking PTEN selectively in the Treg lineage (70) and in transgenic mice overexpressing GLUT1 (62). Other studies support the idea that mitochondrial activity (red) is required for Treg suppressive function: key molecules involved in this regulation are SIRT3/PGC1α (41) the kinase LKB1 (64, 71), and mTOR, which promotes the transition from cTregs into eTregs via mitochondrial activation (67).

a growing glioma leads to significantly delayed tumor growth and longer survival (68). This study suggests that HIF1α, and thus oxygen tension in the microenvironment, may dictate the balance between migration, relaying on glycolysis, and Treg suppressive function, at least *in vitro*. However, it does not seem to affect the physiological Treg development in the thymus, Treg peripheral fitness, or Treg proliferation *in vitro* or *in vivo*, probably because these processes occur in normoxic microenvironments. Kishore et al. have demonstrated the role of glycolysis in Treg migration and dissected the underlying molecular mechanisms: pro-migratory stimuli, like engagement of Lymphocyte function-associated antigen 1 (LFA-1) or of the costimulatory molecule CD28, enhanced glucose uptake and glycolytic rate while promoting migration. These events were mediated by mTORC2 and culminated in the induction of glucokinase (GCK), a hexokinase isoenzyme that interacts with actin and acts as a glycolytic ATP supplier for cytoskeletal rearrangements and cell migration (69). Notably, GCK-silenced Tregs did not show any defect in proliferation or suppression *in vitro*, and their compromised suppressive function *in vivo* could be ascribed to defective recruitment to the inflamed site. These results support the hypothesis of a dichotomy between glycolysis and oxidative phosphorylation being required for distinct activities of Tregs, migration and suppressive function, respectively.

Some pieces of information support the idea that a glycolytic boost may selectively compromise Treg suppressive function and stability *in vivo*, apparently without affecting their development. Mice lacking Phosphatase and tensin homolog (PTEN) selectively in the Treg lineage spontaneously develop systemic lymphoproliferation and lupus-like disease with age, despite increased Treg frequencies. Indeed, PTEN-deficient Tregs lost their suppressive function *in vivo* in the EAE model and displayed signs of functional instability, i.e., the release of pro-inflammatory cytokines in the inflamed site (70). Notably, this "fragile" phenotype correlated with higher glycolytic rates (but normal oxidative profile) of PTEN-KO Tregs compared to control Tregs, at short time (70) but not at later time points (56) after *in vitro* activation. Similar conclusions have been drawn from the analysis of Tregs in transgenic mice overexpressing GLUT1: also in this model, which develops again spontaneous autoimmunity, Tregs were more glycolytic, were expanded in numbers, but were less suppressive and more fragile *in vivo* (62). It may be speculated that glycolysis may promote the proliferation of Tregs that, instead of performing classical immune suppressive functions, are skewed toward a tissue-repair program, which may become relevant in specific conditions and microenvironments *in vivo*. It is important to consider that Treg uptake of glucose may induce T cell suppression by itself, irrespective of the Treg-intrinsic metabolic pathways. Indeed, competition for glucose is a key element for T cell activation especially under glucose restriction, and the proficiency of Tregs to internalize glucose leads to an induced senescence in surrounding T cells (74).

Conversely, other studies support the idea that mitochondrial activity may be required for Treg suppressive function. Tregs have greater mitochondrial mass and reactive oxygen species (ROS) production than conventional T cells, and Tregs

lacking sirtuin 3 (SIRT3) or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), both essential for mitochondrial activities, display weakened suppressive functions *in vitro* and *in vivo* (41). The deletion of histone deacetylase 9 (HDAC9) increases in Tregs the expression of genes related to oxidative phosphorylation (including SIRT3 or PGC1α), enhancing mitochondrial respiration; these cells also display increased suppressive function *in vitro* (41). Further studies have identified the complex I of the electron transport chain as an important element for Treg suppressive function (40). The kinase LKB1, which induces a transcriptional program oriented to mitochondrial metabolism (64, 65), is not only required for proper Treg development and survival, but also implicated in maintaining Treg stability and suppressive function (64, 71), further supporting the idea that proficiency for oxidative phosphorylation may be a prerequisite for Treg suppression. Recent data have revealed that mTOR not only drives Treg expansion through a glycolytic-lipogenic program (63), but may also support Treg suppressive function promoting the transition from cTregs into eTregs, via mitochondrial activation (67). *In vitro*, Treg suppressive function was reduced if Tregs were preactivated under acute pharmacological mTOR inhibition; *in vivo*, the acute deletion of mTOR in Tregs led to spontaneous loss of immune tolerance (67). Notably, mTOR activation was required for the acquisition of an eTreg phenotype through the post-transcriptional regulation of Interferon regulatory factor 4 (IRF4) expression. Following *in vitro* activation, Tregs significantly upregulated genes associated with mitochondrial metabolic pathways such as the tricarboxylic acid (TCA) cycle and the electron transport chain, in an mTOR-dependent fashion. It should be noted that, in many experimental systems including the mTOR-deficient mouse models, it might be quite difficult to discriminate between metabolic pathways involved in Treg expansion/fitness and in Treg effector function, since these two events are often coupled. Therefore, functional links between metabolic deficiencies and Treg alterations should be interpreted with caution.

MAIN IMMUNE SIGNALS AND FACTORS CONTROLLING TREG METABOLISM

Foxp3 is recognized as the major transcription factor underlying Treg identity; however, Foxp3 expression alone may not be sufficient for a stable and complete Treg differentiation and function, which also require continuous TCR stimulation and epigenetic reprogramming (77). Some metabolic pathways have been selectively linked to Foxp3 activity. Indeed, it has been shown that ectopic Foxp3 expression is necessary and sufficient to increase the expression of genes involved in mitochondrial respiration and to enhance fatty acid oxidation; in turn, the ability to catabolize fatty acids protects Foxp3-expressing cells from lipotoxicity, an event that may promote the selection of this population in mixed cultures and in stressed microenvironments (44). Foxp3 expression not only promotes oxidative metabolism but also suppresses glycolysis through the inhibition of MYC, a key factor of T cell metabolism (78), and also favors the oxidation of L-lactate to pyruvate through the modulation of lactate dehydrogenase (LDH) (40, 62). Therefore, Foxp3 expression may prime a default metabolic program that is shifted from glycolysis to oxidation.

Contrary to conventional T cells, Tregs constitutively express a variety of costimulatory, inhibitory, and cytokine receptors, playing diverse roles in Treg maintenance and functions and also impacting on Treg metabolism (**Figure 4**). The IL-2 receptor conveys indispensable signals for Treg thymic expansion and maturation and for Treg peripheral homeostasis (79). Together with the TCR, IL-2 represents one of the predominant signals that promote mTORC1 activity (63); accordingly, peripheral Tregs lacking the high affinity IL-2 receptor displayed a transcriptional program compatible with reduced cholesterol biosynthesis and also disrupted mitochondrial activity (79). Several receptors belonging to the TNFR superfamily, like TNFR2 and OX40, are also constitutively expressed by Tregs, are massively upregulated following Treg activation, and convey key signals for the NFkB-mediated acquisition of an eTreg phenotype (22). A key role for mTOR-driven activation of mitochondrial metabolism has been identified in eTreg differentiation (67). Whether the TNFR-NFkB axis promotes the conversion of cTregs into eTregs through the priming of mitochondrial functions, similarly to the pathway described for another costimulatory molecule, CD28 (35), remains to be understood.

Tregs express a series of Toll-like receptors (TLR), and their stimulation may subvert or promote Treg suppressive and stability depending on the TLR type (80). Gerriets et al. showed that TLR1/TLR2 ligation on murine activated iTregs boosted their proliferation and their glycolysis, but also compromised their suppressive ability (62). This event may explain the destabilization of Tregs occurring at inflamed sites where TLR ligands are abundant, and further corroborates the idea that certain strong inflammatory signals may uncouple glycolysis-related proliferation and oxidation-related immune suppression. Opposite results have been obtained when TLR8 was stimulated in human Tregs: this treatment subverted their suppressive functions, however this event was accompanied by a loss, and not a gain, of GLUT-mediated glucose uptake and mTOR-dependent glycolytic activities (73). Therefore, different TLRs may operate completely divergent functions in Treg expansion and suppression, which may involve opposite metabolic rewiring.

EXTRACELLULAR FATTY ACIDS: NUTRIENTS OR SIGNALING MOLECULES?

Beside internalizing glucose from the outer environment and oxidizing intracellular lipids, T cells can capture and catabolize other molecules as a source of energy and biosynthetic precursors, such as the amino acid glutamine that is processed through glutaminolysis and fuels the TCA cycle, and plays a role in the reciprocal regulation of Th17 and iTreg differentiation (81).

Free long-chain fatty acids represent another potential extracellular nutrient for T cells, following their internalization via specific translocators, such as CD36. Several studies have

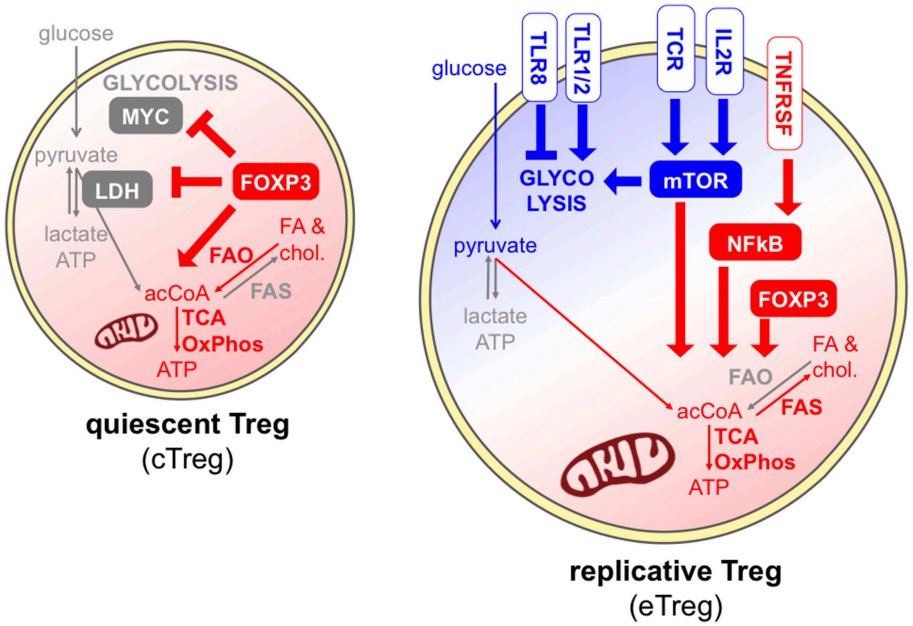

FIGURE 4 | Different signals can promote or antagonize Treg exit from quiescence. In quiescent Tregs, Foxp3 maintains Treg survival through the promotion of fatty acid oxidation and mitochondrial metabolism (red) (44), and the suppression of glycolysis through the inhibition of MYC and the modulation of LDH (40, 62). Effector Tregs, undergoing cell division, can receive multiple signals through the TCR, costimulatory and cytokine receptors, and mostly display a combination of glycolytic (blue) and mitochondrial (red) metabolism. IL-2 receptor and the TCR can directly promote mTORC1 activity (63), and IL-2 signal supports cholesterol biosynthesis and mitochondrial activity through mTOR (79). A key role for mTOR-driven activation of mitochondrial metabolism has been identified in eTreg differentiation (67). However, FAO may not be required for Treg activation (47). Receptors of the TNFR superfamily activate NFkB thus inducing the eTreg phenotype (22), and this may occur also through the priming of mitochondrial functions, similarly to CD28 (35). Finally, different TLRs can have opposite functions on Treg glycolytic activities and proliferation (73, 80).

reported that Tregs can capture fatty acids in culture (39, 42, 44) and also *in vivo*, especially in the tumor microenvironment in a mouse model of glioma (82). Notably, palmitate internalization and glucose uptake occurred in two distinct subpopulations of Tregs, with only minor overlap (82). In the VAT, Tregs acquire a tissue-specific program driven by the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) (83). This factor controls the expression of genes related to fatty acid translocation, biosynthesis, and oxidation; treatment with a PPARγ-agonist induced CD36 upregulation and fatty acid uptake in adipose tissue Tregs, and this event was associated with Treg expansion (83).

The relevance of long-chain fatty acid uptake in Treg differentiation and functions is still unclear. *In vitro*, exogenous BSA-conjugated palmitate was incorporated at high levels into endogenous fatty acids in iTregs, and the inhibition of cellular synthesis enhanced the extracellular fatty acid uptake (42). Supplemented palmitate enhanced oxidation rates in cells ectopically expressing Foxp3 (44) and iTregs *in vitro*, leading to a skewed iTreg development at the expense of Th17 cells (39, 84). Of note, the Foxp3-driven oxidative machinery may protect Tregs from the risk of lipotoxicity induced by high rates of fatty acid internalization (44). Based on these findings, exogenous fatty acids seem to promote iTreg polarization even though BSA-conjugated fatty acids may be internalized irrespective of physiological translocation that is mostly mediated by CD36 and other transporters.

Other data indicate that fatty acid uptake may also favor Treg suppressive function; indeed, the inhibitor of fatty acid translocation, sulfo-N-succinimidyl oleate (SSO), altered the expression of key suppressive molecules of Tregs and their inhibitory activity *in vitro* (68). As a general interpretation, high concentration of free fatty acids in a certain microenvironment may tip the balance toward immune regulation, even though the exact role of this metabolic-immune crosstalk in modulating immune responses deserves further studies.

Extracellular short-chain fatty acids (acetate, butyrate and propionate) exert a well-established role in Treg differentiation and expansion in the intestine; several studies [reviewed in Zeng and Chi (85)] have demonstrated that bacterial species colonizing the gut can break down dietary fibers, thus leading to the production of short-chain fatty acids. These metabolites bind specific G protein-coupled receptors on the cell surface and can promote the conversion of conventional T cells into pTregs or induce the proliferation of colonic tTregs. Therefore, short-chain fatty acids act as a bridge between microbial/dietary metabolism and immune regulation by working as signaling molecules, and less likely by directly affecting intracellular metabolic routes.

TREG EXPANSION IN TISSUES DEVOTED TO METABOLIC FUNCTIONS: THE ADIPOSE TISSUE AND THE LIVER

Since nutrient availability and signaling molecules can impact on Treg cell-intrinsic metabolism, which in turn can dictate Treg expansion and function, it could be predicted that the metabolic context of specific tissue microenvironments may affect immune regulation. This event may become particularly true in those tissues that are devoted to the control of systemic metabolism, such as the VAT and the liver. Presumably, in these tissues, the concentration of particular nutrients in the extracellular environment may vary depending on systemic and local metabolic processes.

Several groups have observed a prominent Treg accumulation in the VAT of healthy lean mice, have characterized the main mechanisms driving their expansion and suppressive function, and have reported a causal link between VAT-Treg deficiencies and metabolic inflammation (26, 83, 86–88). Among the signals driving VAT-Treg accumulation in mice, the TCR, Foxp3, and cytokines like IL-33 seem to play key roles (25, 26, 87). While these studies have demonstrated that Tregs can shape systemic metabolism through the suppression of metabolic inflammation in the VAT, it is not clear whether the local metabolism can affect Treg behavior. As already mentioned above, in the healthy VAT, PPARγ promotes the accumulation of intracellular lipids in Tregs; in obese mice fed with a high-fat diet, the reduction of Treg frequency corresponds to lower lipid content in Tregs (83). This result suggests that intracellular lipid accumulation may occur in alternate conditions in adipocytes (under dietary fat overload) and in Tregs (in healthy conditions), and also indicates that Tregs may not accumulate lipids as a simple consequence of extracellular or systemic lipid abundance. Less is known about Treg accumulation in the VAT of lean or obese humans and of patients with metabolic inflammation or cancer. According to recent data, Tregs express PPARγ also in the omental adipose tissue of humans, and Treg frequency is moderately increased in that tissue compared to the peripheral blood; however, no significant variation was observed in obese subjects or in type-2 diabetes patients compared to healthy controls (89). We observed even an expansion of Tregs in the VAT of obese compared to control subjects, and a mild positive correlation between VAT-Treg frequency and body mass index (90). Therefore, the mechanisms that regulate Treg accumulation in VAT may be completely distinct in mice and humans. Interestingly, we could observe a positive correlation between VAT-Treg percentage and the amount of the polyunsaturated ω6 arachidonic acid in the adipose tissue (90). Whether Tregs and generally immune cells can somehow shape the lipid composition of the tissue, and whether, conversely, different types of fatty acids can have an impact on Tregs and immune populations, remain open questions. It is worth noting that, in mice, VAT-Tregs utilize a catabolite of prostaglandin-E2 (which is synthetized from arachidonic acid) to suppress metabolic inflammation (88), therefore, the local availability of certain lipidic precursors may impact on Treg-mediated control of metabolic inflammation.

The liver is the main organ regulating systemic lipid metabolism and is susceptible to the development of abnormal lipid accumulation and inflammation (steatohepatitis) in pathological conditions induced for instance by high dietary fat intake. Less is known about liver-resident and liver-infiltrating Tregs compared to other tissues. In the early post-natal life of mice, a wave of hepatic Treg colonization occurs that seems dependent on microbiome (91). Of note, the transcriptomic profile of these hepatic neonatal Tregs reveals a high expression of PPARγ and the involvement of lipid handling machinery and oxidative phosphorylation (92). Interestingly, we have observed that such post-natal hepatic Treg expansion was higher in the $Mdr2^{-/-}$ mouse model (spontaneously developing cholangitis and chronic liver disease with time) and was accompanied by an intracellular lipid accumulation in Tregs (93). In adult mice, the identity of liver-resident Tregs remains more elusive with respect to other tissues (94), also because of the significant proportion of Tregs entering this highly vascularized tissue through the blood vessels (95). Like in the adipose tissue, also in the liver Tregs control metabolic inflammation and therefore systemic metabolism. Indeed, post-natal Treg depletion provokes the spontaneous development of steatohepatitis (92); if genetically susceptible adult mice are deprived of Tregs, the hepatic catabolism of lipoproteins is impaired, resulting in hypercholesterolemia and exacerbated atherosclerotic disease (96),

Taken together, the findings in VAT and in liver speak in favor of an inverse relation between external lipid overload and Treg cell-intrinsic lipid accumulation. Indeed, Tregs seem to accumulate lipids, under a PPARγ-driven program and as a consequence of synthesis and/or capture, concomitantly to their own expansion and thus to the control of metabolic inflammation. Conversely, Tregs show impaired lipid metabolism and proliferation in conditions of systemic and local lipid overload. The exact connections between Treg cell-extrinsic and -intrinsic lipid metabolism, and their consequences for Treg suppression and metabolic diseases, have not been clarified. Since Tregs exert several non-immune functions involved in tissue homeostasis, regeneration and repair (97), we cannot exclude that Tregs directly instruct tissue cells for specific metabolic activities, and that the bidirectional crosstalk may be mediated by conventional signaling molecules like amphiregulin (98) or by metabolites, stress signals, and nutrients derived from tissue cells and systemic circulation.

TREG EXPANSION UNDER METABOLIC RESTRICTION: THE TUMOR MICROENVIRONMENT

A hallmark of the tumor identity is represented by the ability evolved by tumor cells to escape immune recognition, and especially to suppress T cell response. Among the mechanisms concurring to this outcome, the local accumulation of Tregs plays an essential role. It is well-established that Treg frequency increases markedly at the tumor site in most solid malignancies and in both experimental models and cancer patients. Several

mechanisms may be involved in this local expansion, including the proliferation of preexisting tissue-resident Tregs, the recruitment of Tregs from the circulation, and the conversion of conventional T cells into pTregs (5). Therefore, the pool of tumor-infiltrating Tregs consists of a mixed population of tTregs and pTregs, possibly recognizing different antigen repertoires, performing specific activities, and showing diverse susceptibility to local signals of proliferation and stabilization (99). Most Tregs at the tumor site display an effector phenotype characterized by high expression of molecules related to their suppressive function and heightened inhibitory activities *ex vivo*. The transcriptomic profile of tumor-infiltrating Tregs results from the combination of tissue-associated signatures with a tumor-specific signature that is shared among different cancer types, and includes costimulatory molecules and chemokine receptors (100). Since the pioneer studies of North and Bursuker on the so-called at that time "suppressor cells" (101), many other studies have clearly demonstrated that Tregs could suppress anti-tumor immunity in experimental models, especially in immunogenic tumors and in certain therapeutic windows. Indeed, CD25+ T cell depletion by means of a specific monoclonal antibody (102), or the inducible genetic ablation of Foxp3+ Tregs (103), could evoke anti-tumor immunity that controlled tumor growth. In the majority of human cancers, a high density of Tregs at the tumor site correlates with a poor prognosis, a finding that confirms the detrimental role of these cells in the battle between host immunity and tumor cells (4). A growing amount of data demonstrate that Tregs play a range of non-immune, tissue-repairing functions that involve the release of amphiregulin, a ligand of epidermal growth factor receptor. Recent work has demonstrated that tissue-infiltrating activated Tregs promote malignancy also through the direct stimulation of epithelial cell growth via amphiregulin (98). Several strategies have been proposed, and have also been tested in some cases, to achieve Treg depletion or functional inactivation in the context of cancer immunotherapy. While CD25-targeted approaches have shown limited success (5), recent studies have highlighted that Tregs are major off-targets of the classical immune checkpoint blockers, being especially sensitive to anti-CTLA-4 antibodies capable of inducing antibody-dependent cell-mediated cytotoxicity (6).

From a metabolic point of view, the tumor microenvironment represents a peculiar and extremely complex context, when multiple metabolic interactions between tumor cells and stromal cells can be established that have not been completely elucidated. The idea that tumor cells evolve the ability to support their proliferative burst by pushing glycolysis was described by Otto Warburg and coworkers in the 1920s and was thus called the Warburg effect. Since then, researchers have accumulated a huge amount of information about the numerous metabolic routes that, together with glycolysis, characterize tumor cell proliferation and survival (104, 105). Besides tumor cells, several stromal and immune cells in the tumor microenvironment are able to reprogram their cellular metabolism when adapting to this peculiar context: for instance, a metabolic crosstalk is established between tumor cells and tumor-associated macrophages, which shapes macrophage functions and finally leads to tumor promotion (106).

This microenvironment poses a series of metabolic hurdles for T cells: (i) hypoxia in poorly vascularized tumor areas can affect T cell functions also through HIF1α induction; (ii) lactate released by both tumor and stromal cells, and the consequent extracellular acidity, can profoundly suppress the effector functions of T cells and compromise anti-tumor immunity; and (iii) the capture of nutrients (glucose, amino acids, and fatty acids) by tumor and stromal cells generates a status of metabolic restriction that ultimately leads to T cell starvation (107). In more detail, competition for glucose between T cells and tumor cells has been identified as a key event in determining the success of anti-tumor T cell activation, through the activity of a glycolytic intermediate, phosphoenolpyruvate, that directly modulates calcium flux downstream TCR signaling (108, 109). Not only glycolysis, but also mitochondrial metabolism is crucial for optimal anti-tumor T cell functions; indeed, a defective mitochondrial biogenesis and oxidative metabolism correlated with a functionally exhausted phenotype (110).

Based on this evidence, the selection of Tregs in the tumor microenvironment may derive from their ability to evolve a metabolic reprogramming that allows their survival and proliferation in such a hostile setting. Several studies have addressed this possibility and have tried to characterize those metabolic pathways that were responsible for tumor-associated Treg expansion and function (**Figure 5**).

Some data point to a key role of glycolysis in Treg proliferation in the tumor setting. First, intra-tumoral Tregs display particularly high glucose uptake compared to other cell types in a mouse model of melanoma (109), and we have shown that Tregs were able to efficiently compete for glucose with effector T cells in another mouse tumor model (93). Our data also indicate that, compared to effector T cells, tumor-infiltrating Tregs display higher levels of GLUT1 on the cell surface, express higher levels of glycolysis-related genes, and engage a higher glycolytic flux as measured in terms of extracellular acidification rate directly *ex vivo*; in human hepatocellular carcinoma, the gene expression profile of effector Tregs (selected as OX40-positive cells) is significantly enriched in glycolysis-related genes (93). Others have shown that pretreatment of human Tregs with a TLR8 agonist (that inhibits glycolysis), or 2-DG, reversed their ability to control anti-tumor T cell response *in vivo*, in a humanized mouse model (73). Of note, the pretreatment impaired Treg ability to induce senescence in responder CD8 T cells. Indeed, competition for glucose is a major trigger for T cell senescence, and Tregs may cooperate with tumor cells in consuming glucose and starving effector T cells in the tumor microenvironment (74). It has been proposed that, in low-glucose contexts such as the tumor site, Tregs may utilize glucokinase instead of hexokinase to perform glycolysis, having the former a much lower affinity for glucose (69). Altogether, these results suggest that Tregs possess the machineries needed for capturing and utilizing glucose in the tumor context, and that glycolysis may be involved in the maintenance of the tumor-associated Treg population *in vivo*.

More elusive is the role of oxidative phosphorylation in proliferation and survival of tumor-infiltrating Tregs. We have observed that Tregs freshly extracted from murine tumors

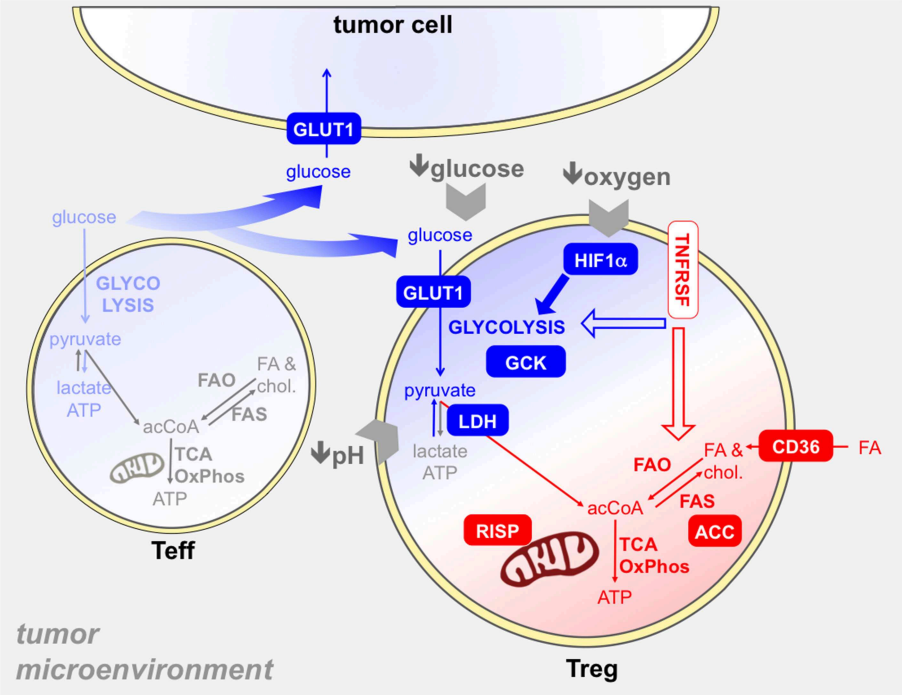

FIGURE 5 | Several metabolic mechanisms may provide an advantage to Tregs in the tumor microenvironment. The tumor microenvironment poses several hurdles to T cells, like hypoxia, glucose restriction, and acidosis. Tregs resist to these obstacles through several mechanisms, which involve both glycolysis (blue) and/or mitochondrial metabolism (red). First, intratumoral Tregs capture glucose at high rates (93, 109), thanks to GLUT1 upregulation, and display a glycolytic program and activity *ex vivo* (93): thus, Tregs may suppress the glycolytic metabolism in effector T cells (light blue) in the tumor microenvironment also through glucose sequestration (74). The usage of GCK instead of hexokinase in glycolysis may endow Tregs to resist in a low-glucose environment (69). Hypoxia triggers HIF1α activation in Tregs, which promotes glycolysis and thus indirectly fosters the oxidation of fatty acids captured through CD36 and other translocators (68). Tregs are more resistant to lactate overload, based on their ability to convert lactate into pyruvate thanks to the modulation of LDH by Foxp3 (40). Mitochondrial metabolism plays a key role in tumor-Treg expansion and function: indeed, mitochondrial complex III (RISP) dictates the ability of Tregs to suppress anti-tumor immunity (66). We have found that intratumoral effector Tregs, expressing TNFR superfamily members, performed fatty acid synthesis through ACC and this pathway was involved in their expansion and function (93). Whether TNFRSF signals can impact on glycolytic and/or mitochondrial metabolism in Tregs remains to be elucidated.

consume oxygen at similar rates as effector T cells (93). Others have shown that mice, where mitochondrial complex III is ablated specifically in Tregs, are protected from tumor growth; however, this effect may be ascribed also to the epigenetic effect of mitochondrial metabolites, besides the defect in mitochondrial respiration (66).

Foxp3 has been shown to directly shift the glycolytic into oxidative metabolism: this program also includes the peculiar ability of Tregs to oxidize lactate into pyruvate also in normal conditions, whereas Tconvs show this activity only in low-glucose, high-lactate conditions. However, Tconvs and Tregs are differentially susceptible to lactate overload and the consequent oxidation through the enzyme LDH: Tconvs rely primarily on glycolysis for their activation, and NAD depletion during lactate oxidation prevents GAPDH activity and suppresses their proliferation; conversely, Tregs are more resistant to the suppressive effect of lactate, possibly being less glycolysis-dependent for their activation, and containing higher levels of NAD continuously regenerated during oxidative phosphorylation (40). These data suggest that in low-glucose, high-lactate conditions, like in the tumor microenvironment, Tregs may have a selective metabolic advantage.

Fatty acids can become a major substrate for oxidative phosphorylation in some conditions. It has been shown that, in hypoxic settings, HIF1α activation diverts glucose away from mitochondria, leaving fatty acids as the main oxidative substrate for Tregs. Therefore, in hypoxic areas of the tumor microenvironment, Tregs may capture and utilize lipids to perform their metabolic functions: accordingly, intra-tumoral Tregs were found to express high levels of the fatty acid transporters CD36, SLC27A1, and SLC27A4, and to perform fatty acid uptake *in vivo*, in a mouse model of glioma (68). In this model, fatty acid oxidation seemed required for Treg suppression: indeed, etomoxir administration to tumor-bearing mice reduced tumor growth while reducing Treg frequencies (68). It has been proposed that the ability of Tregs to oxidize fatty acids may protect them from lipotoxicity (44), thus endowing Tregs with a further metabolic advantage over effector T cells.

In a different mouse tumor model, we have observed that intra-tumoral Tregs accumulated neutral fatty acids, an event

that was not related to fatty acid capture but rather derived from fatty acid synthesis (93). The expression of genes related to this pathway, the metabolite profile, the sensitivity of Treg to specific inhibitors of fatty acid synthesis, and the observation that 2-DG prevents fatty acid accumulation *in vitro* corroborate the hypothesis that tumor-infiltrating Tregs utilize glucose not only as a supply of energy but also as a source of precursors to build macromolecules like fatty acids. We have started to appreciate that many macromolecules can exert several non-energetic and non-structural activities. For instance, fatty acids represent a preferential source of acetyl groups for histone acetylation (111), and thus fatty acid supply or biosynthesis may impact the epigenetic profile of the cells.

CONCLUDING REMARKS

Even though some controversies still remain regarding the metabolic requirements of specific events during the life of a Treg, some general conclusions can be drawn from the existing data. First of all, the view that Tregs are oxidative cells need to be carefully revised; indeed, this assumption mostly comes from studies addressing the development of Tregs *in vitro*, rather than the *in vivo* dynamics of established Tregs. Moreover, many studies are also affected by experimental limitations such as the usage of TGFβ-based protocols and of pharmacological inhibitors with many off-target effects. Based on pieces of evidence coming from many studies, it is clear that Tregs can use different metabolic pathways, including glycolysis, in different phases of their life and in different activities. Generally speaking, while mTOR-driven glycolysis-lipogenesis seems required for Treg development and migration, Foxp3-driven lipolytic-oxidative metabolism is more strictly related to Treg suppressive function. Whether the two axes must coexist in the same Treg cell to achieve a full immune regulatory activity or can be segregated into distinct Treg subtypes or distinct phases of Treg activities, remains to be clarified.

The capacity of Tregs to switch through different metabolic programs may render them particularly able to adapt to hostile microenvironments, and the tumor may represent a prototypical context where Treg adaptation occurs: here, Tregs may be positively selected based on their ability to compete efficiently for glucose, to use alternative glycolytic enzymes, to capture and catabolize lipids thus avoiding incidental lipotoxicity, to better resist to high lactate exposure, and many other mechanisms still to be discovered. Among the signals in the tumor microenvironment supporting metabolic activation in Tregs, the TNFR-related signals may play key roles that have been only incompletely appreciated; these receptors are highly expressed by Tregs at the tumor site from many different tumor histotypes and may drive a switch toward an effector phenotype that includes immunological as well as metabolic features.

Targeting metabolism is considered a promising therapeutic approach for cancer therapy. However, it is now well-established that appropriately designed metabolic interventions can profoundly reshape immune cell functions and rescue anti-tumor immunity (112). Therefore, a holistic assessment of the metabolism of stromal and immune cells, together with tumor cells, would allow a more accurate design of future therapeutic strategies for cancer treatment (112). It should be considered that "metabolic drugs" targeting specific cell types in tumor-bearing hosts are not currently available, thus we are still far from a cell-directed metabolic intervention that would selectively inhibit detrimental immune cells and tumor cells while sparing normal cells. However, it could be predicted that some of these drugs could, at least preferentially, target metabolically active cells at the tumor site. According to this view, Tregs may be preferentially susceptible to metabolic interventions in tumors because of their relative abundance at the tumor site and of their stronger metabolic activation compared to other infiltrating T cells.

AUTHOR CONTRIBUTIONS

IP and SP conceived the concepts behind this review, wrote the manuscript, and prepared the figures.

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REVIEW
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Metabolism and Autoimmune Responses: The microRNA Connection

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Distinct metabolic pathways are known to regulate growth, differentiation, survival, and activation of immune cells by providing energy and specific biosynthetic precursors. Compelling experimental evidence demonstrates that effector T cell functions are coupled with profound changes in cellular metabolism. Importantly, the effector T cell-dependent "anti-self" response characterizing the autoimmune diseases is accompanied by significant metabolic alterations. MicroRNAs (miRNAs), evolutionary conserved small non-coding RNA molecules that affect gene expression by binding to target messenger RNAs, are now known to regulate multiple functions of effector T cells, including the strength of their activation, thus contributing to immune homeostasis. In this review, we will examine the most recent studies that describe miRNA direct involvement in the metabolic reprogramming that marks effector T cell functions. In particular, we will focus on the work showing a connection between miRNA regulatory function and the molecular network dysregulation that leads to metabolic pathway derangement in autoimmunity. Finally, we will also speculate on the possibility that the interplay between miRNAs and metabolism in T cells may help identify novel miRNA-based therapeutic strategies to treat effector T cell immunometabolic alterations in pathological conditions such as autoimmunity and chronic inflammation.

Keywords: T cells, metabolic regulation, immunometabolism, miRNAs, autoimmune diseases

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INTRODUCTION

The immune system encompasses a variety of cellular subsets that are highly dynamic and specialized in several activities essential for host defense and tissue homeostasis (1–4). T cells are a crucial component of the adaptive immune system, with a unique nature that makes them able to respond rapidly to environmental changes (5–7). T cell activation and function are deeply related to specific metabolic programs necessary to regulate T cell signaling and support their growth, differentiation, and effector function. Indeed, the ability of intracellular metabolism to integrate signals and nutrients to produce energy is fundamental to determine a specific T cell fate (8–10).

Over the past decade, increasing studies have highlighted the role of microRNAs (miRNAs) in the metabolic control of immune cells. miRNAs are a class of small non-coding RNAs involved in the fine-tune regulation of gene expression. Several mechanisms have been uncovered by which miRNAs control T cell function, including regulation of intracellular metabolic pathways. Under specific conditions, such as inflammation, the biogenesis of miRNAs may be modified. Aberrant expression of miRNAs may influence T cell metabolic reprogramming, leading to pathological phenomena, including chronic inflammatory disorders and autoimmune conditions.

In this review, we discuss recent literature reporting the involvement of miRNAs in the metabolic control of immune responses, with particular emphasis on those impacting on T lymphocyte differentiation and function, in health and autoimmunity.

METABOLIC REGULATION OF T CELL RESPONSE

T lymphocytes play a crucial role in host defense and coordination of immune response (11). Upon antigen recognition via the T cell receptor (TCR) in the presence of co-stimulatory signals, T lymphocytes clonally expand and produce cytokines to eliminate infected or transformed cells (12, 13). In particular, during an acute infection, antigen-specific T cells proliferate and differentiate into effector T (Teff) cells: $CD8^+$ cytotoxic T lymphocytes (CTLs) that rapidly mediate the clearance of infected cells and $CD4^+$ Teff cells that became functional specialized in distinct T helper (Th) cell subset [Th1, Th2, Th17, follicular Th (Tfh), and regulatory T (Treg) cells] (14, 15). Their dysregulated activation leads to a wide spectrum of autoimmune and inflammatory conditions (16–19).

Compelling evidence indicate that intracellular metabolic programs adopted by T cells finely regulate immune response (20, 21). T cell activation determines an increased biosynthetic demand, which requires rapid changes to generate metabolic intermediates for T cell growth, proliferation, and function (20–22). Both quiescent naïve and memory T cells are characterized by metabolic pathways that supply energy for survival and migration, including oxidative phosphorylation (OXPHOS), fatty acid oxidation (FAO), and amino acid oxidation [**Figure 1**; (10, 23)]. Conversely, activation of T cells drives transcriptional changes, causing downregulation of oxidative metabolism and upregulation of biosynthetic pathways, such as aerobic glycolysis that promotes an increase in biochemical intermediates, necessary for nucleotide, amino acid, and fatty acid synthesis (24). This anabolic program increases nutrient uptake at the expense of ATP production (25, 26). Teff cells require high levels of glucose to proliferate and differentiate in distinct T cell subsets (27). The increased glucose metabolism is controlled by glucose transporters, glycolytic enzymes, multi-protein complexes, and transcriptional factors that coordinate glucose utilization to generate pyruvate (28, 29). Under hypoxic conditions, pyruvate can be converted to lactate by the lactate dehydrogenase (LDH) (anaerobic glycolysis); on the other hand, higher oxygen levels promote pyruvate transfer into the mitochondria to supply intermediates of the tricarboxylic (TCA) cycle (30). TCA cycle starts from acetyl-CoA to generate citrate, which is consumed and regenerated throughout multiple biochemical reactions; nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide ($FADH_2$), and one GTP or ATP molecule are produced in each cycle [**Figure 1**; (31)]. Subsequently, NADH and $FADH_2$ provide electrons to generate ATP via OXPHOS [**Figure 1**; (31)]. Alternatively, glucose-6-phosphate (G6P), derived from the first enzymatic step of glycolysis, can be directed into the pentose phosphate pathway (PPP) that provides ribose for the synthesis of nucleotides and reducing equivalents, such as nicotinamide adenine dinucleotide phosphate (NADPH), for lipid and cholesterol biogenesis [**Figure 1**; (32)].

The increased glucose flow into the pentose phosphate pathway is also accompanied by an increase in glutamine metabolism (glutaminolysis), an energy-producing process required for fast biosynthesis of macromolecules necessary for lipid production (33, 34). Lipids play an important role in the regulation and maintenance of membrane properties, bioenergetic demands, and cell signaling (35). Upon TCR activation, T cells must increase their lipid content at each cell division for new plasma membrane generation; moreover, lipids, especially long fatty acids, are necessary to generate energy through the FAO [**Figure 1**; (36)]. Long-lived memory T cells preferentially use FAO to fuel the TCA cycle and OXPHOS, and maintain ATP production (37). Distinct metabolic and nutrient sensors, including the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, hypoxia-inducible factor 1α (HIF1α), c-Myc, and AMP-activated kinase (AMPK), integrate external stimuli and nutrient availability with intracellular metabolic processes (38–42). mTOR is a serine/threonine kinase with a key role in the regulation of T cell metabolism; it integrates multiple signals in response to nutrients, growth factors, energy, and stress to coordinate immune response upon TCR activation (43, 44). mTOR acts through two main signaling complexes, mTORC1 and mTORC2, which differ for their structure and sensitivity to the inhibitor rapamycin (43–46). PI3K/AKT/mTOR signaling pathway activation induced by TCR engagement, CD28 co-stimulation, or IL-2 receptor leads to an increase of glucose uptake via upregulation of Glucose transporter 1 (Glut1) levels, mediated by mTORC1 complex (45, 47). Instead, mTORC2, which mainly responds to growth signals, controls T cell proliferation and survival by AKT phosphorylation that affects glycogen synthase kinase-3 β (GSK-3β) (48, 49). Moreover, mTORC1 activates glycolytic program in T cells also thanks to the downstream transcription factors HIF1α and its target genes (50). HIF1α is an oxygen sensor rapidly activated under hypoxic conditions, which determines the transcription of target genes including erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glycolytic enzymes (51, 52). HIF1α in turn induces the expression of intermediates required for glycolysis, such as Glut1, in the presence of low oxygen availability (53). HIF1α can also increase glycolysis by inducing the expression of pyruvate dehydrogenase kinase 1 (PDK1), a key metabolic enzyme that favors the conversion of pyruvate to lactate (53).

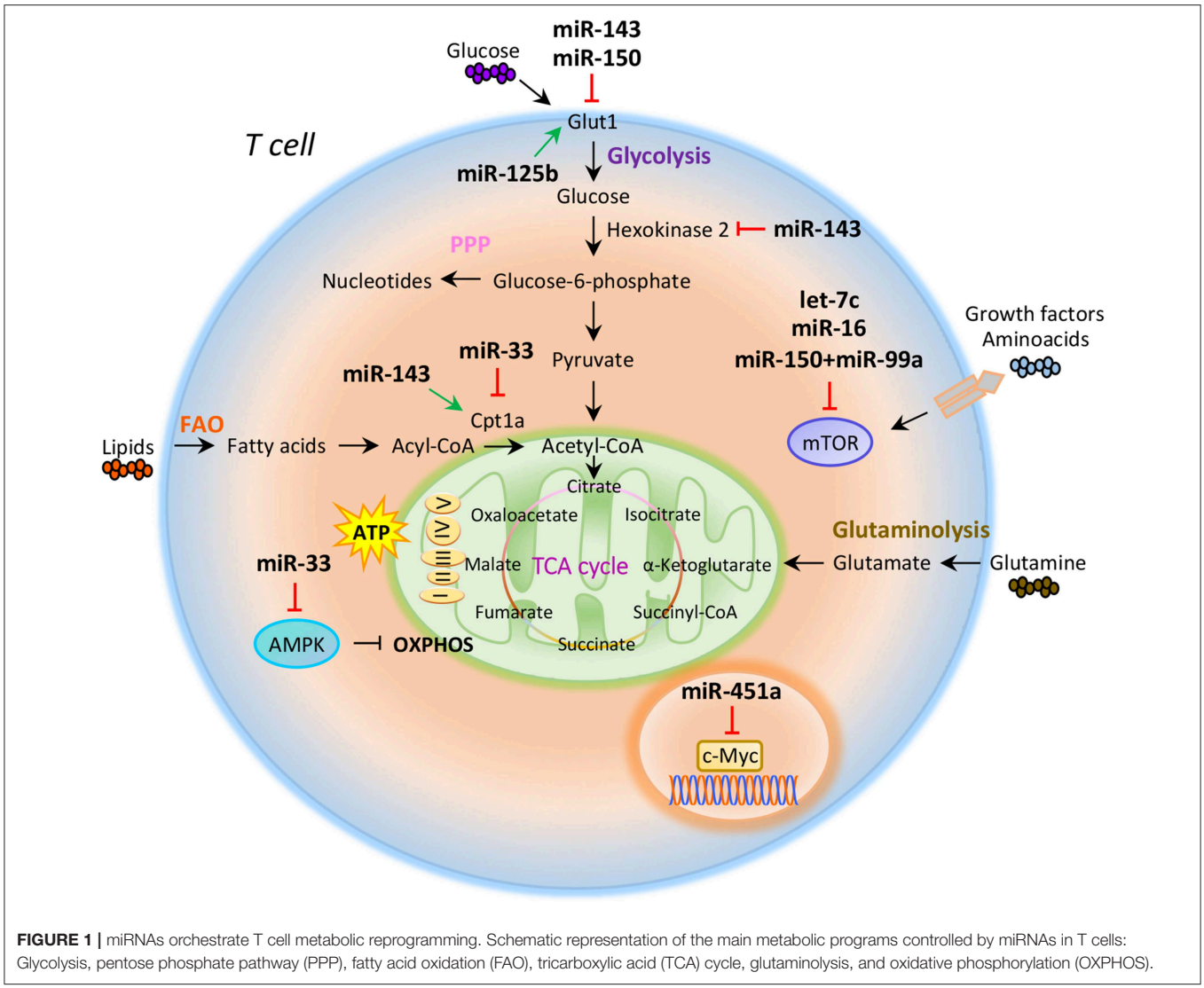

FIGURE 1 | miRNAs orchestrate T cell metabolic reprogramming. Schematic representation of the main metabolic programs controlled by miRNAs in T cells: Glycolysis, pentose phosphate pathway (PPP), fatty acid oxidation (FAO), tricarboxylic acid (TCA) cycle, glutaminolysis, and oxidative phosphorylation (OXPHOS).

mTORC1 is also responsible for the activation of c-Myc, another transcription factor with pleiotropic effects involved in cell proliferation, growth, and metabolism (38, 54). As a metabolic checkpoint, c-Myc is rapidly activated after TCR engagement and induces the transcription of enzymatic mediators of glycolysis, such as lactate dehydrogenase A (LDHA), pyruvate kinase isoenzyme type M2 (PKM2), hexokinase 2 (HK2), and Glut1 (55). c-Myc also induces the expression of transcription factor activating enhancer binding protein 4 (AP-4) which in turn upregulates the abovementioned glycolytic enzymes (56). Conversely, AMPK drives activated T cells to engage OXPHOS and maintain ATP levels under low glucose concentration (57). AMPK is activated in response to energy deprivation and under stress conditions, such as infections, inflammation, and DNA damage (58). AMPK is also a sensor of energy homeostasis and inhibits energy-consuming metabolism by increasing cellular AMP levels during energy deprivation, favoring ATP production (57, 59). Furthermore, AMPK controls catabolic metabolism through the inhibition of acetyl-CoA carboxylase (ACC), a crucial metabolic enzyme of fatty acid biosynthesis, promoting FAO (59). Under nutrient deprivation, AMPK also inhibits mTORC1 complex activity in T cells (57). Indeed, it has been observed that AMPK loss in naïve T cells upregulates mTOR activity and induces glycolysis (57). In all, these findings suggest that metabolism represents a critical checkpoint for T cell activation and function. Distinct levels of regulation (epigenetic, transcriptional, and translational) control and coordinate this cross-talk to ensure the appropriate energetic status underlying the specific immune cell function.

miRNA BIOGENESIS AND BIOLOGICAL FUNCTION

miRNAs are a class of small, non-coding RNAs of 21–25 nucleotides involved in post-transcriptional control of gene expression, through base pairing with complementary sequences in the 3′ untranslated regions (3′UTR) (58). miRNAs are

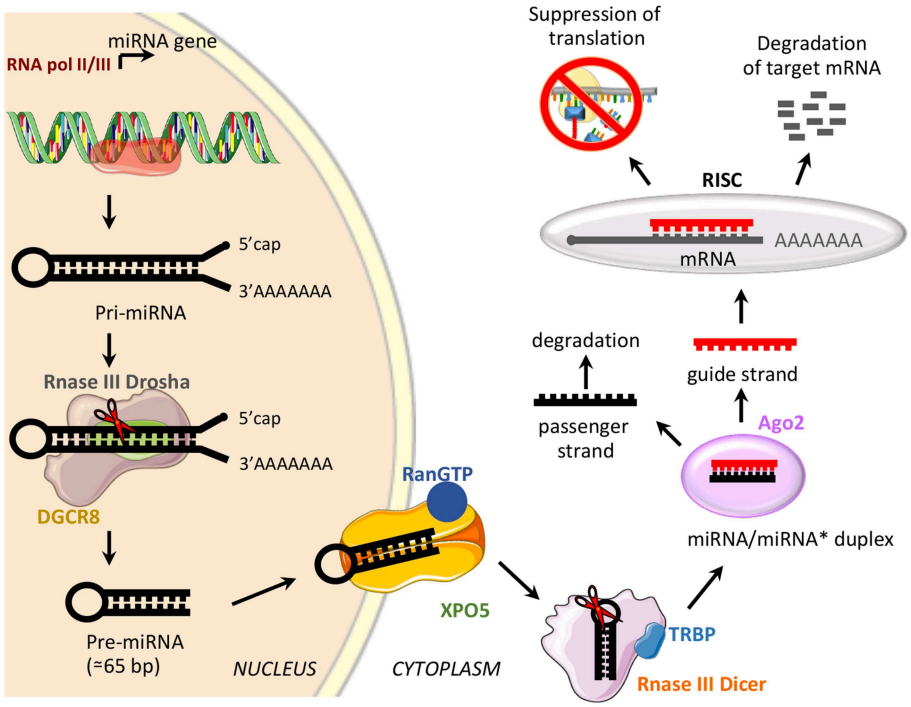

FIGURE 2 | miRNA biogenesis and function. Schematic representation of miRNA biogenesis pathway and biological function. Di George syndrome Critical Region 8 (DGCR8), exportin-5 (XPO5), GTP-binding nuclear protein Ran (RanGTP), trans-activator RNA binding protein (TRBP), Argonaute protein 2 (Ago2), and RNA-induced silencing complex (RISC).

transcribed by RNA polymerase II or III as long variable-length transcripts named primary miRNA transcripts (pri-miRNAs), with a 5′cap and a 3′poly (A) tail [**Figure 2**; (60)]. Pri-miRNAs are processed by Drosha ribonuclease III and its partner named Di George syndrome Critical Region 8 (DGCR8), to produce a hairpin RNA of about 65 nucleotides known as pre-miRNA molecule [**Figure 2**; (61)]. After nuclear processing, the pre-miRNAs are exported from the nucleus to the cytoplasm by the GTP-binding nuclear protein Ran (RanGTP)/exportin-5 (XPO5) complex and cleaved into a miRNA duplex of about 21 nucleotides (ds-miRNAs) by a second endoribonucleolytic Dicer and its cofactor TRBP (trans-activator RNA binding protein) [**Figure 2**; (62)]. ds-miRNAs are loaded into the Argonaute protein (Ago2), which facilitates incorporation of the guide strand into the RNA-induced silencing complex (RISC), while the passenger strand is degraded [**Figure 2**; (63)]. The guide strand binds target messenger RNA (mRNA) based on sequence complementarity; base pairing match induces degradation of target mRNA, while the imperfect complementarity results in suppression of translation [**Figure 2**; (64)].

The biological relevance of miRNAs is highlighted by the discovery that a single miRNA could interact with hundreds of target mRNAs, and each gene transcript may have several sites of miRNA recognition (65, 66). The finely tuned control of gene expression requires the production of the appropriate level of specific miRNAs in a well-defined time frame (65, 66). Since miRNAs are involved in post-transcriptional regulation of several cellular processes, dysregulation or dysfunction of their biogenesis leads to a wide range of human diseases, ranging from cancer to autoimmune disorders (67–69). Specific miRNAs have been described to regulate the function and homeostasis of several immune cell populations (70). Compelling evidence has demonstrated that deletion of Drosha or Dicer within T cell compartment affects T cell development, differentiation and function (71, 72). In this context, it has been shown that Dicer deletion at an early stage of T cell differentiation compromises the survival of TCR alphabeta (α/β) chain cells while it is dispensable for CD4 or CD8 T cell lineage commitment (73, 74). Furthermore, Chong et al. highlighted the essential role of Drosha and Dicer in Treg cells, as specific deletion of one or both of them results in impaired expression of the transcription factor forkhead box P3 (FoxP3), the master gene of Treg cell development and function (71). Among all the miRNAs involved in the control of T cell fate, miR-125b has been shown to restrain the expression of genes encoding molecules important for differentiation of naïve into effector and memory T cells, targeting interferon-γ (IFN-γ), interleukin 2 receptor β (IL-2Rβ), interleukin 10 receptor α (IL-10RA), and Blimp-1 (PRDM1) genes (75).

miR-214 and miR-182 act through different mechanisms to control T lymphocyte activation (76). Specifically, up-regulation of miR-124 in T cells, after TCR stimulation, promotes T cell activation through the inhibition of phosphatase and tensin homolog (PTEN), a negative regulator of T cell activation.

Mechanistically, activation via CD28 induces upregulation of miR-214, which targets the 3′UTR of *Pten*, causing a reduction in PTEN levels promoting T cell activation (76). Constitutive overexpression of miR-182 by IL-2 supports clonal expansion of Th cells, by reducing the transcription factor Foxo1, a suppressor of resting Th cell proliferation (77).

Compelling experimental evidence reports that several miRNAs can also influence T cell differentiation (78–80). In more detail, miR-155, expressed at high levels in Treg cells, is involved in the modulation of the suppressor of cytokine signaling 1 (SOCS1) and the signal transducers and activators of transcription 5 (STAT5) activity, key molecules for Treg cell differentiation and function (81). Data from animal models revealed that mice lacking miR-146a are more susceptible to chronic inflammation and autoimmune disorders secondarily to the loss of peripheral T cell tolerance. These findings suggest that miR-146 may be considered a key regulator of T cell response (82). In all, miRNA network can be considered as a crucial regulator of cell biology and, although studies focusing on miRNAs are progressively increasing, their impact on T cell function is still only partially explored.

ROLE OF miRNAS IN T CELL METABOLIC REPROGRAMMING

The role of miRNAs in the regulation of metabolic reprogramming in cancer cells has been largely investigated, thus opening the study of their potential role in the modulation of T cell metabolism (83–85). Several studies have suggested that miRNAs mainly act on key metabolic enzymes or transporters of energetic nutrients, thus impacting on T cell proliferation and differentiation (86, 87). In this context, Zhang et al. identified miR-143 as a regulator of T cell metabolism that reduces glucose uptake through the inhibition of glucose receptor Glut1. The authors found that, by specifically reducing glycolysis during TCR-dependent activation, miR-143 promotes memory T cell differentiation and metabolic reprogramming (88). In addition, miR-143 stimulates T cell memory differentiation also through the control of two enzymes, hexokinase II and carnitine palmitoyltransferase 1a (Cpt1a), which regulate glucose oxidation and oxidative phosphorylation, respectively [**Figure 1**; (88)]. miR-150 is an additional miRNA that finely tunes T cell glycolytic reprogramming, secondarily to co-stimulatory signals. Recent findings suggest that CD46 signaling, which is activated downstream of CD28 co-stimulation during T cell activation, leads to a rapid downregulation of miR-150 expression, which targets solute carrier family 2 member 1 (SLC2A1) gene, encoding the glucose transporter Glut1 [**Figure 1**; (89)]. Through a direct interference with Glut1-dependent glycolysis, miR-150 profoundly impacts on T cell activation in the absence of a proper co-stimulatory signal (89). On the contrary, metabolic reprogramming of activated T cells seems to be associated with the upregulation of miRNAs promoting glucose uptake and downregulation of those stimulating catabolic pathways. In this context, Liu et al. have studied the role of miR-125b, overexpressed in T cell acute lymphoblastic leukemia (T-ALL) (90). The authors revealed that miR-125b regulates glucose uptake in T cell via Glut1 by reducing the expression of TNF-α-induced protein 3 (TNFAIP3), which inhibits the activation of nuclear factor k B (NF-kB) [**Figure 1**; (90)]. These findings suggest that, through the enhancement of glucose metabolism and oxygen consumption, deregulation of miR-125b contributes to abnormal differentiation of T cell in T cell leukemia (90).

It has been reported that CD28 engagement during T cell activation promotes the expression of Cpt1a, a key enzyme for mitochondrial FAO. This process is finely regulated by miR-33 that, in the absence of CD28 signal, attenuates Cpt1a expression, interfering with the metabolic demand central for future recall of memory T cells [**Figure 1**; (91)]. Several studies have also reported that miRNAs control T cell metabolic reprogramming by targeting important metabolic checkpoints, such as AMPK, mTOR, and c-Myc (92–94). Ouimet et al. unveiled that miR-33 targets AMPK, inducing an unbalance between aerobic glycolysis and mitochondrial OXPHOS (92). miR-33 affects oxidative phosphorylation and induces macrophages M2 polarization through direct targeting of AMPK [**Figure 1**; (92)]. The same authors revealed that miR-33 inhibition increased macrophage expression of the retinoic acid (RA)-producing enzyme aldehyde dehydrogenase family 1, subfamily A2 (ALDH1A2), and of retinal dehydrogenase enzyme, with subsequent production of RA, which favors differentiation of $FoxP3^+$ $CD4^+$ T cells (92).

Several other miRNAs are involved in the control of mTOR pathway; in particular, an increased activation of mTOR, leading to S6 and AKT phosphorylation, has been observed in the absence of co-stimulatory signals in $CD4^+$ T cells deficient for RNaseIII enzyme Dicer, a key component of miRNAs biogenesis (93). Two miRNAs are involved in the fine regulation of mTOR and Rictor mRNA expression. Specifically, the downregulation of let-7c miRNA and miR-16 in Dicer-deficient $CD4^+$ T cells causes TCR signaling amplification and increased IL-2 production, due to overexpression of mTOR and Rictor mRNAs [**Figure 1**; (93)]. Subsequently, restoration of mTOR and Rictor expression by genetic manipulation determines reduction of IL-2 production at levels sufficient to avoid anergy in response to TCR engagement (93, 94). Thus, let-7c miRNA and miR-16 control the balance between activation and anergy through post-transcriptional control of mTOR components in T cells [**Figure 1**; (93, 94)].

mTOR pathway is also regulated by miR-150 expressed at high levels during differentiation of naive $CD4^+$ T cells into Treg cells; miR-150 efficiently represses mTOR in cooperation with miR-99a, and this promotes Treg cell differentiation [**Figure 1**; (87)]. In addition, a recent report showed that miR-451a directly targets c-Myc in Jurkat T cells; indeed, miR-451a negatively correlated with c-Myc expression in $CD4^+$ T cells from dilated cardiomyopathy subjects. These results suggest a role of the miR-451/c-Myc pathway in $CD4^+$ T cell proliferation and activation [**Figure 1**; (95)].

These data support the idea that, through the modulation of intracellular metabolic programs, miRNAs are able to influence T cell fate and differentiation (96). Understanding the mechanism by which miRNAs target metabolism in T cells may lead to therapeutic strategies for immune-related diseases.

T CELL METABOLIC ALTERATION IN AUTOIMMUNITY: THE miRNA CONNECTION

Given the impact of miRNAs in the regulation of T cell metabolic programs, aberrant miRNA expression could interfere with T cell fate with consequent loss of immune homeostasis and autoimmunity (97–100). Autoimmune diseases afflict about 7–9% of the worldwide population; in particular, type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and multiple sclerosis (MS) have been increasing in the last few years (101–104). The role of T cell metabolism in the control of immune function and how its alteration could influence immune response during autoimmunity have been well-characterized (24, 105–111). Naïve T lymphocytes are metabolically inactive, and their differentiation is controlled by metabolic reprogramming, involving glycolysis, FAO, and OXPHOS (10, 112–115). It is well-known that activated T cells require high levels of metabolic compounds to maintain their viability and function (57). In particular, it has been shown that glycolysis sustains cytokine production in Teff cells (57); moreover, FAO and OXPHOS play an important role to increase the inflammatory capacity of memory T cells, and this allows a rapid response upon antigen recall (116, 117). Thus, an aberrant metabolic environment could influence the development of inflammation and autoimmune disorder by fueling the differentiation and activation of pathogenic T cells.

miRNAs were shown to regulate metabolism-related genes in T1D, an autoimmune disease characterized by persistent hyperglycemia secondarily to pancreatic β-cell destruction and insulin deficiency (118, 119). Increased glucose levels in the extracellular microenvironment determine a metabolic reprogramming that fuels autoreactive Teff cell activation and IFN-γ secretion (57, 114, 116). Glucose-activated Teff cells upregulate Glut4 and insulin receptor substrate (IRS)-1 on their surface to sustain glycolytic rate and produce pro-inflammatory cytokines and reactive oxygen species (ROS) that promote the autoimmune response (120). In this context, let-7 family of miRNAs, which control several genes involved in glucose homeostasis, insulin resistance, and cell differentiation, has been reported to control important reguatory mechanisms in T1D subjects [**Table 1**; (121–123)]. Let-7 miRNAs are one of the largest and highly conserved family of miRNAs expressed in T cells, present in multiple copies in the genome; the number of let-7 miRNAs differs between species; for example, in humans, there are 10 mature let-7 miRNAs (124). It has been shown that let-7 miRNAs target multiple genes related to glucose response and the insulin-PI3K-mTOR pathway, such as insulin-like growth factor 1 receptor (IGF1R), insulin receptor (INSR), and IRS-2 but also regulate genes involved in the effector functions of CTLs (i.e., granzyme A, granzyme B, perforin 1, and eomesodermin) (121, 122, 125). In particular, it has been shown that four members of the let-7 miRNAs family (let-7a, let-7e, let-7f, and let-7g) were higher in PBMCs from T1D subjects (126); however, how these miRNAs are involved in T1D pathogenesis is poorly understood. Together, these results suggest that an altered insulin–PI3K–mTOR pathway in T cells of T1D individuals may determine an aberrant glucose uptake as a consequence of a defective regulation of insulin receptor genes. This impaired metabolism could favor the differentiation and activation of pathological Teff cells in T1D patients. Furthermore, serum miR-378 negatively correlates with insulinoma-associated protein 2 (IA2A) and the Zinc transporter 8 (Znt8) autoantibodies in T1D subjects (127). This miRNA is involved in the control of several metabolic processes, such as glycolysis, mitochondrial oxidation, and fatty acid metabolism, through the interference with the expression of mannose-1-phosphate guanylyltransferase (GDP), dimethylarginine dimethylaminohydrolase 1 (DDAH1), LDHA, and carnitine O-acetyltransferase (CRAT) enzymes [**Table 1**; (127–129)]. One hypothesis is that serum levels of miR-378 could reflect metabolic alteration that promotes differentiation of pathogenic T cells in T1D subjects (**Table 1**). In this context, Zhou et al. have investigated by *in silico* analysis that 27 miRNAs out of 530 are located in nine human insulin-dependent diabetes mellitus (IDDM) loci associated with T1D susceptibility (130). Among them, miR-16-2, miR-551b, and miR-877 target specific genes involved in the activation of Teff cells, such as CD28, Fas ligand (FasL), and the autoimmune regulator (AIRE), respectively [**Table 1**; (130)].

miR-26a is an additional miRNA associated with T cell dysfunction, glucose metabolism, and autoimmune disease development (131, 132). Its expression in T cells is regulated by glucose availability, and it is able to target the epigenetic regulator enhancer of zeste homolog 2 (EZH2), a histone-lysine-N-methyltransferase, well-known to improve effector T cell function by inhibiting Notch signaling repressors [**Table 1**; (133, 147)]. In SLE subjects, the levels of miR-26a in $CD4^+$ T cells negatively correlate with disease severity; this suggests that the reduced miRNA regulation of EZH2, secondarily to an increased glycolytic activity in $CD4^+$ T cells, sustains their activation (134, 148). Several defects in metabolic pathways

TABLE 1 | miRNAs involved in T cell metabolic reprogramming, during autoimmune diseases: type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and multiple sclerosis (MS).

miRNAs	Diseases	Targets	References
let-7 family	T1D	IGF1R; INSR; IRS-2	(121–123, 126)
miR-378	T1D	GDP; DDAH1; LDHA; CRAT	(127–129)
miR-16-2	T1D	CD28	(130)
miR-551b	T1D	FasL	(130)
miR-877	T1D	AIRE	(130)
miR-26a	SLE	EZH2	(131–134)
miR-633	SLE	AKT1	(135)
miR-766-3p	SLE	IRS-2; PI3K receptor 1	(136)
NovelmiRNA-25	SLE	AMPD-2	(137)
miR-19b	MS	PTEN	(138)
miR-99b-5p	MS	IGF1R; mTOR; AKT1	(138–142)
miR-21	MS	SMAD7	(143–146)

of T cells from SLE subjects have been described, especially those related to mitochondrial dysfunction (149–151). SLE is a complex multifactorial autoimmune disease where loss of tolerance determines the generation of antinuclear antibodies produced by B lymphocytes and tissue damage by autoreactive Teff cells (150, 152). Mitochondrial membrane hyperpolarization occurs in $CD4^+$ T cells from SLE subjects, leading to increase of ROS and depletion of ATP and glutathione, determining an impaired T cell activation and cell death (149, 153, 154). Of note, mounting evidence show a role for mTOR as a sensor of mitochondrial dysfunction in Teff cell differentiation during SLE (155, 156). It has been recently reported that miR-633 that targets the AKT/mTOR pathway is significantly reduced in $CD4^+$ T cells from SLE-affected subjects, and its expression negatively correlates with disease activity [**Table 1**; (135)]. In more detail, miR-633 inhibits the AKT/mTOR signaling and increases the induction of several cytokines, such as IL-4, IL-17, and IFN-γ, thus contributing to disease pathogenesis [**Table 1**; (135)]. Together with the abovementioned study, others reported an increase of miR-766-3p in $CD4^+$ T cells from SLE subjects. Potential target genes of this miRNA are IRS-2 and PI3K receptor 1, both involved in the PI3K/AKT/mTOR pathway [**Table 1**; (136)]. These data suggest that $CD4^+$ T cells of SLE subjects have an impaired PI3K signaling, which could affect their differentiation and function, leading to the development of autoimmunity. Recently, Guo et al. reported the upregulation of several miRNAs associated to metabolic pathways in PBMCs from SLE patients, such as NovelmiRNA-25 and miR-1273h-5p (137). NovelmiRNA-25 targets the enzyme adenosine monophosphate deaminase (AMPD)-2 involved in purine nucleobase or nucleotide metabolism by converting AMP to inosine monophosphate (IMP) [**Table 1**; (137)]. The overexpression of NovelmiRNA-25 associates with a downregulation of AMPD-2 protein in PBMCs from SLE subjects, contributing to AMP accumulation that improved the activation of pro-inflammatory pathways [**Table 1**; (137, 157)]. Moreover, increased levels of AMP contribute to activate AMPK, a key enzyme for cellular metabolic reprogramming (42). Of note, NovelmiRNA-25 positively correlates with disease activity, suggesting an important role for this miRNA as a biomarker to predict the activation of pathogenic T cells.

The role of miRNAs in the regulation of autoreactive T cell function has been reported also in MS (158–160). MS is the most common chronic inflammatory demyelinating disease of the central nervous system (CNS), characterized by autoreactive T cells able to target myelin-based antigens, leading to demyelinating lesions and neuronal degeneration (161). During MS, the demyelinating process associates with metabolic reprogramming in neuronal cell bodies sustaining chronic inflammation through the release of pro-inflammatory cytokines (162, 163). These events promote the activation of $CD4^+$ T cells, which further increase neuronal damage (162). Mounting evidence highlights the close relationship between T cell metabolic alterations and neurodegeneration in MS (105, 164, 165). Impaired glycolytic engagement has been described in Tconv cells from naïve-to-treatment relapsing remitting (RR)-MS subjects during the generation of induced (i) Treg cells, which contributes to loss of immune tolerance (105). In more detail, the glycolytic enzyme enolase-1 accumulates in the nuclei—secondarily to the reduced engagement in the glycolytic cascade—and constrains the induction of FoxP3 expression during the generation of Treg cells (105). Moreover, other key enzymes involved in glycolysis and mitochondrial respiration are reduced in $CD4^+$ T cells from RR-MS subjects, such as aldolase, hexokinase 1, Glut1, dihydrolipoamide S-acetyltransferase (DLAT), and dihydrolipoamide S-succinyltransferase (DLST); interestingly, restoration of these enzymes after IFN-β-1a treatment correlates with disease amelioration (165). Several studies also reported that alterations in the PI3K/AKT/mTOR pathway, which controls T cell activation and metabolism, ameliorate the clinical course of MS (166–169). Also, rapamycin—an immunosuppressant drug that inhibits mTOR by destabilizing the mTOR–Raptor complex—controls disease progression in experimental autoimmune encephalomyelitis (EAE) mice by suppressing Teff cell functions (170, 171). In this context, miR-19b and the miR-99 family are associated with the mTOR pathway, affecting effector T cell activation during MS [**Table 1**; (138)]. By targeting PTEN, the negative regulator of the PI3K/AKT/mTOR signaling pathway, miR-19b enhances mTOR activity sustaining pathogenic Th17 cell development [**Table 1**; (138)]. Other reports revealed also that the miR-99 family modulates the PI3K/AKT/mTOR signaling pathways. In particular, the miR-99b-5p, a member of this miRNA family, is significantly higher in splenocytes of EAE mice; *in silico* analysis confirmed that it may target multiple genes, such as IGF1R, mTOR, and AKT1 [**Table 1**; (139–141)]. Combined miRNA and mRNA expression analysis confirmed these data also in human disease; indeed, miR-99b-5p levels are upregulated in PBMCs from pediatric MS subjects (142). These results suggest an important role for miR-99 family, in particular the miR-99b-5p, in T cell activation during MS through a hyper-activation of the mTOR pathway in pathogenic lymphocytes.

Furthermore, recent reports suggest that fumaric acid ester (FAE)—a Krebs cycle intermediate used for MS therapy—induces hypermethylation of the miR-21 locus in $CD4^+$ T cells, and this constrains Th17 cell differentiation and function [**Table 1**; (143)]. In more detail, FAE treatment reduces Th17 cells, by direct hypermethylation of CpG sites spanning the MIR-21 promoter. Several studies have shown that miR-21 is upregulated in PBMCs from MS subjects and also in CNS-infiltrating T cells of EAE mice (144, 145). As a therapeutic tool in MS subjects, FAE selectively reduces miR-21 transcripts in Th17 cells and indirectly increases its target—the small mothers against decapentaplegic homolog 7 (SMAD7)—an inhibitor of their differentiation [**Table 1**; (144, 146)].

Taken together, these findings support the existence of a cross-talk between metabolic programs and miRNA network in T cells. Through a strong impact on the intracellular molecular pathways that control T cell differentiation and function, miRNA dysregulation leads to an imbalance between autoreactive T cell activation and regulatory function with consequent loss of immunological tolerance.

CONCLUDING REMARKS

Our understanding of the link between T cell metabolism and miRNA expression has substantially increased in the past decade. The ability of several miRNAs to regulate and reprogram metabolic pathways that drive T cell function and differentiation may represent a hallmark to improve the comprehension of the molecular processes underlying the pathogenesis of autoimmune disorders. However, further studies are required to better understand the connection among miRNAs, T cell metabolism, and loss of immunological tolerance. The precise mechanisms by which miRNAs target the genes encoding for enzymes, multi-protein complex, and transcription factors related to T cell metabolism and how their alteration associates with the development of autoimmune disorders remain to be dissected. Considering the increasing important role of miRNAs in the immune homeostasis, therapeutic approaches could represent an innovative way to control the aberrant metabolism sustaining autoreactive T cell clones.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Metabolic Requirements of Th2 Cell Differentiation

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Upon activation, naïve CD4$^+$ T cells differentiate into a number of specialized T helper (Th) cell subsets. Th2 cells are central players in immunity to helminths and are implicated in mediating the inflammatory pathology associated with allergies. The differentiation of Th2 cells is dependent on transcription factors such as GATA3 and STAT6, which prime Th2 cells for the secretion of interleukin- (IL-) 4, IL-5, and IL-13. Several lines of work now suggest that differentiating Th2 cells in the lymph node are potent IL-4 cytokine producers, but do not become competent IL-5- and IL-13-producing cells until after receiving cues from non-lymphoid tissue. It is evident that Th2 cells that enter tissues undergo considerable changes in chromatin architecture and gene expression, and that over this time, the metabolic requirements of these cells change considerably. Herein, we discuss the metabolic requirements of Th2 cells during their early and late differentiation, focusing on the impact of glucose and lipid metabolism, mTOR activation, the nuclear receptor PPAR-γ and several metabolites.

Keywords: Th2, PPAR-γ, lipid metabolism, glycolysis, mTOR

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INTRODUCTION

CD4 T cells are central mediators of immunity to infections and cancers. Pioneering studies by Mosmann and Coffman identified mouse CD4 T cell clones with distinct functional properties that they termed T helper (Th) 1 and Th2 cells (1). Over 30 years of research has since defined several additional subsets of CD4 T cells including Th17, Tfh, and T regulatory (Treg) cells. Th2 cells are defined by the expression of lineage-defining transcription factors including GATA3 and STAT6, surface molecules such as IL-33R and CCR8 and the effector cytokines IL-4, IL-5, and IL-13 (2). Through the secretion of IL-4, IL-5, and IL-13, Th2 cells promote B cell isotype class switching to IgG1 and IgE (3), induce the alternative activation (M2) phenotype in macrophages (4, 5), induce eosinophil recruitment and promote mucus secretion through the process of goblet cell metaplasia (6, 7). These effector functions have been shown to support immunity to helminths, venoms, certain bacterial infections, and are also beneficial in tissue healing (8, 9). However, Th2 cell-mediated immune responses are also implicated in allergic disorders including asthma, atopic dermatitis, chronic rhinitis, and some forms of gut disorders including ulcerative colitis (10–12). The rise in Th2 cell-mediated disorders has become especially apparent in the past 50 years and represents a significant and growing health and economic challenge.

THE PROCESS OF TH2 CELL DIFFERENTIATION

Th2 cell differentiation from naïve CD4 T cells is typically dependent on the presence of interleukin-4 (IL-4) in the local cytokine milieu. Ligation of the IL-4R induces JAK1/3 mediated phosphorylation and dimerization of Signal Transducer and Activator of Transcription-6 (STAT6) (13). pSTAT6 dimers then translocate to the nucleus and

induce expression of GATA3; the so-called "master" regulator of the Th2 cell lineage. GATA3 is sufficient to induce the Th2 cell phenotype, since for instance, enforced retroviral expression of GATA3 results in IL-4 production in Th1 cells (14, 15). Furthermore, GATA3-deficient T helper cells have impaired Th2 cell differentiation as shown in *in vitro* and *in vivo* studies (16–19). Expression of GATA3 results in profound modifications to the chromatin landscape across the *Il4/Il5/Rad50/Il13* locus at a number of well-characterized sites including several enhancer sites and a locus control region located in *Rad50* (20–23). Together with STAT6, this creates an "active" chromatin hub that allows co-ordinated expression of Th2 cell effector cytokines and a positive feedback loop through which GATA3-induced IL-4 maintains Th2 cell identity (24–26). Other genes important in the later stages of Th2 cell differentiation are also bound by GATA3 including the *Il1rl1* gene encoding a subunit of the IL-33R known as ST2, and the chemokine receptor *Ccr8* (27, 28).

Although the canonical pathway of Th2 cell differentiation is thought to proceed through GATA3 and STAT6, a number of non-classical pathways are also thought to be important during the early stages of Th2 cell differentiation, exemplified by the presence of IL-4^+ and IL-13^+ Th2 cells in STAT6-deficient mice (29). IL-2, induced upon TCR activation, has been shown to be capable of driving IL-4 production in T helper cells in an IL-4R-independent manner (30, 31). Triggering of the IL-2R results in activation of STAT5, with STAT5A being the most dominant isoform inducing downstream IL-4 expression (16). Support for the role of STAT5A in Th2 cell differentiation comes from studies of double STAT5A/STAT6-deficient mice that have further impairments in Th2 cell responses when compared to single STAT6-deficient mice (31, 32). STAT3 was also shown to be important for Th2 cell differentiation by guiding STAT6 to critical Th2 cell gene loci (33). Other studies have shown roles for a number of transcription factors in type 2 cytokine production including c-Maf, NF-κB, and IRF4 during the early stages of Th2 cell differentiation (34–36). Therefore, Th2 cell fate is determined by a complex network of transcription factors that together shape and promote naïve cells to adopt and maintain the Th2 cell phenotype.

TIMED CYTOKINE EXPRESSION IN TH2 CELLS

The Th2 cell effector cytokine genes *Il4*, *Il13,* and *Il5* are positioned together with *Rad50* (Chromosome 5 in humans; Chromosome 11 in mice), which contains a locus control region that co-ordinates at least *Il4* and *Il13* expression (24, 37). Despite the close proximity of these genes, their expression is exquisitely timed and not always concomitant. IL-4 expression is clearly detected in activated CD4 T cells in the lymph node, although several studies have shown that these cytokine-secreting cells are a mixed population of Th2 cells and Tfh cells, which require only low levels of GATA3 expression together with c-Maf (38–40). Meanwhile, IL-5 and IL-13 expression is a feature of Th2 cells only once these cells enter inflamed tissues such as the lungs (39, 41, 42). In response to house dust mite (HDM) allergens, airway Th2 cells tended to express less *Il4* mRNA than their lymph node counterparts, suggesting that IL-4 is the dominant cytokine in the lymph node, while IL-5 and IL-13 are the dominant Th2 cell-derived cytokines in tissues. This distinct timing means that the absence of IL-4 or IL-13 has distinct functional consequences (37, 39, 41, 42). For instance, IL-4-deficient mice were found to clear the helminth *Nippostrongylus brasiliensis* more rapidly despite reduced IgE titers (43). In contrast, IL-13-deficient mice had significantly higher worm counts and took longer to clear infections despite no defect in IgE production. Similar responses were observed in models of *Trichuris muris* and *Heligmosomoides polygyrus* infection (44–46). Hence, the quality of Th2 cells changes over time and their function depends on the tissue context.

METABOLIC PATHWAYS IMPORTANT TO T HELPER CELLS

Generation of energy and biosynthesis of metabolites is critical to the activation, proliferation and differentiation of T helper cells (47). Naïve CD4 T cells favor the generation of energy via mitochondrial pathways (48). The tricarboxylic acid (TCA) cycle is a highly efficient means of converting acetyl-CoA into carbon dioxide and ATP and leads to the generation of NADH and $FADH_2$ in the inner membrane of the mitochondria (47). These two products are vital for the transfer of electrons in the electron transfer chain (ETC) via complexes I–IV. Given its greater efficiency in terms of ATP generation compared to glycolysis, the TCA cycle is able to meet the energy needs of long lived cells such as naïve CD4 T cells (49, 50).

Fatty acid oxidation is a means by which T helper cells can convert fatty acids for the generation of significant amounts of energy. The initial steps occur in the cytosol using ATP to generate fatty acid acyl-CoA, which is transported into the mitochondria via carnitine palmitoyltransferase I (CPT-1). Beta oxidation of fatty acids then produces acetyl-CoA, NADH and $FADH_2$ (47), which all help to fuel the TCA cycle.

During initial activation, glycolysis becomes the dominant metabolic pathway in T helper cells (51). Under the control of transcription factors such as c-Myc and HIF-1α, extracellular glucose is taken up and catabolized to pyruvate, which yields 2 ATP per molecule of glucose (52–54) and provides a source of acetyl-CoA for the TCA cycle. Glycolysis also rapidly provides NADH and a range of intermediates, which are useful in anabolic pathways including nucleotide, amino acid and fatty acid biosynthesis (47). Reduction of pyruvate to lactate is also important to replenish NAD^+ levels within the cell.

In addition to oxidation of lipids, *de novo* fatty acid synthesis needs to take place and is controlled by enzymes including sterol regulatory element-binding protein (SREBP), fatty acid synthase (FAS) and Acetyl-CoA carboxylase (ACC) (55). Straight chain and branched fatty acids are produced from products generated during glycolysis, the TCA cycle and the pentose phosphate pathway (47). For straight chain fatty acids, citrate is exported from the mitochondria and converted into acetyl-CoA in the

cytosol. Following carboxylation by ACC, it can be further extended by FAS in a NADPH-dependent mechanism to varying chain lengths. In order to produce branched forms, amino acids such as leucine or valine are required while fatty acids may also be combined with glycerol to form triacylglycerides and phospholipids (56).

All metabolic pathways are highly intertwined since products and intermediates from one pathway can function as key synthetic precursors in other pathways. These pathways not only promote cell division, survival and expansion, but metabolites and co-factors can also directly influence gene expression by modifying chromatin, acting as ligands for transcription factors and influencing the stability of cytokine mRNAs in the cytosol.

Below, we review the literature on the metabolic demands of CD4 T cells, in particular as they relate to Th2 cells. We address what is known of early Th2 cell differentiation, which primarily occurs in the context of the lymph node and then address metabolic adaptations of Th2 cells in the context of tissue immunity.

METABOLIC CHANGES DURING EARLY ACTIVATION OF TH2 CELLS

Promotion of Th2 Cell Differentiation by Mammalian Target of Rapamycin (mTOR)

In early and probably also later differentiation of T helper cells, coordination of cell growth, proliferation and metabolism is mediated by the kinase mTOR (57) (**Figure 1**). The mTOR complex monitors nutrient availability and integrates signals from growth factors and cytokine receptors to regulate glucose, amino acid and lipid metabolism. mTOR complex1 (mTORc1) is formed with the scaffolding protein regulatory associated protein of mTOR (RAPTOR) while mTOR complex 2 (mTORc2) uses Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) as a scaffold (58). Differentiation toward the effector Th cell lineages Th1, Th2, and Th17 is known to be reliant on mTOR activity, while inhibition of mTOR with rapamycin has been shown to favor Treg cell differentiation (59–61). All effector lineages including Th2 cells have been shown to require mTORc1 activation, since deletion of RAPTOR and thereby mTORc1 potently inhibits effector differentiation (62).

A number of studies have also highlighted a specific role for mTORc2 in Th2 cell differentiation. mTORc2 inhibits suppressor of cytokine signaling-5 (SOCS5) (63), which in turn suppresses IL-4-dependent STAT6 signaling to block Th2 cell differentiation (64). SGK1, a downstream target of mTORc2, promotes Th2 cell lineage commitment while blocking Th1 cell development (65). Furthermore, deletion of the GTPase RhoA, another mTORc2 target leads to decreased glycolysis and IL-4 production (66). Thus, while there is a clear requirement for mTORc1 in early Th2 cell development, signals downstream of mTORc2 seems to have distinct positive effects on the differentiation of Th2 cells.

Early Induction of Glycolysis in T helper Cells

Naïve T cells rely primarily on oxidative lipid metabolism as they recirculate among lymph nodes (51, 52). However, naïve T cells are poised for a rapid switch to effector cell metabolism by accumulating untranslated mRNAs required for glycolysis and fatty acid synthesis (67). Activation of T cells through the T cell receptor, co-stimulatory ligands and cytokine receptors is followed by expansion, differentiation and production of effector cytokines; processes which place a great metabolic pressure on cells.

Cells can upregulate glycolysis at faster rates than oxidative phosphorylation, as glycolysis requires no mitochondrial growth (47). The high rate of glycolysis in effector T cells requires activation of mTOR, HIF-1α and increased expression of glucose transporters like Glut1, which is essential for CD4 but not for CD8 T cells (68, 69). Glut1 is translocated to the cell surface upon activation, a process mediated through the phosphatidylinositol 3-kinase (PI3K)-AKT pathway (70) and Myc expression (53). Glycolysis supports T cell activation in many ways; for instance by supporting epigenetic modifications through lactate dehydrogenase A (54) and by supplying dividing cells with many side products required for division and growth. Effector T helper cell subsets including Th1, Th2, and Th17 are all highly dependent on glycolysis for growth and function, and a small subset of Treg cells has also been shown to be highly glycolytic (61). Effector T helper cells have been shown to undergo various levels of glycolysis in *in vitro* assays and Th2 cells express the most Glut1 and appear the most glycolytic, when analyzed via Seahorse Analyzer (41, 51), suggestive of a more prominent role for the glycolytic machinery in these cells.

Fatty Acid Metabolism in Early T helper Cell Activation

A critical aspect of early T cell activation is the upregulation of lipid metabolism, especially lipid synthesis pathways, which enables cells to grow and divide. Typically, mTORc1 promotes lipid synthesis pathways by activating SREBP transcription factors (71). One of many targets of SREBPs is an enzyme essential in *de novo* synthesis of fatty acids, ACC1. In studies by Berod et al. (72), inhibition of ACC1 genetically or pharmaceutically prevented the differentiation of all effector lineages, while Treg cells preferentially differentiated from cultures of Th17 cells. This demonstrates that fatty acid synthesis is an essential feature of early T helper cell differentiation. In a separate study, it was suggested that early T helper cell activation, proliferation and growth may also rely on fatty acid uptake, orchestrated by the nuclear receptor, peroxisome proliferator activated receptor gamma (PPAR-γ) (73). However, the early division and proliferation of T helper cells was shown to be unaffected by genetic loss of PPAR-γ in another study (74) and the increase in PPAR-γ expression under neutral conditions is minor (73, 74). PPAR-γ becomes highly expressed specifically in Th2 cells and likely regulates fatty acid metabolism later in the Th2 cell differentiation program.

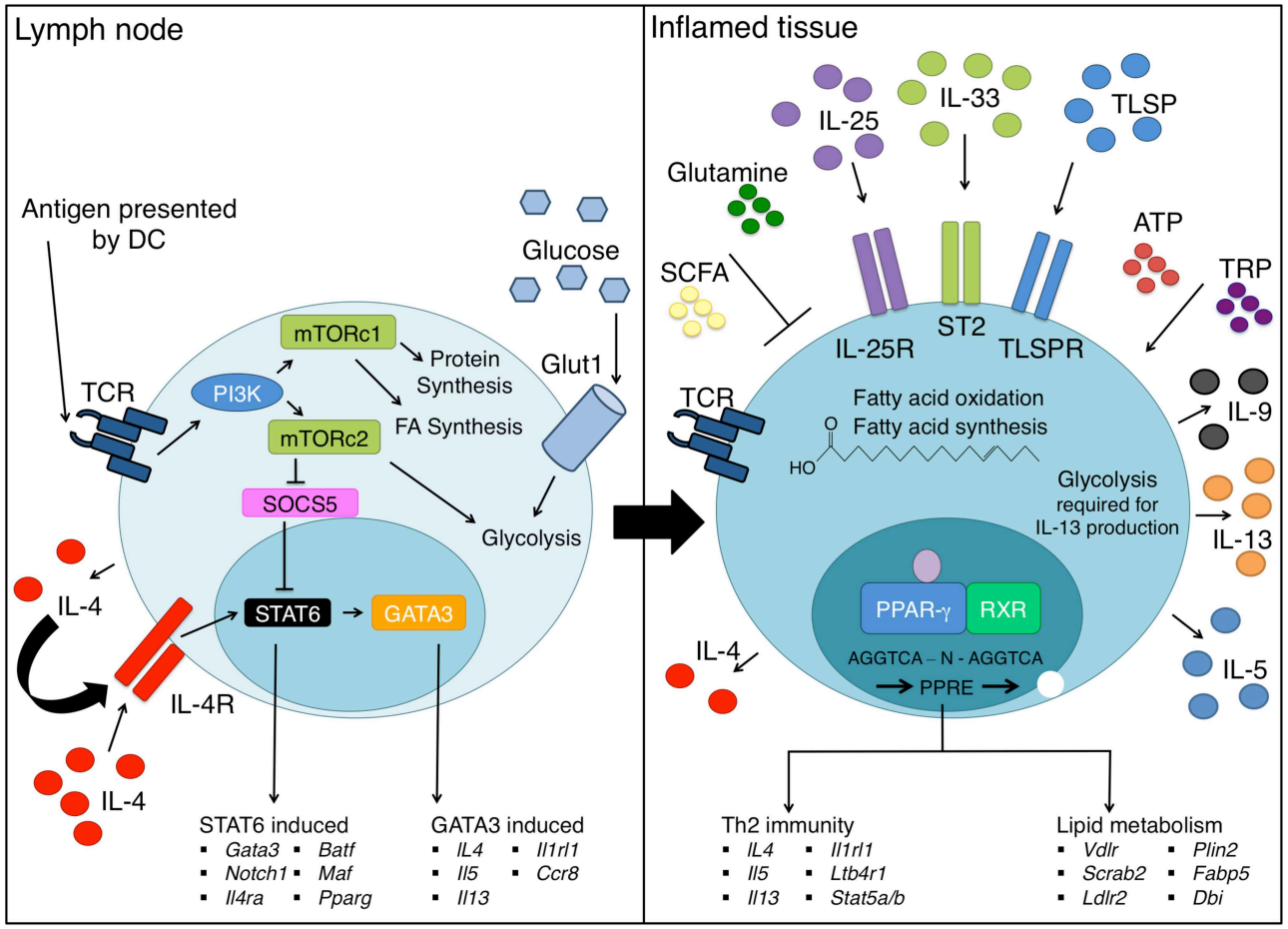

FIGURE 1 | Th2 cell differentiation requires extensive metabolic reprogramming. Upon encountering cognate antigen in the lymph node, naive CD4 T helper cells differentiate into Th2 cells under the influence of the IL-4-STAT6-GATA3 axis. Concurrently, changes in energy requirements occur as a Th2 cell differentiates, which requires an increase in glucose uptake via GLUT1 and marked upregulation of glycolysis. mTORc1 senses nutrient availability and co-ordinates metabolism in T helper cells, and mTORc2 promotes Th2 cell differentiation through several mechanisms. As Th2 cells enter inflamed tissue sites such as the lung, they continue to differentiate through exposure to a range of inflammatory cytokines including TSLP, IL-25, and IL-33 that promote production of effector cytokines such as IL-5, IL-9, and IL-13. In addition, PPAR-γ drives expression of lipid metabolic genes such as *Fabp4* and *Vdlr* as well as genes critical to Th2 cell effector functions, such as ST2 (*Il1rl1*) and *Il5*. Extracellular metabolites present in the tissues including SCFA, ATP, and TRP can further promote Th2 cell differentiation and/or function. On the other hand, glutamine obtained from the diet potentiates Th1 cells at the expense of Th2 cells.

METABOLIC CHANGES DURING LATER ACTIVATION OF TH2 CELLS

Inflammatory Cytokines in the Tissue Potentiate Th2 Cell Differentiation

Priming of CD4 T cells toward the Th2 cell subset in the lymph node induces the production of IL-4. However, several studies in the context of infection to *N. brasiliensis* and *H. polygyrus*, or to the allergen house dust mite (HDM) have shown that a large portion of IL-4-producing cells in these settings are Tfh cells (38, 75, 76). Full Th2 cell effector functions in the *N. brasiliensis* and *HDM* models, are not observed until T helper cells reach the lung tissue.

It is increasingly appreciated that the activation of epithelial and innate cells at the site of allergen, venom or pathogen entry plays an important role in shaping Th2 cell responses (77). Impaired barrier function, exposure to damage associated molecular patterns (DAMPs) and microbial products can trigger receptors like Toll Like Receptor-4 (TLR4) and Protease Activated Receptor-2 (PAR2) in epithelial and tuft cells that line the surface of the airways (78, 79). These cells as well as innate lymphoid cells (ILCs), macrophages and dendritic cells (DCs), in turn secrete a range of potent inflammatory cytokines including IL-1, IL-18, IL-25, IL-33, GM-CSF, M-CSF, and thymic stromal lymphopoietin (TSLP) (42, 77). In the case of DCs, cytokines such as TLSP and IL-33 can promote expression of OX40L and suppression of IL-12 which further promotes Th2 cell differentiation and function (80–85). These signals from epithelial cells and innate cells in the lung have been shown to contribute

significantly to the final identity of Th2 cells in the tissue environment (41, 42).

Studies of infection with the helminth *N. brasiliensis* suggest that cytokines such as IL-25, IL-33, and TSLP are not required for the initial stages of Th2 cell differentiation within the lymph node but function to shape those "primed" Th2 effector cells upon entry to the inflamed sites such as the lung (42). Furthermore, our recent work depicted that activated T cells entering the lung were exposed to type I interferons in the context of HDM challenge, although how this cytokine may impact on Th2 cell functions remains unclear (41).

Comparison of the transcriptional and chromatin landscapes of lung Th2 cells to Th2 cells or naïve CD4 T cells from the lymph nodes demonstrated stark differences in lung Th2 cells (41, 42), suggesting that these cells more closely aligned with type 2 ILC (ILC2) from the lung than their lymph node counterparts (86). Thus, striking changes in cellular identity occur when T helper cells reach inflammatory tissues, and this is likely coupled with important metabolic changes.

Activation of Lipid Metabolism Pathways Is a Prominent Feature of Tissue Th2 Cells

A distinguishing feature of Th2 cells in the airways of mice administered HDM antigens was a striking upregulation in the expression of genes related to lipid oxidation and synthesis (41) (**Figure 1**). Comparison of DNA accessibility by ATAC-Sequencing also revealed more open chromatin at many gene loci associated with lipid metabolism in Th2 cells, compared with other T helper cell subsets in the lung, or naïve CD4 T cells in the lymph node (41). This feature of Th2 cells appears to be shared by ILC2 in the gut and lungs (87, 88). Using etomoxir and orlistat *in vivo* to block fatty acid oxidation, synthesis and uptake, Wilhelm and colleagues demonstrated that ILC2 were highly dependent on fatty acid metabolism both for their expansion and function (88). Similarly, blockade of these pathways in Th2 cell-mediated inflammation of the airways reduced Th2 cell pathologies such as airway eosinophilia and goblet cell metaplasia, and appeared to reduce T helper cell expansion and the production of IL-5 and IL-13 by Th2 cells to some extent (41).

PPAR-γ: Linking Th2 Cell Function and Cellular Metabolism

A feature of Th2 cells, ILC2, and alternatively activated M2 macrophages is the expression of PPAR-γ, a master regulator of adipocyte differentiation and regulator of lipid metabolism in various cell types (89–93). PPAR-γ belongs to a superfamily of nuclear receptors whose transcriptional effects are regulated by many natural ligands and dependent on co-factors such as CEBP, RXRα and other transcription factors (94). In macrophages and dendritic cells, PPAR-γ expression is induced by IL-4R ligation and STAT6 activation and it is likely the same mechanism at play in Th2 cells (95–97). The absence of PPAR-γ prevents the acquisition of the M2 phenotype, with impaired fatty acid uptake and mitochondria biogenesis (96, 98). The absence of PPAR-γ in CD4 T cells ameliorated Th2 cell-associated pathology in airway inflammation models and impaired Th2 cell-mediated immunity to *H. polygyrus* (91, 93). An important facet of this phenotype was that PPAR-γ appeared to be particularly important for the pathogenic phenotype of Th2 cells in the lung (91, 93). Early activation of Th2 cells in lung-draining lymph nodes did not appear to be greatly affected (91). This suggests that PPAR-γ becomes important in sensing ligands in inflamed tissue. It remains unclear how the loss of PPAR-γ impacts on ILC2, although its high expression specifically in this subset of ILC, and its important role in M2 macrophages and Th2 cells implies that it could be important for ILC2 functions.

PPAR-γ Directly Promotes Th2 Cell Functions

The impact of PPAR-γ on the expression of the early Th2 cell effector cytokine IL-4 is ambiguous. Studies have characterized that the absence of PPAR-γ reduces (93), increases (74) or has no effect (91) on CD4 T cell-derived IL-4, thus making it apparent that the impact of PPAR-γ on IL-4 production is context and assay dependent. A clearer impact of PPAR-γ has been demonstrated for features of Th2 cells in lung tissue. For instance, the absence of PPAR-γ in CD4 T cells impairs the expression of ST2 in lung and airway Th2 cells, and significantly impairs the expression of IL-5 and IL-13 by CD4 T cells (91). In humans, PPAR-γ is highly expressed in $CRTH2^+$ Th2 cells thought to harbor the pathogenic Th2 cell subset (93). It has also been linked to IL-9 production by a subset of pathogenic Th2 cells, which are prevalent in lesions taken from the skin of contact dermatitis patients (99). Inhibition of PPAR-γ profoundly suppressed the frequency of $IL\text{-}9^+$ Th2 cell clones.

Mechanistic studies have pinpointed an enrichment for PPAR-γ binding sites at open chromatin regions in Th2 cells (41), and chromatin immunoprecipitation-sequencing (CHIP-Seq) has identified a number of critical target genes for PPAR-γ binding including *Ap1*, *Ets1*, *Runx1*, *Gata3*, *Stat5, Il5,* and *Il13* (100). Since PPAR-γ is a potent repressor as well as activator of gene transcription, it is difficult to predict the impact of this nuclear receptor through CHIP-Seq and ATAC-Seq analysis. Our own work demonstrated that the addition of PPAR-γ ligands to *in vitro* cultures had little direct impact on effector cytokine production by Th2 cells, but potently upregulated ST2 expression (91). For instance, the prostaglandin derivative 15dΔ12,14- PGJ_2 (15d-PGJ_2) was able to induce ST2 expression, as did synthetic agonists such as pioglitazone (101, 102), a member of the class of clinically-approved compounds known as thiazoldinediones (TZDs). Thus, PPAR-γ plays an important role in shaping the chromatin architecture of Th2 cells and appears particularly important for late stage effector functions of Th2 cells.

A Role for PPAR-γ in Modulating Th2 Cell Metabolism

While PPAR-γ is a well-characterized regulator of cellular metabolism in macrophages, dendritic cells, tumor cells and adipocytes (103–105), its impact on Th2 cell metabolism is less well-understood. In co-operation with STAT6, PPAR-γ is thought to regulate lipid metabolism in DC and macrophages through the regulation of genes including *Fabp4* (97).

In Th2 cells, arrays of PPAR-γ-deficient $ST2^+$ T helper cells suggested that a range of metabolic pathways may be affected by the absence of PPAR-γ including carbohydrate synthesis, metabolite transport, lipid storage and lipolysis (91). However, these gene expression arrays are complicated by the fact that $ST2^+$ Th2 cells have difficulty differentiating into fully pathogenic Th2 cells. In the study by Angela and colleagues, PPAR-γ was shown to be induced by mTORc1 activation and particular important for the expression of genes associated with fatty acid uptake and lipolysis including *Ldlr, Scrab2*, *Vdlr, Plin2, and Fabp5* (73). In this study, silencing of PPAR-γ impaired oxidative metabolism and glycolysis suggesting that PPAR-γ may not only promote lipid metabolism.

Thus, PPAR-γ plays important roles in promoting the expression of critical Th2 cell-associated factors such as ST2, but also likely contributes to regulating the lipid metabolism in these cells, especially in the tissue context. More mechanistic studies are required to dissect the impact of PPAR-γ on cellular metabolism in T cells, potentially in the context of overexpression systems.

Glycolysis and Th2 Effector Cell Function *in situ*

Glycolysis is not only important in the early phases of T cell activation but may also play a direct role in shaping T cell effector functions in inflamed tissues such as the lung. Active glycolysis has been shown to promote production of IFN-γ by Th1 cells and CD8 T cells *in vitro* and in the tumor microenvironment (106, 107). In the absence of active glycolysis, the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) bound the 3'UTR of *Ifng* mRNA, impeding its translation (106). Our own recent studies depicted that following *in vivo* or *in vitro* blockade of glycolysis, the expression of IL-13 and IL-5 was significantly reduced (41). Whether this is also mediated through GAPDH remains to be determined.

High concentrations of extracellular lactate, a byproduct of glycolysis, has also been shown to reduce the CCL5-induced motility of CD4 T cells. This effect is mediated by the sodium lactate transporter Slc5a12 and has been proposed as a mechanism retaining effector cells at sites of inflammation (108). It has also been shown to potentiate CD8 T cell function (109). Whether these mechanisms play a role in Th2 cell function and the pathology of asthma remains to be shown.

In summary, while glycolysis appears to be important for effector cytokine production by T helper cells, Th2 cells in tissues appear enriched for pathways associated with lipid metabolism compared to other T helper cell subsets and naïve cells. This is typified by the expression of genes associated with fatty acid metabolism, the open chromatin state of Th2 cells at several key genes associated with lipid metabolism, and the requirement for PPAR-γ in mediating robust Th2 cell-mediated immune responses.

THE ROLE OF EXTRA CELLULAR METABOLITES ON TH2 CELL DIFFERENTIATION AND FUNCTION

In addition to the activation of PPAR-γ by derivatives of prostaglandins and medium chain fatty acids, a range of other metabolites have been postulated to regulate Th2 cell differentiation and function.

Extracellular ATP

ATP found in the extracellular environment operates as a potent DAMP due to its almost complete absence from healthy tissues and its quick release following cell damage (110). It is sensed via P2X and P2Y receptors, expressed throughout the immune system. It has been widely shown that levels of extracellular ATP are elevated in the bronchoalveolar lavage fluid (BALF) of asthmatic patients in comparison to healthy controls (111) and it is thought to induce migration of eosinophils and activation of mast cells in the lung and airways (112). Unlike Treg cells, Th2 cells appear to be relatively insensitive to cell death induced by extracellular ATP (113). Exposure of mice to inhaled allergens such as ovalbumin (OVA) results in an increase of ATP in the airways (111). Non-degradable forms of ATP, which cannot be metabolized by ectonucleases CD39 and CD73, are capable of breaking tolerance and inducing type 2 responses to inert antigens such as OVA (111). It can also be induced in airway epithelium in response to allergens such as the Cockroach allergen, Per a 10, or aeroallergens derived from *Alternaria alternata*. It can drive IL-33 release which further supports Th2 cell differentiation and metabolic reprogramming *in situ* (114, 115). Autocrine ATP is sensed via the $P2Y_2$ receptor, which increases intracellular Ca^{2+} concentrations that in turn increase IL-33 release. Blockade of the $P2Y_2$ receptor is sufficient to halt Th2 cell induction (115). ATP has also been shown to induce DCs that promote Th2 cell responses. Interestingly, CD39-deficient mice have defective Th2 cell responses to both OVA and HDM (116). In the absence of CD39, DCs in these mice have impaired purinergic receptor activity, appear less able to upregulate co-stimulatory molecules and exhibit defects in chemotaxis. These studies suggest an important inflammatory role for ATP in driving Th2 cell responses to a range of allergens.

Short Chain Fatty Acids (SCFAs)

The gut microbiome greatly influences the composition of metabolites that is in our circulation. One important immune regulatory product of fermenting bacteria in the intestines are SCFAs. SCFAs can be transported into cells via various receptors and have been shown to contribute to epigenetic modifications (117). SCFAs also bind to G-Protein Coupled Receptor 41 (GPR41, also known as FFAR3) and GPR43 (FFAR2) (118) and can modulate immune cell functions through these receptors. Typically, SCFAs have been shown to suppress inflammation and promote tolerance by various mechanisms. In line with this, mice fed a high fiber diet and hence with high circulating levels of SCFAs, have been shown to develop reduced airway inflammation in OVA/alum and HDM models of allergic airway disease. In one study, SCFAs appeared to suppress

dendritic cell activation and migration through GPR41 (118). In another study, GPR41 was proposed to promote Treg cell IL-10 production, thereby suppressing allergic airway inflammation (119). Administration of a high-fiber or high-acetate diet in pregnant mice was also shown to reduce allergic airway disease in progeny, by promoting acetylation of the Foxp3 locus in Treg cells, highlighting the potent immune modulatory effects of these molecules (120). Studies of ILC2 have also shown that type 2 cytokine production and GATA3 expression may be dampened by SCFAs (121, 122). Despite the known anti-inflammatory function of SCFAs, a recent study of Th2 cells in people with eosinophilic esophagitis and in a mouse model of fungal infection revealed that SCFAs may also potentiate Th2 cell cytokine production (123). Thus, the impact of SCFAs on Th2 cell differentiation and function requires further investigation.

Glutamine

The conditionally essential amino acid glutamine is found at relatively high concentrations in the plasma and is capable of providing cells with a potential source of energy. However, under conditions of catabolic stress such as tissue damage or infection demand for glutamine rises with immune cells consuming particular high levels (124). Upon activation, T cells increase their uptake of glutamine 5–10-fold through the glutamine specific transporters SNAT-1/2 (124, 125). It serves as an important source of nitrogen and as an anapleurotic substrate for the TCA cycle and production of ribose in T cells (126–128). A lack of glutamine results in a failure for sustained proliferation and impairs cytokine release from T cells (125). Although glutamine plays an essential role in T cell activation, the addition of glutamine to the diet has been shown to favor Th1 cell responses over Th2 cells (129). Similarly, addition of high concentrations *in vitro* impairs Th2 cell differentiation in human PBMC cultures (130). This is at least in part due to the ability of high concentrations of glutamine to inhibit cytosolic phospholipase A2 (cPLA2), a key enzyme in releasing arachidonic acid from glycerophospholipids (131). This in turn provides the precursor molecules for a number of eicosanoids including well-known inflammatory mediators such as leukotrienes (LTs), prostaglandins and platelet-activating factor (PAF), which are important for Th2 cell functions. Thus, glutamine has the ability to regulate Th2 cell responses.

Indoamine 2,3-dioxygenase (IDO)

IDO is the rate-limiting enzyme required for tryptophan (TRP) metabolism (132). Within tissues such as the lung, IDO expression is high on epithelial cells and certain DC subpopulations (133). Given its constitutively high expression on Treg cells and cancer, IDO has been widely linked to immune suppression although some studies have indicated that it can in fact promote Th2 cell function. For instance it has been observed that 3-hyrdroxyanthranilic and quinolinic acids, metabolites of the KYN pathway, are capable of inducing apoptosis in Th1 cells without affecting Th2 cells (134). IDO expression by eosinophils is capable of inhibiting IFN-γ production by Th1 cells with no effect on Th2 cell function (135).

It has also been shown that IDO can potentiate Th2 cell cytokine production during *in vitro* differentiation of Th2 cells (133). In the context of airway inflammation, IDO-deficient mice appeared to have reduced Th2 cell responses and reduced levels of circulating IgE. Thus, IDO may aid in potentiating the polarization of Th2 cells and possibly inhibit bystander Th1 cells. Reduced expression of IDO during pregnancy results in enhanced ratios of Th1:Th2 cells (136). Interestingly, KYN-TRP levels are profoundly influenced by the composition of the gut microbiota via activation of AhR and TLRs in the host (137). Thus, regulation of TRP metabolism by IDO appears to promote Th2 cell responses.

METABOLIC INTERVENTIONS TARGETING TYPE 2 INFLAMMATION

Clinical and epidemiological studies have long indicated that type 2 inflammation, allergies and metabolic disorders are highly linked. For instance, a strong link between obesity and asthma has been reported in many studies (138, 139). In addition to BMI, other abnormalities in metabolism are also thought to predispose children and adults to asthma (138, 139). One obvious solution to reducing asthma linked to obesity is weight loss through exercise, although it can be difficult for asthmatics due to exercise-induced exacerbations. Nonetheless, aerobic exercise is known to reduce lung and airway inflammation, and reduce pathogenic cytokine secretion (140), which suggests that the right form of exercise could alleviate symptoms of asthma, in some patients.

Despite recent evidence that PPAR-γ promotes Th2 cell functions in mice and humans (91, 93), preclinical studies had repeatedly shown that PPAR-γ agonists reduced goblet cell metaplasia, alarmin release and airway hyperresonsiveness in mouse models of asthma (141–144). For this reason, several trials of TZDs were initiated in asthma and COPD. However, one recent trial using pioglitazone resulted in exacerbations in 14% of severe asthmatics. No patients experienced improvements in their disease symptoms, resulting in a premature cessation of this trial (145, 146).

In the last few years, a number of trials of putative anti-inflammatory dietary compounds has been initiated. A recent trial of polyunsaturated fatty acids conducted in pregnant women in Denmark showed that infants born to mothers on this supplement had a reduced absolute risk of developing wheeze and asthma in the first 3 years of life (147). Thus, modulating inflammation through the diet of mothers shows promise as a way to prevent allergy in infants. Furthermore, the anti-inflammatory effects of high fiber diets in preclinical studies have led to the commencement of trials in various disease settings including diabetes and asthma (148, 149). The results of these trials are eagerly anticipated.

CONCLUDING REMARKS

As Th2 cells differentiate, their metabolic requirements and exposure to nutrients changes dramatically. In the early activation of CD4 T cells, strong induction of glycolysis and

lipid metabolism is required to kick start the differentiation of effector T helper cell lineages including Th2 cells, seemingly at the expense of Treg cell differentiation. These pathways drive cell division and proliferation in essentially all T helper cell subsets, although mTORc2 appears to promote Th2 cell differentiation via several specific mechanisms. The extracellular environment changes profoundly when Th2 cells move from the draining lymph node to inflamed tissues via the vasculature. In the blood and in non-lymphoid tissues, T helper cells become exposed to levels of glucose, lipids, SCFAs and amino acids that are known to vary highly between individuals, and can have an important impact on Th2 cell differentiation. Important sensors for these factors include solute carrier proteins, ectonucleotidases, G-protein coupled receptors and nuclear receptors like PPAR-γ. In tissues, Th2 cells appear insensitive to death induced by ATP-sensing receptors, rely on lipids either as a source of energy or as ligands for PPAR-γ, and are susceptible to regulation by SCFAs released by the microbiota. Hence, sensors of the extracellular environment influence the metabolism and function of T helper cell subsets in peripheral tissues and can have a strong bearing on the cytokine balance of an individual.

A major challenge going forward is whether we can understand precisely how all of the metabolic components in our blood and tissues work together to regulate T helper cell responses in humans. An issue with current studies in metabolomics is that they are conducted in patients with disease, who can have metabolic disruptions for multiple reasons. An important goal for the future is to conduct prospective cohort studies of healthy individuals, in order to understand how the metabolome shapes the T helper cell balance and impacts on the development of allergic diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Metabolic Profile of Tumor and Virally Infected Cells Shapes Their Microenvironment Counteracting T Cell Immunity

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Upon activation naïve T cells undergo metabolic changes to support the differentiation into subsets of effector or regulatory cells, and enable subsequent metabolic adaptations to form memory. Interfering with these metabolic alterations leads to abrogation or reprogramming of T cell differentiation, demonstrating the importance of these pathways in T cell development. It has long been appreciated that the conversion of a healthy cell to a cancerous cell is accompanied by metabolic changes, which support uncontrolled proliferation. Especially in solid tumors these metabolic changes significantly influence the tumor microenvironment (TME) and affect tumor infiltrating immune cells. The TME is often hypoxic and nutrient depleted, additionally tumor cells produce co-inhibitory signals, together suppressing the immune response. Interestingly, viruses can stimulate a metabolism akin to that seen in tumor cells in their host cells and even in neighboring cells (e.g., via transfer of virally modified extracellular vesicles). Thus, viruses create their own niche which favors viral persistence and propagation, while again keeping the immune response at bay. In this review we will focus on the mechanisms employed by tumor cells and viruses influencing T cell metabolic regulation and the impact they have on shaping T cell fate.

Keywords: metabolism, tumor, T cell, virus, hypoxia

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INTRODUCTION

In recent years the fundamental importance of energy regulation in immune cells has been appreciated and has created the research field of "Immunometabolism." Since there are excellent current reviews discussing the metabolic regulation of T cells in detail (1–3), we will here only give a short overview and then focus on the role of the tumor or virally infected target cell in manipulating T cell fate. The reader might also find **Table 1** helpful, summarizing the main mechanisms discussed for a quick overview. Naïve T cells are relatively quiescent cells that have a low energetic demand. They predominantly make use of mitochondrial oxidative phosphorylation (OXPHOS). Upon antigen encounter and activation by professional antigen presenting cells, T cells increase the expression of nutrient transporters, especially glucose transporters 1 and 3 (GLUT1 and 3) (26) and start utilizing glycolysis even in the presence of sufficient oxygen. Glycolysis provides fast energy and biological building blocks required for cell division and effector function. Alongside,

mitochondrial biogenesis is also activated and mitochondrial mass increased, allowing for increased respiration. Furthermore, a recent study highlights the important role of distinct mitochondrial metabolic pathways in regulating T cell proliferation and effector differentiation (27). Mitochondrial function is critical, and its disruption seems to be an underlying mechanism of T cell exhaustion (28–30). Upon the resolution of an acute insult (such as an acute viral infection) a proportion of the effector T cell pool differentiates into memory cells. This conversion is accompanied by the cell's metabolism refocussing on OXPHOS and fatty acid metabolism, while reducing glycolysis. Memory T cells have an increased mitochondrial mass and contain mitochondria with densely packed cristae linked to more efficient OXPHOS (31), poised to mount a fast recall response. In the situation where the immune response fails to deal with the insult effectively and T cells are subjected to persisting antigenic challenge like in cancer and chronic viral infection, effector responses become less vigorous and instead of developing into classical memory cells, formation is skewed toward exhaustion. Here we will discuss aspects of how the metabolic profile and signaling by target cells shapes their microenvironment and the impacts on T cell function, differentiation and fate decision.

TABLE 1 | Table summarizing metabolic pathways and their role in tumors/TME and viral infection.

Mechanism	Tumor	Virus
Glycolysis	(i) Glucose depleted TME (ii) Inhibition of effector T cells (4, 5)	Stimulation of increased glycolysis in host cell for viral production and in neighboring cells via exosomes e.g., HTLV, HIV, KSHV, EBV (6, 7)
Hypoxia/ pseudohypoxia	Stabilization of HIF1-α: (i) Enhances glycolysis and acidosis of TME (8) (ii) Expression of ectoenzymes CD39/CD73 increasing extracellular adenosine levels (9) (iii) Upregulation of PD-L1 (10)	Stabilization of HIF1-α mimicking the effect of hypoxia (termed: pseudohypoxia) e.g., KSHV, EBV, HCV, HCMV, HPV (11, 12)
Lactate production	(i) Acidification of TME (ii) Inhibition of effector T cells (13, 14) (iii) Inhibition of CD4+ Th1 T cells (15) (iv) Promotion of Treg (15) (v) Induction of regulatory macrophages (16) (vi) Upregulation of PD-L1 (17)	Lactate secretion from (i) infected cells and (ii) neighboring cells stimulated by virally infected cells, creates microenvironment supporting viral propagation (e.g., KSHV)? (4, 18)
Amino acid depletion	(i) Increased glutaminolysis leads to glutamine depletion in TME (19) (ii) Expression of indoleamine 2,3-dioxygenase leads to depletion of tryptophan (20) (iii) Recruitment/induction of MDSC, which can deplete the essential amino acids cysteine and arginine Inhibition of effector T cells Induction of Treg (23)	Recruitment of MDSC e.g., to HBV infected liver (21) Inhibition of amino acid uptake mediated by HIV Vpu protein (22)
Lipid metabolism	Induction of increased release of fatty acids by adipocytes to fuel tumor (24)	Induction/modulation of fatty acid production e.g., CMV, KSHV, HCV, Zika, Dengue (25)

THE INCREASED GLYCOLYSIS IN CANCER CELLS IMPACTS ON T CELLS

Reprogramming of energy metabolism has been recognized as one of the hallmarks of cancer cells (32). It is well established that cancer cells have increased glucose uptake, which is fermented to lactate even in the presence of oxygen, a process known as aerobic glycolysis (or Warburg effect). Of note, glucose can also be further metabolized through the mitochondrial tricarbolyxic acid (TCA) cycle in tumors (33).

Glucose is an important source of carbon for the production of amino acids, nucleotides, and fatty acids. Oncogenic mutations of the phosphatase and tensin homolog (PTEN) and the phosphoinositide 3-kinase (PI3K) pathway lead to the reprogramming of glucose metabolism and increased glucose uptake via stimulation of glucose transporters in cancer cells [recently reviewed by Marbaniang et al. (34)]. Increased transcription of glycolysis genes in KRAS mutated colorectal cancer cell lines (35) is another example of the impact of oncogenic mutations on the cellular metabolic state. Mutations in the tumor suppressor gene p53 was shown to play a role in glucose metabolism, and interestingly also in mitochondrial activity and lipid metabolism (36).

The sustained consumption of glucose by tumor cells eventually leads to a decrease of glucose levels in the TME. Competition for glucose between tumor and T cells has been shown to decrease IFN-γ production by CD8+ T cells and to limit T cell antitumor functions (4, 5). Conversion of glycolysis intermediates by the pentose phosphate pathway generates NADPH (that can serve as an electron acceptor), which is needed for tumor cells to scavenge reactive oxygen species (ROS) and maintain redox homeostasis. ROS produced by tumor cells participate in the oxidative stress T cells encounter in the TME, and interestingly, Tregs are more resistant than conventional CD4+ T cells to oxidative stress-induced cell death (37).

THE INFLUENCE OF HYPOXIA ON T CELL FUNCTION

Reduced blood flow in some tumor areas results in low oxygen levels (hypoxia) and acidification as discussed below and shown in **Figure 1**. Hypoxia leads to the stabilization of the transcription factor hypoxia-inducible factor 1-α (HIF1-α). HIF1-α in cancer

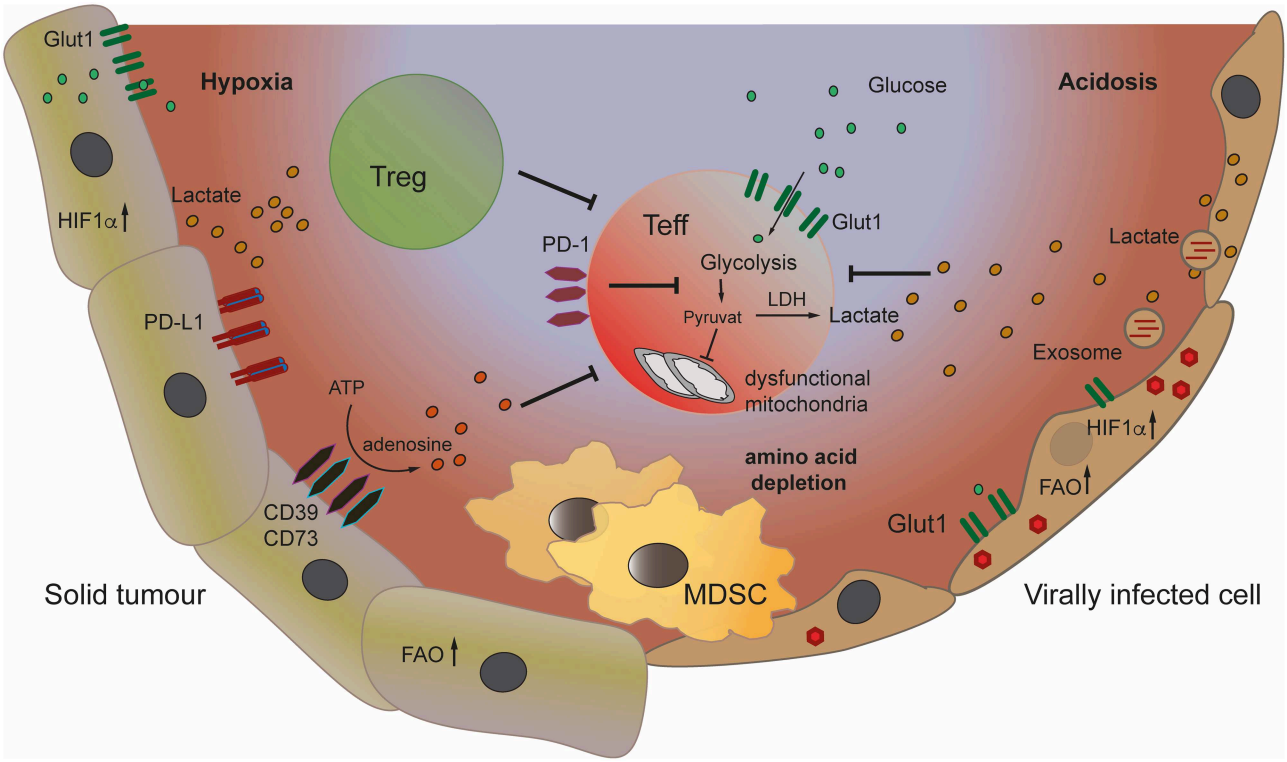

FIGURE 1 | Metabolic changes in tumor and virally infected cells can create a suppressive microenvironment leading to inhibition of effector T cells. The architecture of the tumor microenvironment (TME) can create hypoxic areas, leading to stabilization of HIF1-α in tumor cells and increase of glycolysis, depleting the TME of glucose. Increased glycolysis and production of lactate leads to acidification of the TME. Lactate inhibits effector T cells, while promoting Treg. Tumor cells convert ATP to immune suppressive adenosine by expressing the ectoenzymes CD39/CD73. The recruitment and induction of myeloid derived suppressor cells (MDSC) to the TME and to virally infected organs increases immune inhibition. Viruses also induce glycolysis and lactate production in infected and neighboring cells through the transfer of viral signaling molecules to their target cells via exosomes.

cells promotes glucose uptake by upregulation of GLUT1, GLUT3, and increased expression of glycolytic enzymes further promoting glycolysis and acidosis (8). In an elegant study Walton et al. have demonstrated that high acidity in the context of hypoxia results in inhibition of mTOR signaling in T cells and induction of T cell anergy. mTOR is a key sensor of nutrients and a major regulator of cellular metabolism and effector T cell function (38). Earlier studies had demonstrated that mTOR inhibition in the presence of TCR triggering drives T cell anergy (39), while promoting Treg development (40). Taken together these mechanisms might conceivably play a role in the tumor microenvironment *in vivo*, with both T effector cell inhibition and increased Treg numbers detrimental to tumor control. Various viruses (e.g., HCV and human papillomaviruses) have also been shown to manipulate the host cell's metabolism by promoting through different mechanisms HIF1-α stability and activity (11, 12) in the absence of hypoxia leading to subsequent increased glycolysis. In a recent report, influenza A (H1N1) virus led to proteasome inhibition and in turn stabilization of HIF1-α in normoxic conditions, however the impact on viral replication remains to be determined (41).

Apart from driving increased glycolysis HIF1-α induces expression of the ectoenzyme CD73 (9) and the expression by cancer cells and Tregs of the tandem ectoenzymes CD39/CD73 which generate extracellular adenosine from the degradation of extracellular ATP. The binding of adenosine to the 2A2-adenosine receptor (A2AR) expressed by many immune cells including T cells, inhibits anti-tumor T cells (42). Finally, HIF1-α is also involved in the upregulation of PD-L1 by tumor cells (10). Interestingly, a deregulated oxidative metabolism in tumors and the associated hypoxia in the TME, correlated with resistance to PD-1 treatment (43).

A recent publication shows that hypoxia and glucose deprivation lead to down-regulation of MHC class I molecules on tumor cells facilitating immune escape. This finding was accompanied by tumor cells losing their sensitivity to IFN-γ mediated induction of MHC upregulation (44). Consequently, tumor cells evade killing by activated IFN-γ producing T cells creating another hurdle for T cell therapies.

TUMOR CELLS PRODUCE LACTATE THAT PROMOTES IMMUNE SUPPRESSION

In addition, the lactate secreted by glycolytic tumor cells into the TME adversely impacts on effector T cells. Lactate inhibits

CD8+ T cell proliferation, cytokine (IL-2 and IFN-γ) production, and cytotoxicity (13, 14), but also induces T cell apoptosis (45). These effects on activated CD8+ T cells by increased lactate levels are mediated through inhibition of NFAT upregulation and inhibition of phosphorylation of p38 MAP kinase and JNK (14, 45). A recent report demonstrated that exogenous lactate can reduce the frequency of Th1 CD4+ T cells and their IFN-γ production, while increasing the expression of FoxP3 and the frequency of regulatory CD4+ T cells (Tregs) (15). Expression of cell surface GLUT1 is lower in Tregs as compared to other CD4+ T cell subsets, and Tregs appear not to rely on glycolysis but rather lipid oxidation and OXPHOS (46). FoxP3 was shown to play a role in inducing resistance to the suppressive effects of lactate by mediating distinct metabolic adaptation in Tregs (18). The accumulation of lactate in the TME leads to TME acidification known to decrease T cell proliferative capacity and inducing anergy (47) (**Figure 1**). Interestingly, expression of the lactate receptor GPR81 by macrophages was shown to play a role in inducing immune regulatory genes and regulating inflammation (16).

Lactate was also shown to upregulate programmed cell death ligand-1 (PD-L1) expression in lung cancer cells (17). Interestingly, PD-L1 blockade on tumor cells inhibited their mTOR kinase activity and decreased the expression of glycolytic enzymes (4), which might contribute to the success of anti-PD-L1 checkpoint blockade.

Finally, the increased concentration of lactate in the TME seems to be advantageous to some tumors, as lactate can be converted to pyruvate to fuel the TCA cycle. Glycolytic tumor cells can therefore produce the fuel for neighboring cells in heterogenous tumors (48), while at the same time suppressing the immune response.

TUMOR CELLS DO NOT SOLELY RELY ON GLUCOSE AS A SOURCE OF ENERGY BUT UTILIZE AMINO ACIDS LEAVING THE TME DEPLETED

TCA activity can be replenished by mitochondrial metabolism of various amino acids such as alanine, cysteine, leucine, and fatty acids (33). Recent data shows that at least some tumors depend on the non-essential amino acid glutamine (in humans, the most abundant amino acid in the circulation), as a source of nitrogen needed for nucleotide biosynthesis, and of carbon to fuel the TCA cycle (49). Metanalysis of studies assessing the metabolic profile in patients with cancer have revealed that in addition to the expected increase in lactate in tumor tissues, other metabolic changes can be identified (50, 51). Glutamate and kynurenine were the two most frequently elevated metabolites when 343 pairs of tumor/normal samples were compared.

Glutamine metabolism is upregulated by many oncogenic mutations (52). The *MYC* oncogene is one of the most frequently amplified genes in human tumors. MYC upregulates glutamine transporters, and MYC-transformed cells are dependent on glutamine metabolism (19). This can lead to reduced levels of glutamine in the TME, compared to normal tissues, resulting in limited availability for T cells. In order to sustain the energetic demands of cell proliferation and differentiation following T cells activation, T cells upregulate glutamine uptake, and enzymes for glutamine metabolism. Interestingly, extracellular glutamine deprivation and subsequent decreased intracellular pool of the glutamine-derived α-ketoglutarate promotes a shift in murine CD4+ T cells toward Treg differentiation (53). This was supported by data using human T cells where inhibition of glutaminolysis (conversion of glutamine into TCA cycle metabolites) promoted Treg differentiation (54).

Competition for glutamine may therefore represent an additional mechanism of immunosuppression in the TME.

Furthermore, many tumors constitutively express indoleamine 2,3-dioxygenase (IDO) which catabolizes the essential amino acid tryptophan depleting it from the TME inhibiting T cell proliferation (20). Depletion of tryptophan suppresses CD8+ effector T cell proliferation but again promotes Treg differentiation via activation of the GCN2 kinase (55). Tryptophan metabolism also releases the immunosuppressive catabolite kynurenine that activates the aryl hydrocarbon receptor which also promotes Treg differentiation (56). IDO inhibitors have been tested in clinical trials, but responses were overall disappointing either as single agents, or in combination with anti-PD1 therapy leading to a halt of some combination therapy phase III trials (57).

Tumors are well known to induce and attract myeloid derived suppressor cells (MDSC), which crucially can suppress both innate and adaptive immune responses (**Figure 1**). One mechanism being nutrient depletion by the sequestration of cysteine and the production of arginase-1, an enzyme leading to the break down of arginine, both amino acids being essential for T cells. In contrast to other cells T cells cannot convert the oxidized precursor cystine to the reduced amino acid cysteine and are dependent on extracellular levels (23). The depletion of arginine which has been demonstrated to contribute to suppression of T cell responses in cancer (58) is also operative in chronic viral infection. The HIV protein Vpu antagonizes amino acid uptake into CD4+ T cells (22), while in chronic hepatitis B virus (HBV) increased numbers of MDSC found in the infected liver correlate with low levels of arginine (21). As a consequence of the above combined mechanisms T cells in the TME and in chronic viral infections can find themselves depleted of essential amino acids, leaving them little ability to function effectively.

THE ROLE OF LIPID METABOLISM IN THE REGULATION OF T CELL RESPONSES

An enhanced lipid metabolism is crucially required for the synthesis of cell membranes in blasting and proliferating T cells (59) and highly organized lipid rafts in the membrane of effector T cells which enable the organization of the immunological synapse (12). A perturbation of the cholesterol and fatty acid homeostasis leads to a reduction in effector T cells. Furthermore, the development of T cell memory has been shown to be dependent on increased mitochondrial fatty acid oxidation (60, 61). Like proliferating T cells, proliferating cancer cells

require fatty acids for the synthesis of membranes and other molecules. Many tumor cells acquire fatty acids through *de novo* synthesis, however some ovarian, prostate and breast cancers rely on the uptake of exogenous fatty acids (62). Tumor cells have been shown to communicate with adipocytes to enhance provision of fatty acids (24), establishing a link between obesity and increased risk of cancer. Viruses likewise manipulate their host cell's lipid metabolism, for example human cytomegalovirus (CMV) induces an increase in fatty acid production to synthesize lipids for incorporation into the viral envelope (63). Hepatocytes infected by Hepatitis C virus (HCV) are forced to increase lipogenesis and gluconeogenesis to support viral particle production via sophisticated mechanisms involving viral proteins and interference with host miRNAs (64, 65). Since hepatocytes are vital in regulating systemic glucose and lipid homeostasis these manipulations by HCV lead to a significantly increased risk for patients to develop metabolic disorders. Pathogenic Flaviviruses, such as Zika and Dengue virus also rely on their host's lipid metabolism to complete their life cycle and thus interfere with this pathway to remodel intracellular membranes to allow virion biogenesis (25).

The importance of lipid metabolism in both cancer and viral infection make these pathways interesting candidates for therapeutic intervention.

TUMORS, TME AND T CELLS ARE HETEROGENOUS

One should keep in mind that tumor cells are proliferating, rapidly evolving cells, and metabolic changes are very heterogenous across different cancers, between patients with the same type of cancer, and within the same patient since spatial and temporal tumor cell heterogeneity also occur (66). In addition to intrinsic tumor cell differences, the heterogeneity of the tumor microenvironment (including oxygen levels/perfusion levels) influences the metabolic changes in tumor cells, which in turn as well shows heterogeneity. Tumor metabolomic analysis provides important information, however more efforts for instance in standardization of sampling, data analysis, choice of sample (cell lines, tissues, or blood), to ensure that only cancer specific changes are detected, are needed. These caveats are also true for the study of many chronic viral infections, especially where culture models are hard to establish and/or *in vivo* studies are limited to humanized models or primates where humans are the exclusive natural host of a virus as in HIV, HBV, HCV, and EBV.

As discussed above, metabolic suppression in the TME is important in inhibiting effector T cells and many solid tumors are devoid of much T cell infiltrate. Since T cell memory formation is equally dependent on metabolic programs, this can also be inhibited and skewed toward T cell deletion or dysfunction. Indeed, T cell exhaustion or Treg development occur in the TME instead of classical memory differentiation. It could be argued that exhaustion is a distinct type of memory, since exhausted T cells can be long lived, do retain limited effector functions and exert control over persistent viral infections (67–69). Thus, anti-viral CD8+ T cells with exhausted phenotype(PD-1 intermediate/high, low IFN-γ/effector cytokine production) can for example maintain a low viral load in patients with untreated chronic HBV (70). Furthermore exhausted T cells can be reinvigorated by stimulation with cytokines such as IL-12 (71) and are targeted in immune checkpoint inhibitor therapy (69).

VIRUSES MANIPULATE THE METABOLISM OF BOTH THEIR HOST CELL AND CELLS IN THEIR MICROENVIRONMENT

Viruses acquire both the energy and the building blocks needed to synthesize progeny virions from their host. For this reason, it is not surprising that many viruses manipulate their host cell metabolic pathways and associated signaling cascades [reviewed in (6, 7)]. Interestingly, many of these metabolic changes mimic those found in cancer. This suggests that in the case of oncogenic viruses, these metabolic alterations also contribute to cellular transformation. An example of shared metabolic alteration between viruses and cancer is the induction of the Warburg effect. Similar to many cancer types, different viruses were shown to shift glucose metabolism and redirect the glycolysis end product, pyruvate, away from mitochondrial OXPHOS. Interestingly different viruses developed diverse mechanisms to manipulate glucose metabolism in their host cells. Moreover, increased glycolysis and reduced OXPHOS were shown to support both viral replication and latency, by activating biosynthetic pathways supporting viral propagation.

In the last decade, there is accumulating evidence that viruses not only manipulate the infected cells but also communicate and manipulate other cells in their microenvironment. One method viruses use for this is manipulation of extracellular vesicle (EV) secretion from the host cell (**Figure 1**). An increasing number of viruses has been shown to manipulate EV-secretion and cargo(72–84).

The field of EV has been extensively studied in the last years, mainly in cancer. Tumor-derived EVs were shown to have a dramatic effect on tumor growth and metastasis [reviewed in (85–87)]. Interestingly, it was shown that both cancer cells and viruses use EV to alter the metabolism of cells in their microenvironment. A fascinating example of this phenomenon comes from the two oncogenic gammaherpesviruses Epstein Barr Virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV). Both viruses establish latency quickly after primary infection. Though during latency, these viruses express only a small subset of their genome, both viruses were shown to have a complex effect on their host metabolism (88). KSHV was shown to shift glucose metabolism from mitochondrial OXPHOS to aerobic glycolysis and to induce fatty acid synthesis and glutaminolysis (89–94). One of the driving forces for these metabolic alterations is the virally encoded microRNAs, which are thought to downregulate different genes which are involved in the regulation of OXPHOS and by that shift cells to more glycolytic metabolism (94). Importantly it was shown that these microRNAs are also transferred from infected cells to non-infected cells in the microenvironment using EVs (95–97) and

that the viral microRNAs are active in these cells to induce similar metabolic phenotype (97) Similar to KSHV, EBV was also shown to alter its host cell metabolism (98). Specifically, the latent protein LMP1 was shown to shift host cell metabolism from OXPHOS to aerobic glycolysis (98, 99) by inducing expression of multiple genes, such as GLUT1. In EBV-induced carcinomas this increased glycolysis promotes MDSC expansion (100) leading to tumor immunosuppression as discussed. Additionally, LMP1, which is expressed in around 30% of EBV-driven Hodgkin Lymphomas, is also involved in stimulating regulatory T cell responses (101). LMP1 can be transferred in EV secreted from infected cells and thus manipulate EV-recipient cells (2, 102, 103). This suggests that similarly to KSHV, EBV can use EV to manipulate neighboring cells and thereby modulate its microenvironment.

Why do viruses alter their host's metabolism? One clear advantage is the activation of biosynthetic pathways to support viral replication. Redirecting pyruvate away from mitochondria and reducing OXPHOS can free different carbon molecules for the synthesis of nucleotides, amino acids and lipids or for protein glycosylation. Permissiveness of CD4+ T cells to HIV has been shown to be strongly influenced by the metabolic activation status of the T cells. CD4+ T cells with high rates of OXPHOS and glutaminolysis where the most susceptible (104, 105). Indeed, HIV infection could be significantly reduced by blocking glutaminolysis (105). In the case of latent viruses, the advantage of altering their host cell's metabolism is less obvious. Since during latency, there is a minimal expression of viral proteins, these viruses are completely dependent on cellular replication to maintain and replicate their genome. Adopting a "cancer-like" metabolism might support uncontrolled cell division, which results in maintenance and amplification of the viral genome.

Altering the metabolic state of cells in the microenvironment might suggest other advantages for infected cells. For example, in KSHV infection, it was shown that altering the metabolism of non-infected cells leads to the secretion of high-energy metabolites. These metabolites are being taken up by infected cells supporting their growth. Therefore, it is suggested that viruses can use EVs to create a specific niche which supports infected host cell growth (97).

Moreover, altering the metabolic phenotype of the niche could also allow viruses to escape the immune system. Since, T cells as part of their differentiation and activation need to undergo dramatic reprogramming of their cellular metabolism (3, 106) a low glucose high lactate microenvironment restricts T cells, dampening their effector function (4, 18). This raises the intriguing hypothesis that by manipulating their host cell's metabolism viruses attenuate T cell function by creating a suppressive microenvironment.

OUTLOOK AND THERAPEUTIC IMPLICATIONS

A better understanding of tumor metabolism is obviously important in order to target tumor cells as well as to counteract their immunosuppressive impact on anti-tumor T cell responses. As described below different strategies are therefore being explored. One approach consists in the intratumor delivery by nanoparticles of RNA interference that silences lactate dehydrogenase A (LDHA) (107). The observation in preclinical models that the effect of anti-PD1 treatment in a model of melanoma, was improved in mice with LDH-A deficient tumors (108), and that, the deletion of LDHA in myeloid cells was shown to induce T cell antitumor immunity against lung carcinoma (109), further validates the targeting of LDHA. Expression of catalase by chimeric antigen receptor (CAR) T cells improved the protection of CAR T cells against oxidative stress induced in part by ROS in the TME (110). A recent report showed that acetate could be used as an alternative carbone source and rescue the functions (e.g., IFN-γ production) of exhausted tumor infiltrating T cells, and glucose-restricted CD8+ T cells (111).

Autophagy is a catabolic process that allows cell survival and maintenance of cell metabolism in face of stressful conditions such as nutrient starvation. In tumor cells, autophagy appears to play different roles by promoting tumor suppression but also tumor initiation (112). A better understanding of autophagy in tumors could therefore potentially be exploited to develop novel anticancer treatments. Note that in the context of viral infections, autophagy can again play a dual role by promoting or limiting viral replication (113).

Despite viruses amending the immune response, as discussed above, anti-viral responses often successfully eliminate the infecting agents or keep them life-long under control as evidenced by the rare occurrence of CMV or EBV-mediated disease in healthy individuals despite up to 90% of the human population being persistently infected (12). This has led to the interesting idea of repurposing anti-viral T cells against (114). In a recent study Rosato et al. demonstrated that anti-viral T cells can target tumors when these were loaded with exogenous viral peptide. This strategy was made even more efficient when combined with check-point blockade (114) potentially opening up new therapeutic avenues. It remains to be determined if such a strategy impacts on anti-viral control. An important question will be whether utilizing anti-viral T cells could over time lead to their exhaustion and/or reprogramming into Treg in the suppressive TME. Thus, the choice of viral peptide targets and combination with other strategies will be critical; especially considering that a persistent common virus such as EBV is oncogenic if uncontrolled (115).

Altering cell metabolism is one of the hallmarks of cancer. However, it is becoming clear that this effect is not limited to the tumor cells, and as part of tumor development, the metabolic phenotype of the TME is also dramatically changed. Additionally, viruses can mimic this phenotype, affecting the metabolism of both their host cells and cells in their microenvironment. Despite considerable advances, we still have some way to go in understanding how these metabolic alterations affect T cell response and how they could successfully be used to target cancer and chronic viral infection. However, it is clear that

the metabolic profiling of antigen specific T cells and their target cells should now be part of the development of new therapeutic strategies.

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All authors have contributed to writing the article, and have read the manuscript.

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REVIEW
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Metabolic Programming of Macrophages: Implications in the Pathogenesis of Granulomatous Disease

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Metabolic reprogramming is rapidly gaining appreciation in the etiology of immune cell dysfunction in a variety of diseases. Tuberculosis, schistosomiasis, and sarcoidosis represent an important class of diseases characterized by the formation of granulomas, where macrophages are causatively implicated in disease pathogenesis. Recent studies support the incidence of macrophage metabolic reprogramming in granulomas of both infectious and non-infectious origin. These publications identify the mechanistic target of rapamycin (mTOR), as well as the major regulators of lipid metabolism and cellular energy balance, peroxisome proliferator receptor gamma (PPAR-γ) and adenosine monophosphate-activated protein kinase (AMPK), respectively, as key players in the pathological progression of granulomas. In this review, we present a comprehensive breakdown of emerging research on the link between macrophage cell metabolism and granulomas of different etiology, and how parallels can be drawn between different forms of granulomatous disease. In particular, we discuss the role of PPAR-γ signaling and lipid metabolism, which are currently the best-represented metabolic pathways in this context, and we highlight dysregulated lipid metabolism as a common denominator in granulomatous disease progression. This review therefore aims to highlight metabolic mechanisms of granuloma immune cell fate and open up research questions for the identification of potential therapeutic targets in the future.

Keywords: macrophage, immunometabolism, granuloma, tuberculosis, schistosomiasis, sarcoidosis

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INTRODUCTION

At its core, a granuloma is a compact and organized structure formed by the initial aggregation of macrophages in response to a persistent stimulus (1, 2). A multitude of stimuli have been reported to evoke a granulomatous reaction, including infectious agents, the best studied of which are *Mycobacterium tuberculosis* and the parasitic trematode genus *Schistosoma*, non-infectious foreign bodies such as silica, pollutants, dust and biomedical implants (3, 4), as well as autoimmune or inflammatory diseases of unknown etiology, including sarcoidosis and Crohn's disease. It has also been suggested, particularly in the case of sarcoidosis, that a combination of infectious and non-infectious factors could be responsible for disease pathogenesis, with evidence suggesting *Propionibacterium acnes* or mycobacterial infection as potential environmental triggers (5).

Depending on the nature and persistence of the inciting stimulus, the macrophages at the core of the granuloma can undergo a series of distinct morphological changes, the most prominent involving epithelioid cell differentiation; an enigmatic process for which the exact trigger and molecular mechanisms have yet to be elucidated. The resultant macrophages, termed "epithelioid," are characterized by a hypertrophic, flattened appearance, diffuse cytoplasm and elongated nuclei, as well as epithelial-like interdigitated cell membranes, enabling the cells to interlace and form an epithelioid barrier to wall off the persistent antigen (1, 6, 7). These epithelioid macrophages can further develop into multinucleated giant cells via as yet undefined mechanisms; however, cell-cell fusion (8, 9) and cytokinesis failure (10) have been proposed. Furthermore, in the case of tuberculosis granulomas, macrophages can transform into foam cells as a result of their enhanced accumulation of lipids as the infection progresses (11).

As the granuloma matures, a number of additional cells can be recruited to the structure—a process that depends heavily on the nature of the inciting agent. Such cells include granulocytes, monocytes, dendritic cells, B and T cells, NK cells and fibroblasts (1, 12, 13), which surround the macrophage core resulting in a complex and highly organized structure. A subsequent and final stage of granuloma maturation involves the development of pathological structures as a result of dysregulated inflammation, which are associated with tissue damage and morbidity. One such feature is the formation of a fibrotic outer capsule that occurs in granulomas of diverse etiology, including sarcoidosis and schistosomiasis. Another pathological feature, which develops primarily in granulomas of infectious origin, is the formation of necrotic regions within the central core of the granuloma. This is a characteristic that is well-studied in the case of tuberculosis, during which necrosis is associated with a failure of the immune response and results in bacterial dissemination and patient morbidity (14).

Due to the major clinical role of granulomas in an array of disease pathologies, the morphological properties of granuloma formation have been extensively studied. However, the fundamental and molecular core mechanisms involved in the granulomatous immune response are only starting to emerge. Our group recently identified the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling pathway as a molecular mechanism in the initiation and maintenance of granulomas in mice, as well as its activation in sarcoidosis patients suffering from a progressive form of the disease (15). mTOR is well-known to sense and integrate a range of environmental signals to regulate cellular metabolism and cell growth in many cell types (16, 17). However, our work specifically revealed an involvement of macrophage mTORC1 signaling in the context of granuloma formation and development (15), thus highlighting the potential importance of macrophage immunometabolic responses in granulomatous disease. While mTORC1 was also recently identified as a driver of foam cell biogenesis in tuberculosis (18), the relevance of mTOR signaling in schistosomiasis remains to be elucidated. Likewise, adenosine monophosphate-activated protein kinase (AMPK) has been reported to orchestrate lipid catabolism and oxidative phosphorylation (OXHPOS) in tuberculosis (19, 20); however, an involvement of this metabolic regulator in schistosomiasis and sarcoidosis has barely been defined. Interestingly, peroxisome proliferator receptor gamma (PPAR-γ)-signaling has been implicated in the progression of all three diseases. As PPAR-γ is a master regulator of lipid homeostasis (21), this strongly suggests an involvement of lipid metabolism in the pathogenesis of granulomas.

Indeed, the significance of macrophage metabolic plasticity dependent on disease pathology is now widely accepted and the field of immunometabolism has gained momentum in recent years. New findings have emerged that shed light on the complex molecular mechanisms underpinning macrophage function across numerous diseases states; however, at present, such responses are exquisitely linked to the current M1/M2 framework of macrophage polarization. In this review, we focus on the current literature describing the immunometabolic programming of macrophages—as the workhorses at the forefront of granuloma formation, development and maintenance (1, 13)—in the best characterized examples of granulomatous disease: tuberculosis, schistosomiasis and sarcoidosis. In particular, we highlight literature pertaining to the role of PPAR-γ and lipid metabolism in the pathogenesis of these diseases, which suggests dysregulated lipid metabolism as a key contributor to granuloma fate.

A PRELUDE TO M1/M2 POLARIZATION AND ITS IMMUNOMETABOLIC NATURE

At present, the concept of M1/M2 macrophage polarization, albeit oversimplified and based primarily on *in vitro* data, still remains one of the best means by which macrophage activation can be described. Classically activated M1 macrophages function at the crux of host defense by eliciting essential pro-inflammatory responses and bridging innate and adaptive immunity. In contrast, alternatively activated M2 macrophages are crucial to immune regulation by promoting the resolution of inflammation and preventing an exacerbated, chronic inflammatory state, as well as the maintenance of tissue homeostasis by inducing tissue healing and remodeling (22–24). M1 and M2 polarization states are additionally defined by distinct metabolic profiles that are crucial in driving the differential activation of macrophages (25). It is important to note, however, that while the scheme of M1/M2 polarization has been very useful in furthering our understanding of macrophage function and biology, *in vivo* and human studies point toward a larger, less distinct spectrum of polarization states (26, 27). Accordingly, it is becoming more common to refer to the subcategories, which include M1a, M1b, and M2a-c that are, in part, defined by their differential expression of chemokine receptors (28). Transcriptomic analyses have revealed that there are six main polarization states in humans that are highly sensitive to certain stimuli, and thus the nomenclature has also been adapted to "M(LPS)," "M(IL-4)" and others (29). In addition, there is significant evidence to suggest that these polarization states have a high plasticity and do not represent a terminal differentiation (30).

Nevertheless, the pathological consequences of the immunometabolic nature of macrophage polarization in granulomas are only just starting to emerge, with the tubercle granuloma currently constituting the best-studied example. Thus, while the metabolic landscape of granuloma macrophages in a human disease scenario will be much more complex, the simplified M1/M2 dogma and the use of *in vivo* models currently comprise the primary framework for discussing macrophage metabolism in the context of granulomatous disease. Below, we therefore provide a brief overview of the key immunometabolic features of M1/M2 macrophage polarization as an introduction to the subsequent discussion on their contributing roles in the pathology of tuberculosis, schistosomiasis and sarcoidosis.

Classically Activated (M1) Macrophages

M1 macrophage polarization is typically stimulated by inflammatory cytokines including interferon-gamma (IFN-γ) and/or tumor necrosis factor (TNF) in combination with Toll-like receptor (TLR) ligation (31). This leads to NF-κB signaling via the phosphorylation of Inhibitor of (I)κB kinase, and the activation of several interferon-regulatory factors (IRFs) (32), which are signature genes of the M1 macrophage phenotype. Although both TLR ligation and IFN-γ signaling can individually and redundantly induce the phenotype (31), it was recently shown in tumor-associated macrophages that both mechanisms may need to work synergistically to produce an effective, highly pro-inflammatory M1 switch (33). Metabolically, M1 macrophage activation classically involves the Warburg effect. This metabolic switch is well-known in oncology and refers to a distinct metabolic profile in tumor cells undergoing uncontrolled division, during which cells utilize glycolysis for rapid ATP generation in the presence of oxygen, termed "aerobic glycolysis" (34). In macrophages, this switch drives the essential increase in ATP production required to support the innate immune response to infectious insult and/or tissue injury, supporting the rapid production of inflammatory cytokines and enhancing phagocytosis (25). Both TLR ligation and LPS-induced increased hypoxia-inducible factor 1-α (HIF-1α) expression are associated with the Warburg effect in macrophages (35, 36). Specifically, HIF-1α links the effector functions of M1 macrophages to their metabolic profile by both activating glycolytic enzymes and directly promoting transcription of the inflammatory mediator IL-1β (36). Moreover, when HIF-1α is overexpressed in macrophages, mitochondrial OXPHOS is suppressed and the cells enter a highly glycolytic and inflammatory state with a clear M1 phenotype (37). It must be noted, however, that the Warburg effect has almost exclusively been observed *in vitro* and in cancer cells, and it has been proposed that M1 macrophages only exhibit a "Warburg-like" phenotype (38).

Alternatively Activated (M2) Macrophages

M2 macrophage polarization is brought about by the cytokines IL-4 and IL-13 released from $CD4^+$ Th2 cells (39, 40). The IL-4/13 receptor activates insulin receptor substrate 2 (IRS2), leading to the upregulation of key M2 markers: Arg-1, RELMα, and Ym1. The IL-4/13 receptors signal via signal transducer and activator of transcription 6 (STAT6) to activate phospholipases and GATA binding protein 3 (GATA3), which are required for the production of chemokines and the anti-inflammatory cytokine IL-10 (41, 42). There is also evidence that IL-10 can produce an M2 or M2-like phenotype via the phosphorylation of STAT3, leading to the production of IL-10 in an autocrine manner (43, 44). Unlike their M1 counterparts, M2 macrophages are defined by their intact TCA cycle, which allows for the generation of FADH2 and NADH and the production of high ATP yields through OXPHOS. Concomitantly, M2 macrophages display increased glycolysis as a carbon source (15, 45). Importantly, an intact TCA cycle allows for the glycosylation of M2-specific mannose and lectin receptors by UDP-GlcNAc (46). OXPHOS in M2 macrophages is supported by fatty acid oxidation (FAO), and M2 polarization is marked by increased expression of the lipid scavenger receptor CD36, as well as a dependence on cell-intrinsic lysosomal lipolysis (38, 45, 47). M2 macrophages are also known to consume more glutamine than other macrophage phenotypes (48). Glutamine augments the production of UDP-GlcNAc (46) and glutaminolysis has been shown to produce α-ketoglutarate, a major metabolite and component of the TCA cycle (49). Glutamine-derived α-ketoglutarate promotes specific histone demethylation and transcription of M2 genes (49, 50).

TUBERCULOSIS AND THE IMMUNOMETABOLIC RESPONSE: A BALANCING ACT

Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (Mtb) and is one of the most frequent etiological triggers of granuloma formation. It is the leading cause of mortality from a single infectious agent and was responsible for an estimated 1.7 million deaths worldwide in 2017 (51). However, only a small proportion (5–10%) of the infected population will develop active tuberculosis, with a greater incidence in individuals infected with HIV or suffering from malnutrition or diabetes (51). It has therefore long been established that the outcome of Mtb infection depends upon a complex and dynamic interplay between host and pathogen (52–54), with the degree of bacterial virulence and host resistance defining the pathogenesis of disease (55–57).

Initial Host Responses to Mtb Infection

Tuberculosis is typically a disease of the lungs initiated upon inhalation of airborne droplets containing the tubercle bacilli at an estimated infectious dose of just a single bacterium (58, 59). The bacteria are subsequently deposited in the airways where they are phagocytosed by resident alveolar macrophages and transported into the lung parenchyma (60, 61). The generation of TNF-α and inflammatory chemokines by the infected macrophages then drives a localized inflammatory response involving the recruitment of additional mononuclear cells (52), including interstitial macrophages, monocytes, neutrophils, and dendritic cells, many of which Mtb subsequently infects (54). Dendritic cells transfer Mtb from the lungs to the local draining lymph nodes (62) for subsequent priming of naive T cells and

the adaptive immune response (63), the development of which is unusually delayed during Mtb infection, taking up to 5–6 weeks in humans (64, 65). The latter has been attributed to successful immune evasion by the bacteria, which is thought to seek refuge within lung phagocytes (54). Thus, although Mtb is known to infect multiple immune cells in the lung, macrophages are considered to be the key players in the balance between bacterial containment and disease progression (66). Moreover, increasing evidence from non-human primate and/or human patient granulomas suggests that mycobacterial burden, as well as advancement to active disease, is associated with an increased proportion of alternatively activated M2 to classically activated M1 macrophages (67–69). We will explore this further in the following subsections, with a particular focus on the emerging importance of the immunometabolic characteristics of M1- vs. M2-like macrophages on the outcome of mycobacterial infection.

Distinct Immunometabolic Responses of Pulmonary Macrophages to Mtb

Tissue resident alveolar macrophages are the first cells to encounter invading mycobacteria after aerosol infection (54). Several studies in diverse model systems, encompassing mycobacterial infection of human alveolar macrophages (70–72), mouse models (73, 74) and zebrafish larvae (75), demonstrate an initial mycobactericidal role that is conserved in multiple tissue resident macrophages across species. However, these reports contend with numerous contradictory studies that render the alveolar macrophage as a favorable niche for the growth and survival of mycobacteria, acting as a shield against the bactericidal activity of subsequently recruited immune cells, as well as activation of the Th1 response (54).

At steady state, alveolar macrophages do not conform to the classical definition of M1 or M2 polarization, but instead exhibit a hybrid phenotype that affords a degree plasticity in keeping with their critical role in maintaining lung homeostasis (76, 77). It is logical that as sentinels of the lung, alveolar macrophages would mount an initial pro-inflammatory response to invading pathogens. Taking into account their potential to switch between activation states, it may not be surprising if they then re-polarize to a more M2-like status in response to a persistent pathogen to protect against hyperinflammation and exacerbated lung immunopathology. Such a switch toward an M2 state is particularly pertinent in the case of Mtb infection, considering the ability of mycobacteria to manipulate host immune responses to favor their own survival (52). Recent data suggesting alveolar macrophages are more permissive to Mtb replication than recruited monocyte-derived interstitial macrophages (78) highlight distinct metabolic programming as a critical factor in the differential antimycobacterial capacity of these two macrophage populations. This study utilized transcriptomic and subsequent pathway enrichment analyses, as well as functional *in vivo* and *in vitro* experiments employing inhibitors of different metabolic pathways, to reveal the metabolic preferences of Mtb-infected alveolar vs. interstitial macrophages. This work demonstrated that Mtb-infected alveolar macrophages preferentially utilize FAO and exhibit enhanced fatty acid uptake, as well as higher bacterial replication rates, than interstitial macrophages. As it is well-known that Mtb accesses host-derived fatty acids and cholesterol as a primary carbon source (79–82), the fact that alveolar macrophages exhibit enhanced fatty acid uptake may explain why they are more permissive to Mtb replication. Furthermore, Huang et al. (78) showed that a FAO inhibitor, etomoxir, almost entirely abolished the production of IFN-β in Mtb-infected BMDMs. As type I interferon responses have been shown in multiple studies to be detrimental during tuberculosis [reviewed by (83)], this may explain how FAO can also contribute to Mtb survival. Conversely, interstitial macrophages were primarily glycolytic, which was shown to restrict mycobacterial growth (78), presumably due to the pro-inflammatory cytokine signals associated with glycolysis as well as the comparatively lower uptake of fatty acids by these cells. This is in agreement with several studies that demonstrate the requirement of glycolysis for Mtb growth control (84–86). Huang et al. (78) also showed that depletion of alveolar macrophages in Mtb-infected mice reduced bacterial burden, whereas depletion of the interstitial macrophage population was detrimental, resulting in enhanced bacterial survival. Thus, in this setting, alveolar macrophages appear to function as M2-like nutritionally permissive hosts in which mycobacteria can evade pro-inflammatory action and replicate relatively unperturbed, which is in contrast to the more growth-restrictive environment within pro-inflammatory interstitial macrophages. These data are supported by additional studies of alveolar or M2 macrophage depletion in animal models of pulmonary tuberculosis, which was associated with an enhanced Th1 response, reduced bacterial burden in the lung and protection against tuberculosis-induced lethality (87, 88). Granuloma formation was also found to be defective in mice deficient in alveolar macrophages, while the attraction and activation of T cells in the lung, as well as the numbers of polymorphonuclear cells, were enhanced (87). This is in contrast to the depletion of activated M1-like macrophages, which was detrimental and resulted in enhanced bacterial survival (88).

Such immunometabolic divergence of the two pulmonary macrophage populations in response to Mtb infection suggests that the developmental origin of granuloma macrophages plays a role in disease progression. Indeed, the role of ontogeny in macrophage functionality and metabolism during infectious insult has been discussed in a recent review (89). Furthermore, it should be noted that while the Warburg effect is considered to be primarily an *in vitro* phenomenon, the *in vivo* relevance of this bioenergetic phenotype is starting to emerge in the literature. Specifically, during the establishment of chronic Mtb infection in mice (up to 30 days after low-dose aerosol infection), transcriptomic and histological analysis of Mtb-infected lung tissue revealed a HIF-1α-dependent enhancement of glucose uptake and glycolysis, as well as lactate formation and export (84). This was coupled with a concomitant down-regulation of pyruvate dehydrogenase complex (PDC), TCA cycle enzymes and OXPHOS. Moreover, defects in macrophage glycolytic capacity have been associated with the enhanced

susceptibility of cigarette smokers to Mtb infection (86). In this study, extracellular flux analysis of Mtb-infected human alveolar macrophages isolated from the bronchoalveolar lavage (BAL) fluid of smokers and non-smokers revealed an impairment of metabolic activity in the alveolar macrophages of the smokers, including reduced glycolytic response and spare respiratory capacity, which was accompanied by a weakened inflammatory response.

Biphasic Immunometabolic Response of Individual Mtb-Infected Macrophages

Further to the immunometabolic distinction between pulmonary macrophages of different lineages, increasing evidence points toward a time-dependent blend of M1 and M2 responses to Mtb infection within individual granuloma macrophages (90–92), which may further explain the paradox of alveolar macrophage functionality during infection. One such study utilized high-throughput capped analysis of gene expression (deepCAGE) technology to investigate the promoter-based transcriptional landscape of Mtb-infected macrophages (91). This work revealed drastic gene expression alterations that included up-regulation of genes involved in M1-related immune response and inflammation, as well as M2-related cell wounding and apoptosis. Furthermore, to delineate macrophage responses to Mtb infection, authors of a recent review (92) comprehensively analyzed the metabolic patterns reported in transcriptome databases and supplementary data files from studies of primary macrophage Mtb infection in the literature. The authors report on a biphasic response marked by a defensive M1 phenotype during the early phase of *in vitro* infection (up to 8 h post-infection), followed by a switch to an M2-driven adaptation/resolution phase as the infection progresses (24–48 h after infection). The early pro-inflammatory phase was characterized by upregulation of genes indicating a classical Warburg shift in metabolism, including *Hif1α*, as well as genes encoding glucose uptake transporters (GLUT1 and GLUT6), hexokinases (HK1 and HK2), phosphofructokinase liver (PFKL; from the phosphofructokinase-1 (PFK-1) family), 6-phosphofructo-2-kinase/fructose-2,6 biphosphatase 3 (PFKFB3; an essential enzyme from the PFK-2 family that is responsible for elevated glycolytic flux), and the major lactate secretion transporter member 4 (MCT4). Consistent with the profile of M1 macrophage polarization, this up-regulation of Warburg-associated genes was coupled with the downregulation of genes encoding mitochondrial enzymes and proteins, including the PDC, TCA cycle enzymes (aconitase 2 [ACO2], isocitrate dehydrogenase 2 [IDH2] and subunits of the succinate dehydrogenase [SDH] complex), as well as multiple components of respiratory chain complexes.

These findings are consistent with the current knowledge of the molecular mechanisms underpinning the metabolic switch in M1 macrophages, which are brought about by two distinct "breakpoints" in the TCA cycle (25). The first breakpoint occurs at the conversion of citrate to α-ketoglutarate, which is catalyzed by isocitrate dehydrogenase 1 (IDH1). IDH1 is downregulated 7-fold in M1 macrophages compared to non-polarized macrophages, while the ratio of citrate to α-ketoglutarate is tripled (46). Itaconate, generated from the citrate accumulated as a result of this metabolic break (93), is a powerful bactericidal agent produced by M1 macrophages that functions as a potent inhibitor of bacterial isocitrate lyase (ICL). ICL, an enzyme that facilitates retention of carbon from fatty acids via the glyoxylate shunt (94), has been implicated in the control of Mtb infection (95, 96) due to its requirement for fatty acid catabolism by the bacteria (96). In this study, Muñoz-Elías and McKinney show that deletion of the genes that encode two ICL isoforms in Mtb (*icl1* and *icl2*) reduced growth and survival of the bacteria in murine macrophages and in human monocyte-derived macrophages, as well as the bacterial load in mouse lungs.

The second breakpoint in the TCA cycle occurs at the succinate-fumarate conversion step, which is catalyzed by SDH, as demonstrated by the accumulation of mitochondrial succinate in LPS-stimulated macrophages (36). Succinate accumulation occurs partially as a result of the aforementioned citrate-induced generation of itaconate, which downregulates SDH directly (97). Succinate can also be produced directly from glutamine in a TCA cycle-independent, γ-aminobutyric acid (GABA)-dependent manner via the GABA shunt (36, 46, 49, 50). Increasing evidence supports the role of succinate as an important metabolic signal linking metabolism and immunity (92, 98), as succinate has been shown to stabilize HIF-1α and its proinflammatory effects, in particular its induction of IL-1β expression (99, 100). In addition to the identification of genes involved in these classical pathways, Shi et al. (92) report the modulation of a diverse array of genes associated with a number of pro-inflammatory processes affected as a result of these TCA cycle breaks. Such processes include an augmented oxidative stress response coupled with an increase in antioxidant defense, as well as the synthesis of pro-inflammatory bioactive lipids (including long chain fatty acyl-CoAs, phospholipids and prostaglandins), and arginine uptake and metabolism.

Consistent with the metabolic profile of alternative macrophage activation, the adaptation/resolution phase transition in Mtb-infected macrophages was marked by gene expression changes signifying a reduction in glucose uptake and dampened glycolytic metabolism, with a concomitant recovery of the TCA cycle and OXPHOS (92). The latter coincided with induction of *Pgc1b* (92), which encodes PPAR-γ coactivator-1β (PGC-1β), a key player in mitochondrial biogenesis and oxidative metabolism that has been implicated in alternative macrophage activation (47). The PPAR-γ coactivator family of transcription factors (PGC-1) are potent anti-inflammatory regulators and enhancers of OXPHOS in various cell types, including macrophages, where retroviral transfection with PGC-1β drives non-polarized cells toward an anti-inflammatory M2 phenotype characterized by enhanced OXPHOS and arginase-I expression (47).

Studies focusing on the behavior of Mtb during its infection cycle are also suggestive of a biphasic immunometabolic response of individual macrophages to Mtb infection. Following macrophage invasion, Mtb undergoes a series of physiological adaptations that accompany distinct phases of its infection process, from initial infection to intra-macrophage adaptation

and eventual establishment of successful, productive disease. Transcriptional profiling of Mtb, in conjunction with the use of a clock plasmid to measure bacterial replication and death rates, revealed a clear distinction in bacterial survival and response to host phagocyte function in a 2-week *in vitro* infection model encompassing this multi-phasic infection process (101). For the first 2 days post-infection of primary BMDMs, the bacteria encountered a "bottleneck" during which Mtb killing outweighed its relatively high replication rate (101); a finding that is emulated *in vivo* by rapid bacterial growth and pronounced bacterial killing in the mouse lung during the first 2 weeks of infection (102). Reduced survival of Mtb during early *in vitro* infection was attributed to Mtb stress due to the initial macrophage arsenal, inferred by up-regulation of Mtb genes involved in general stress response, carbon metabolism, oxidative stress, iron storage, as well as hypoxia and nitrosative stress (101). These findings are in accordance with an earlier transcriptomic dataset that was also acquired 2-day after Mtb infection of murine BMDMs (103).

The bottleneck observed by Rohde et al. (101) was followed by a period of reduced replication but enhanced bacterial survival (intracellular adaptation phase), which progressed over time to a period of extended growth resulting in established macrophage infection. Interestingly, only a subset of Mtb genes were upregulated early (by day 2 post-infection) and remained elevated over the remaining 2-week infection period, including genes involved in fatty acid and cholesterol metabolism, secreted antigens and regulators. Furthermore, many of the general stress response genes observed during early infection were markedly down-regulated during the adaptation and establishment phases. Such modifications of the Mtb response over time are consistent with an M1-M2 switch in macrophage activation, and suggest that Mtb exploits the proposed biphasic response of host macrophages in order to establish productive infection.

Foamy Macrophages and Lipid Metabolism in Tuberculosis

A characteristic feature of tuberculosis pathogenesis is the formation of foam cells (lipid-laden macrophages) that are associated with tubercle granuloma necrosis (11) due to their contribution to caseum formation, which is defined as an accumulation of necrotic debris at the core of the granuloma. The latter promotes inflammation, tissue injury, and the eventual cavitation of the granulomatous structure, resulting in transmission of live bacilli and tuberculosis disease [reviewed by (104)]. Although the role of foamy macrophages in the progression of tuberculosis is clear, the mechanisms controlling foam cell biogenesis remain ill-defined for this disease. However, a recent study using multiple Mtb-infection models has shed light on these mechanisms, as well as the lipid composition of tuberculosis foam cells (18). This work demonstrated that foamy macrophages in necrotizing granulomas in tuberculosis lung lesions accumulate triglycerides (TAG), and exhibit a multispecies TAG profile that is conserved in rabbits, non-human primates and humans. Elevated levels of TAG were also detected in Mtb-infected human monocyte-derived macrophages, and were accompanied by higher lipid content and enhanced expression of TAG biosynthesis genes. Mechanistically, this TAG accumulation was mediated by TNFR signaling via downstream activation of the caspase cascade and mTORC1. Inhibition of these pathways was shown to significantly reduce lipid content in these cells, and up-regulation of the pathways was also observed in transcriptomic data from human tuberculosis lung tissue (18). Interestingly, an involvement of mTORC1 was previously implied by histological analysis of a human tuberculosis lung sample, which revealed mTORC1 activation in foamy macrophages (105). Furthermore, this key metabolic sensor has been shown to promote lipogenesis, in particular TAG biosynthesis, in obesity, diabetes, cancer and neurodegenerative disorders, while blocking lipolysis and β-oxidation (106). Indeed, Mtb and other mycobacterial species induce mTOR in macrophages (107, 108), and rapamycin inhibition of mTOR has been shown to decrease mycobacterial viability (107).

It is also interesting to note that mycobacterial infection of macrophages induces the expression and activation of PPAR-γ (109), a hallmark of alternative macrophage activation and a master regulator of lipid metabolism that controls fatty acid uptake, storage and lipogenesis (21). PPAR-γ has been implicated in mycobacterial disease progression by modulating host cell metabolism toward lipid droplet formation, as well as diminishing the pro-inflammatory immune response to favor bacterial survival (109–113). Moreover, a link between mycobacterial virulence and host PPAR-γ expression and activation during infection has been proposed. For instance, the virulent H37Rv strain of Mtb was shown to induce PPAR-γ expression, and attenuated growth of virulent Mtb was observed in human macrophages following PPAR-γ deletion (111), as well as in the lungs of macrophage-specific PPAR-γ-deficient mice (113). In contrast, attenuated *M. bovis* bacillus Calmette-Guerin (BCG) up-regulated PPAR-γ to a lesser extent (111) and *M. smegmatis*, an avirulent mycobacterial strain, failed to induce PPAR-γ expression (110, 111). Taken together, these findings suggest that PPAR-γ may also contribute to foam cell biogenesis in tuberculosis granulomas. This may additionally explain the link between PPAR-γ and tuberculosis pathogenesis, which is suggested by the correlation between Mtb virulence and PPAR-γ activity. Furthermore, enhanced expression of PPAR-γ has been reported in adipogenesis, and this was shown to be mTOR-dependent (114, 115), which raises the question as to whether PPAR-γ plays a role in mTORC1-mediated foam cell formation in tuberculosis. Interestingly, PPAR-α, one of the three PPAR isoforms alongside PPAR-γ and PPAR-β and a key driver of fatty acid β-oxidation (116), was recently reported to play an essential role in host innate immune defense against Mtb and BCG (117). In this study, the PPAR-α-mediated antimycobacterial response of BMDMs was attributed to enhanced autophagy, lysosomal biogenesis and phagosome maturation, as well as suppression of exacerbated inflammation via activation of transcription factor EB (TFEB). Moreover, PPAR-α promoted lipid catabolism, mitochondrial respiration and fatty acid β-oxidation in mycobacteria-infected macrophages (117). Thus, it appears that PPAR-γ and PPAR-α play opposing roles during

mycobacterial infection, which is due, in part, to their distinct effects on host lipid homeostasis.

AMPK is another key metabolic regulator that has gained traction in tuberculosis research, although the literature on its role in other granulomatous diseases is sparse. AMPK is an important coordinator of M2 polarization and lipid catabolism, and its effects can overlap, enhance or antagonize those of mTOR and the PPAR family [reviewed by (118)]. During Mtb infection, AMPK activation increases OXPHOS, FAO and also antimicrobial autophagy by interacting with PGC-1α, as well as inhibiting Mtb-induced mTOR activation (19, 20). It is well-established that autophagy constitutes a successful antimycobacterial host response (119), therefore it is no surprise that Mtb has developed mechanisms to protect itself against this process. One such mechanism is the induction of microRNA expression in macrophages that directly silence AMPK (20). A second mechanism could be the activation of mTOR (19, 120), which is a well-known inhibitor of autophagy (121, 122). Indeed, mTORC1 has been reported to inhibit PPAR-α (123), which may constitute a mechanism by which mTORC1 inhibits autophagy in Mtb-infected cells. In comparison, mTORC1 is known to enhance the expression of PPAR-γ (114, 115), which supports an interplay between mTORC1 and PPAR-γ in the formation of foamy macrophages during Mtb infection.

These findings highlight not only the importance of macrophage lipid metabolism in the pathogenesis of tuberculosis, but also the critical involvement of mTORC1 and PPAR-γ in the survival and maintenance of Mtb during disease progression. The additional identification of AMPK and PPAR- α as host protective metabolic signaling pathways further accentuates the important link between immunometabolic signaling and the outcome of granulomatous disease. An overview of the Mtb-macrophage interactions described in this section is provided in **Figure 1**.

SCHISTOSOMIASIS: A STORY OF EGGS AND FATS

The second major cause of infectious granulomas, schistosomiasis (or bilharzia), is an endemic tropical disease of significant morbidity, mortality and socioeconomic impact. Like other helminth-borne infections, schistosomiasis occurs mainly in the southern hemisphere, where it is considered endemic in over 50 countries and affects over 200 million people, making it the most significant parasitic infection after malaria (124). Schistosomiasis is transmitted to humans via the aquatic larvae of several geographically distinct species of the *Schistosoma* trematodes. The most prominent causative agents of human schistosomiasis are *S. mansoni*, *S. japonicum*, and *S. haematobium*, all of which employ freshwater snails as intermediate hosts. Upon subcutaneous infection with the swimming larval stage, known as cercariae, the worms mature in the lung over the course of a month. During this time, the infection elicits an acute type-1 inflammatory response, which, in the case of *S. mansoni* and *S. japonicum*, subsides when the adult schistosomes travel to the hepatic and mesenteric vasculature to mate and lay eggs (125). *S. haematobium* instead colonizes the urogenital tract. Egg deposition into perivascular tissues is accompanied by an acute type-2 response about 2 months post-infection, which finally becomes a lower-grade chronic disease marked by chronic pain, anemia, diarrhea, hepatomegaly and malnutrition (126). During this type-2 antihelminthic response, schistosome egg antigens induce rapid periovular granuloma formation that encases the eggs. Periovular granulomas protect the host from continuous exposure to toxic egg antigens and reduce immunopathology, but hepatic granulomas frequently become fibrotic if untreated, leading to portal hypertension and eventually liver cirrhosis with significant morbidity (127). Schistosome granulomas therefore have advantages and disadvantages for the host and their usefulness as a containment strategy has been discussed at length (128–130).

Cellular Composition of Schistosome Granulomas

Typically, periovular granulomas consist of alternatively activated M2 macrophages, Th2 $CD4^+$ T cells and eosinophils (128). Although M2 macrophages are considered the key component of schistosome granulomas (131), granuloma composition is variable, with hepatic granulomas showing a higher cell heterogeneity and intestinal granulomas containing largely macrophages (132). Based on inverse proportions of eosinophils, macrophage numbers also appear to be higher in the granulomas of naturally-infected wild water rats (*Nectomys squamipes*) than in chronic granulomas of experimentally-infected mice (133), indicating that there may be significant differences between experimental and natural granuloma formation that must be taken into account. Schistosome egg-derived antigens such as omega-1 play a major role in driving a highly Th2-skewed antihelminthic immune response at 5–6 weeks after the initial infection, causing local $CD4^+$ T helper cells to release IL-4 and IL-13 (134, 135). At the same time, intercellular adhesion molecule 1 (ICAM-1) is upregulated on sinusoidal endothelial cells surrounding newly deposited eggs (136). This milieu of cytokines and chemokines results in the T cell and STAT6-dependent recruitment of $Ly6C^{hi}$ monocytes to the periovular space, where they differentiate into granuloma macrophages with only a minor contribution from resident hepatic macrophages (131, 137–139). In the case of *S. japonicum* infection, parasite antigens can also signal via TLR2 to promote M2 polarization (140). Contrastingly, Tundup et al. (141) showed that the TLR co-receptor CD14 is highly upregulated in hepatic macrophages upon *S. mansoni* infection and acts as a crucial negative regulator of M2 polarization, possibly as part of a parasitic defense mechanism against granuloma formation (141). CD14, in turn, induces uptake of oxidized low-density lipoproteins (oxLDL), cholesterol and lipids into macrophages (142).

Lipid Metabolism and PPAR-γ-Signaling in Schistosomiasis

S. mansoni infection was recently linked to specific metabolic changes in hepatic macrophages, where granuloma formation around schistosome eggs was associated with differential lipid

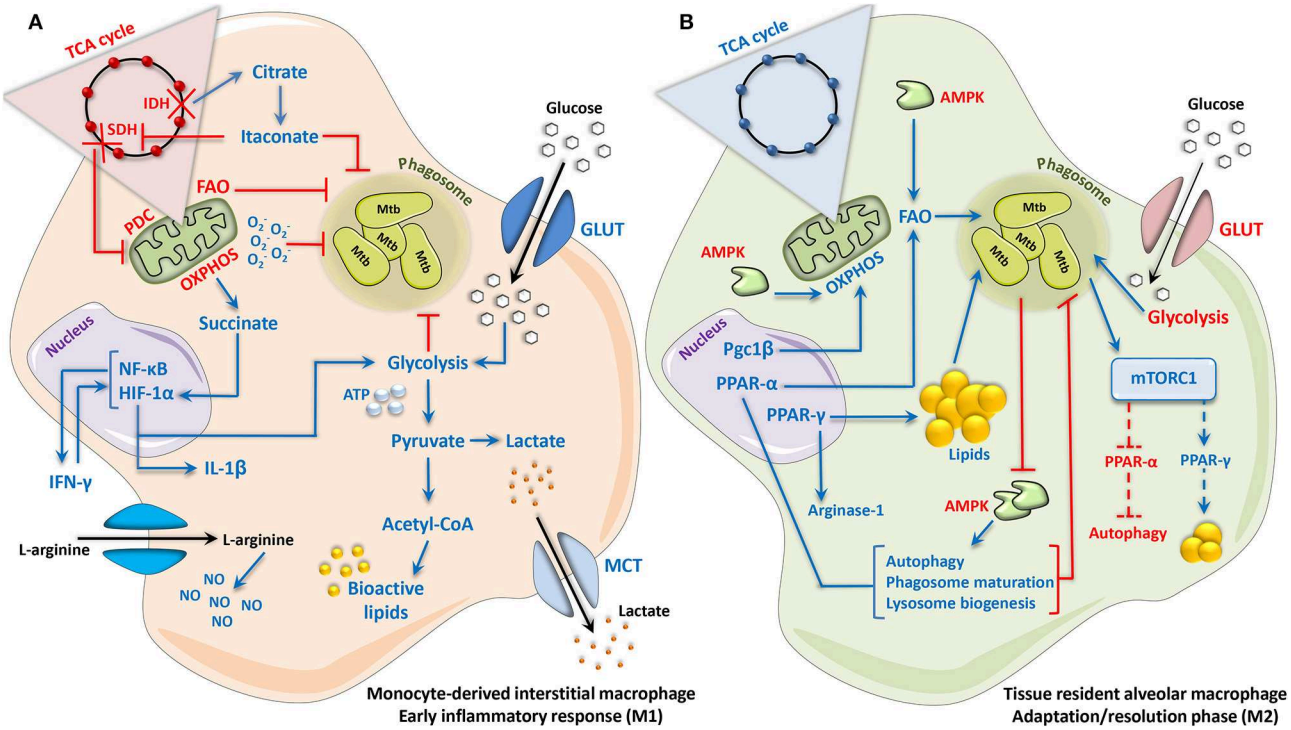

FIGURE 1 | *Mycobacterium tuberculosis* elicits a biphasic immunometabolic response in host macrophages. **(A)** Monocyte-derived interstitial macrophages, and macrophages during the acute phase response to Mtb infection, adopt an M1-like phenotype characterized by a Warburg-like switch in metabolism. Enhanced HIF-1α-mediated glycolytic activity and glucose uptake results in the rapid generation of ATP to support the pro-inflammatory reaction, which occurs simultaneous to the increased generation and export of lactate. Concomitant reductions in OXPHOS, PDC, and FAO occur as result of two consecutive breaks in the TCA cycle at the conversion of citrate to α-ketoglutarate (α-KG) and succinate to fumarate, as well as the down-regulation of the TCA cycle enzymes involved in these reactions: IDH and SDH, respectively. Accumulation of the TCA intermediate citrate as a result of the first breakpoint in the cycle (citrate to α-KG) leads to the generation of itaconate, which inhibits SDH directly as well as Mtb survival by blocking bacterial isocitrate lyase and consequently fatty acid catabolism by the bacteria. Accumulation of succinate as a result of the second break in the TCA cycle (succinate to fumarate), as well as direct inhibition of SDH by itaconate, stabilizes HIF-1α and its pro-inflammatory activity. The uptake and metabolism of arginine are additionally increased, promoting the production of NO via iNOS/NOS2. Together, these events culminate in the generation of a potent antimycobacterial response marked by the generation of pro-inflammatory cytokines, ROS/RNS and bioactive lipids, which in parallel with increased glycolysis and reduced FAO, result in Mtb growth control. **(B)** Tissue-resident alveolar macrophages, and macrophages entering an adaptation/resolution phase during chronic Mtb infection, adopt an M2-like phenotype characterized by an intact TCA cycle and enhanced FAO-driven OXPHOS, with a concomitant reduction in glycolytic activity and glucose uptake. This coincides with increased expression and activity of Pgc1β, which promotes mitochondrial biogenesis and oxidative metabolism. During the infection process, Mtb augments PPAR-γ expression, resulting in a weakened inflammatory response, as well the formation and accumulation of lipid droplets within the cell, thus providing a favorable niche for the growth and survival of Mtb. PPAR-α can also be activated in macrophages during Mtb infection, which enhances autophagic, lysosomal and phagosomal processes that contribute to Mtb growth control. Likewise, cytosolic AMPK, while enhancing OXPHOS and FAO, also promotes antimycobacterial autophagy, and is therefore directly inhibited by Mtb. mTORC1, induced by Mtb, supports bacterial survival by promoting lipogenesis and blocking autophagy, potentially due to its interactions with PPAR-γ and PPAR-α, respectively. Dotted lines represent interactions inferred from the literature. Up- and downregulation are indicated by blue and red, respectively. Acetyl-CoA, acetyl coenzyme A; AMPK, adenosine monophosphate activated protein kinase; ATP, adenosine triphosphate; FAO, fatty acid oxidation; GLUT, glucose transporter; HIF-1α, hypoxia-inducible factor 1-alpha; IDH, isocitrate dehydrogenase; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; MCT, monocarboxylate transporter; Mtb, *Mycobacterium tuberculosis*; mTORC1, mammalian/mechanistic target of rapamycin complex 1; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; NO, nitric oxide; NOS2, NO synthase 2; OXPHOS, oxidative phosphorylation; PDC, pyruvate dehydrogenase complex; Pgc1β, peroxisome proliferator-activated receptor gamma coactivator 1-beta; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDH, succinate dehydrogenase; TCA, tricarboxylic acid.

and cholesterol metabolism (143). This is supported by multiple studies that have highlighted the significance of PPAR-γ in the context of schistosomiasis. PPAR-γ is a hallmark of M2 polarization and globally regulates lipid uptake and metabolism (144, 145). It is activated by a multitude of immunometabolic ligands, including various unsaturated fatty acids, lipoproteins, eicosanoids, flavonoids and the amino acids glutamine and arginine (146). Although the precise role of PPAR-γ in macrophages depends on their lineage, polarization state and post-translational modifications (145, 147), PPAR-γ is generally considered to be an anti-inflammatory receptor known to limit M1 activation, for instance by transrepression and ubiquitination of NF-κB (145, 148). Importantly, lipids secreted from the tegument of *S. mansoni* adults were shown to directly activate PPAR-γ in macrophages, leading to upregulation of arginase-1 expression and other M2 markers *in vitro* (149). This study did

not clarify whether this effect is only achieved in the presence of adult worms, or whether similar PPAR-γ-activating lipids are also found on the eggs. However, both live and inactivated *S. mansoni* eggs have been reported to induce a 7-fold increase in PPAR-γ expression in human liver cell cultures (150). PPAR-γ activation is a classical M2 macrophage marker and M2 macrophages are known to preferentially utilize FAO. While mice with a macrophage-specific PPAR-γ knockout show a downregulation of genes for both synthesis and oxidation of fatty acids in non-infectious settings (144), *S. mansoni*-infected human liver biopsies revealed a specific downregulation of FAO-related genes, including acetyl-CoA acyltransferase 2 (ACAA2) and acyl-CoA synthetase long-chain family member 1 (ACSL1) (151). Additionally, a secretory protein of *S. japonicum* inhibited PPAR-α, a key promoter of FAO, in a colitis mouse model (152), indicating that downregulation of FAO may be directed by the parasite itself. These findings support the notion that macrophage polarization during schistosomiasis is complex, with a potential decrease in FAO despite many classical M2 signatures (153).

It is now well-established that infection with *S. mansoni* alters host lipid metabolism globally by reducing total cholesterol, low-density lipoprotein (LDL) and triglycerides in the plasma of humans and apolipoprotein E (apoE)-deficient or high-fat diet-fed mice (154–156). In particular, PPAR-γ activation during schistosomiasis has been shown to counteract atherosclerosis and other high-fat diet-induced pathologies (157, 158). PPAR-γ has been strongly linked to these conditions, and mice with macrophage-specific PPAR-γ knockouts become susceptible to diet-induced obesity and insulin resistance (144). Peritoneal macrophages with a conditional PPAR-γ knockout have markedly reduced expression of cholesterol transport genes, such as the ATP-binding cassette transporters (ABC) A1 and G1, as well as reduced cholesterol efflux (157, 159, 160). Similar effects were shown in hepatic macrophages during schistosomiasis, where a recent study reports downregulation of a number of genes involved in cholesterol metabolism, including *APOC1* and *APOC3* (143), which are known contributors to inflammatory atherosclerosis (161, 162). A recent RNA interference study revealed that ApoC1 in particular is a key promoter of oxLDL cholesterol uptake into macrophages via the lectin-like oxLDL receptor-1 (LOX-1), which is in turn inhibited by PPAR-γ (163, 164). Thus, *S. mansoni* infection blocks macrophage oxLDL cholesterol uptake by inhibiting ApoC1 and ApoC3 and may at the same time facilitate cholesterol efflux via ABCA1 and ABCG1. This suggests that unlike in tuberculosis, schistosomiasis macrophages do not accumulate lipids (see **Figure 2**). In accordance with this, there are no reports to date of *S. mansoni* or *S. japonicum* leading to foam cell formation. The rarer *S. mekongi*, which does not promote fibrosis, appears to be the only member of its genus to induce foam cells, although this phenomenon has not yet been investigated further (165).

Mechanistically, *S. mansoni* infection leads to significant upregulation of CD14, which induces macrophage uptake of oxLDL cholesterol and lipids (141, 142). Additionally, PPAR-γ is known to mediate transcription of the *Cd36* scavenger receptor gene in macrophages (166), which promotes uptake of oxLDL cholesterol and provides a stepping stone for the development of atherosclerosis (158). A PPAR-γ response element in the *Cd36* gene means that PPAR-γ directly promotes CD36 transcription following PPAR-γ activation by oxLDL, constituting a positive feedback loop for cholesterol accumulation (158, 167, 168). In mice infected with *S. mansoni* while on an atherogenic high-fat diet, significant lipid uptake was found to be induced in the outermost cell layers of hepatic periovular granulomas; however, the plasma ratio of HDL to LDL was still improved (156). Unfortunately, this study did not determine which cells were responsible for the increased lipid uptake. However, as the experiments were performed only 7 weeks after *S. mansoni* infection, when granulomas are still somewhat immature and not yet associated with additional leukocytes (128), it is likely that the responsible cells were either eosinophils or macrophages. Another study showed that *S. mansoni* infection can induce lipid uptake and retention in hepatic stellate cells (150). Therefore, the lipid droplet-positive cells identified by Stanley et al. (156) could be non-granulomatous hepatic cells, although this would not account for their circular distribution around the granulomas. Since lipid uptake was only induced in the outermost cell layers of granulomas (156), there may be an as-of-yet unknown significance of physical proximity between the egg and the surrounding macrophages. While it is known that female schistosomes take up large amounts of fatty acids in order to produce eggs, it is not established whether eggs, which themselves contain large amounts of fatty acids, could extract and ingest lipids from surrounding macrophages (169, 170). It may be the case that CD36-induced oxLDL uptake, while pro-atherogenic in arterial walls, allows some sequestration of oxLDL cholesterol in non-migratory macrophages, thus contributing to the anti-atherogenic effect of schistosomiasis (171).

Overall, *S. mansoni* is characterized by modifications in the PPAR-γ network of lipid metabolism, during which oxLDL cholesterol uptake via ApoC1 and ApoC3 is directly suppressed, while uptake via CD14 and CD36 may be enhanced due to PPAR-γ signaling. Since PPAR-γ can directly activate multiple HDL cholesterol efflux channels, and there appears to be limited lipid accumulation and no evidence of foam cell formation in schistosome macrophages, this suggests that there may be a significant efflux of cholesterol from granuloma macrophages during schistosomiasis. The expulsion of HDL cholesterol could explain the anti-diabetic and anti-atherogenic effects observed in response to PPAR-γ agonists such as rosiglitazone (172–174).

PPAR-γ in *Schistosoma*-Induced Fibrosis

Macrophage lipid metabolism may also be involved in the formation of fibrosis, the major pathology of chronic schistosomiasis, as suggested by several studies using pharmacological PPAR-γ induction. For instance, in a mouse model of *S. japonicum*-induced liver fibrosis, the PPAR-γ agonist rosiglitazone was able to reduce liver fibrosis and extracellular matrix (ECM) deposition, which was attributed to decreased inflammatory signaling (175). Although this study did not link these effects to granuloma macrophages, but rather to hepatic

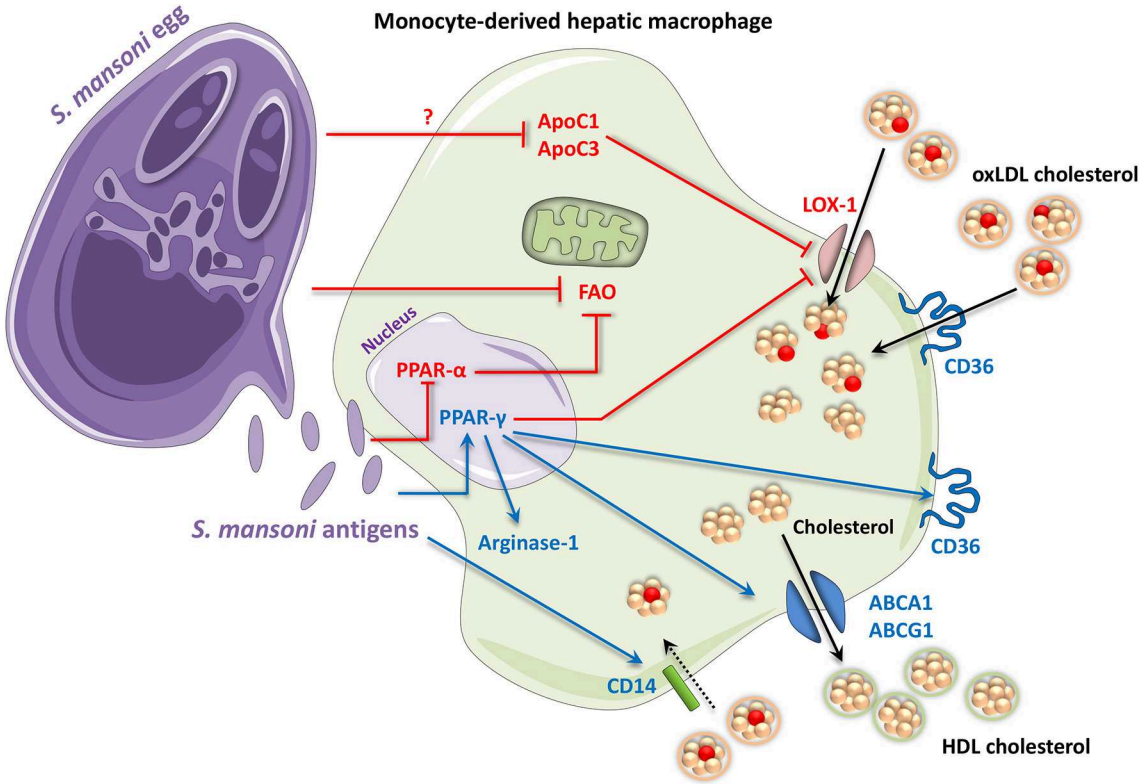

FIGURE 2 | Periovular granuloma macrophages in schistosomiasis assume an M2-like phenotype with altered lipid metabolism. Antigens from *Schistosoma mansoni* eggs (and adults) activate PPAR-γ signaling. Some *Schistosoma* products have also been shown to inhibit signaling by the FAO inducer, PPAR-α. PPAR-γ contributes to an M2 phenotype by upregulating M2 markers including arginase-1. It also promotes expression of the scavenger receptor CD36, which takes up free fatty acids and oxidized LDL cholesterol. In contrast, PPAR-γ inhibits oxLDL cholesterol uptake via the LOX-1 receptor. *S. mansoni* itself also attenuates LOX-1 by decreasing ApoC1 and ApoC3 via an unknown mechanism. Additionally, *S. mansoni* infection also leads to an upregulation of CD14, which has in turn been shown to promote uptake of oxLDL and other lipids in macrophages. HDL cholesterol efflux may also be increased through the PPAR-γ-mediated upregulation of ABCA1 and ABCG1, contributing to an anti-atherogenic phenotype. Up- and downregulation are indicated by blue and red, respectively. ABC, adenosine triphosphate-binding cassette transporter; Apo, apolipoprotein; CD, cluster of differentiation; FAO, fatty acid oxidation; HDL, high-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1; oxLDL, oxidized low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor.

stellate cells and myofibroblasts, ECM deposition and local synthesis have been described as key components in both sarcoid and infectious lung granulomas (176). Similar to the effects of rosiglitazone, the PPAR-γ agonist pioglitazone reduced hepatic and splenic histopathology in a mouse model of *S. japonicum* infection by increasing the proportion of regulatory T cells and inducing the polarization of mannose receptor-positive M2 macrophages (177). Another PPAR-γ agonist, telmisartan, reduced both hepatic fibrosis and granuloma diameter in *S. mansoni*-infected mice, although it did not synergistically increase the efficacy of praziquantel treatment, an antihelminthic drug commonly used to treat schistosomiasis (178). These findings may seem contradictory, seeing as PPAR-γ is known to induce M2 polarization, which is necessary for host survival during initial infection (179, 180), and also contributes to fibrosis by promoting fibroblast hyperactivity through TGF-β signaling (181, 182). At the same time, pharmacological activation of PPAR-γ has been shown multiple times to decrease histopathology and fibrosis in schistosomiasis and some renal pathologies (183). Where PPAR-γ agonists have failed to ameliorate fibrosis, this has been speculatively attributed to the simultaneous activation of pro-fibrotic M2 macrophages (184, 185). It is therefore likely that this dual role of PPAR-γ is due to its activity in various cell types. For instance, activation and overexpression of PPAR-γ has been shown to act directly on both human and mouse fibroblasts to decrease fibrosis by reducing growth factor expression, mitosis, collagen secretion, and responsiveness to TGF-β (186, 187).

While these studies do not explicitly link the anti-fibrotic effects of different PPAR-γ agonists to immune cell lipid metabolism, it is clear that PPAR-γ plays a significant role in schistosomiasis pathogenesis and future work investigating how macrophage lipid metabolism affects fibrosis should prove interesting. Taken together, it is becoming apparent that *Schistosoma* infection has highly complex consequences for lipid metabolism, at both the cellular and global level (summarized in **Figure 2**), and more work is required to elucidate the exact metabolic pathways that characterize schistosome granulomas.

SARCOIDOSIS: A DISEASE LACKING AN IMMUNOMETABOLIC SWITCH?

Unlike tuberculosis and schistosomiasis, which are triggered by known pathogens and are typically organ-specific, sarcoidosis is an enigmatic multi-systemic disease of unknown origin. It is characterized by the development and accumulation of epithelioid, non-caseating (non-necrotic) granulomas typically found in the lungs; however, sarcoid granulomas can present almost anywhere in the body, with other notable sites including the eyes, skin and lymph nodes (188). The disease is increasingly difficult to treat once it develops from a self-limiting to progressive state, with pulmonary and cardiac involvement representing the most frequent causes of patient morbidity and mortality (189). Due to the unknown etiology of sarcoidosis, there is currently no therapeutic approach targeting the pathogenetic mechanisms (190). However, the occurrence of familial forms of the disease (191, 192) suggests that a genetic background may play a pathological role, and pine pollen (193), microbial infection (specifically *P. acnes* and mycobacteria) (5) as well as air pollutants (comprising mineral, micro or nanoparticles) (3) are increasingly regarded as strong environmental trigger candidates (188, 194, 195). Pollution, in particular, gained momentum among clinicians and biologists when a recrudescence of sarcoidosis was observed in the aftermath of the World Trade Center tragedy in subjects exposed to particulate matter (3). Although the exact relative contributions of genetic and environmental factors are unknown, it has been suggested that genetic factors account for 66% of disease susceptibility in monozygotic twins (191).

Immunological Profile of Sarcoidosis

At the cellular level, sarcoid granulomas are characterized by a strong Th1/Th17 phenotype (196). Once again, this sets sarcoidosis apart from tuberculosis and schistosomiasis, which are both marked by an acute Th1 and M1 phase that later progresses to a predominantly M2 phenotype. It has long been known that the pro-inflammatory milieu of IL-12, IL-17, TNFα, and IFNγ upregulate adhesion molecules on macrophages to promote aggregation, cell-to-cell contact and fusion (197, 198). In 2011, however, macrophages and multinucleated giant cells from granulomas of patients with systemic neuromuscular sarcoidosis were shown for the first time to be M2-polarized with high CCL18 expression and resistance to conversion by Th1 cytokines (199). More recently, immunohistochemical analysis also revealed M2 macrophage activation in lung and lymph node samples from pulmonary sarcoidosis patients (200). Advanced sequencing techniques confirmed that a CD163-positive macrophage fraction of peripheral blood mononuclear cells (PBMCs), isolated from sarcoidosis patients and treated with purified protein derivative (PPD), exhibits a partial M2 profile with upregulation of IL-13 downstream pathways (201, 202). Furthermore, the pro-inflammatory cytokine IL-17, well-established in sarcoidosis pathology, has also been shown to induce an M2-like phenotype in macrophages (203, 204). It is important to note that sarcoidosis is frequently linked to clinical anergy, which presents as the loss of skin test reactivity to PPD and other antigens in sarcoidosis patients. While $CD4^+$ T cells and dendritic cells have been implicated in this anergic response [reviewed by (205)], the findings described above suggest that anti-inflammatory macrophages may also contribute to the paradoxical diminished immunity often observed in sarcoidosis patients.

Dysregulation of Lipid Metabolism in Sarcoidosis

The M2 profile of macrophages reported for some sarcoid granulomas may also be associated with the expression and activity of PPAR-γ, as PPAR-γ deficiency was observed in alveolar macrophages of pulmonary sarcoidosis patients (206) and its expression was shown to negatively correlate with disease severity (207). Furthermore, polymorphisms in the *Pparg* gene and the gene encoding its transcriptional coactivator PPAR-γ coactivator 1-α (*Ppargc1a*) were identified at a higher frequency in sarcoidosis patients compared with healthy subjects (208). Thus, a genetic defect in PPAR-γ signaling may be a predisposing factor for the development of severe sarcoidosis. Additional work utilizing diverse mouse models supports the role of PPAR-γ in lung immunopathology (157, 159, 209–212). In one such study employing a multiwall carbon nanoparticle (MWCNT) model, which recapitulates the chronicity of human granulomas (211), PPAR-γ expression and activity was significantly reduced in alveolar macrophages following oropharyngeal instillation of MWCNT in wild type animals. Accordingly, increased pulmonary granuloma formation, as well as expression of pro-inflammatory markers in granulomatous lung tissue and BAL, were observed in macrophage-specific PPAR-γ-deficient mice (termed *PPAR-γ*$^{fl/fl}$, *Lyz2*-Cre) (153). Moreover, increased numbers of Th1 lymphocytes were observed in BAL fluid extracted from *PPAR-γ*$^{fl/fl}$, *Lyz2*-Cre mice compared to wild type controls. PPAR-γ deficient BAL cells also displayed elevated expression of inducible nitric oxide synthase (iNOS) and IFN-γ, as well as Th1-associated cytokines (209). Reconstitution of PPAR-γ by lentiviral transduction in the same study significantly reduced the expression of pro-inflammatory mediators and decreased the number of BAL lymphocytes by 90%. Taken together, these data suggest an important role of PPAR-γ in the regulation of pulmonary inflammation and maintenance of lung homeostasis.

As mentioned earlier, chronic inflammation typically triggers a heightened activation state in fibroblasts, lending to substantial deposition of ECM components at the site of injury and consequent development of fibrosis (213, 214). Thus, PPAR-γ deficiency in sarcoid granulomas of patients presenting severe forms of the disease may play a role in the characteristic Th1/M1 bias, and consequently the development of fibrosis that is typically associated with disease pathogenesis. This notion correlates with the observation that NF-κB activation is increased in BAL samples from sarcoidosis patients (215). As described previously in this review, transrepression of NF-κB by PPAR-γ is well-established, thus increased and/or uncontrolled activation of NF-κB as a result of PPAR-γ deficiency could be one mechanism by

which continuous inflammation occurs. Indeed, in a study coupling a computational model of granuloma formation and function, termed *GranSim* (216), with macrophage polarization data from non-human primate tuberculosis granulomas, continuous or increased NF-κB signaling was shown to exacerbate inflammation, resulting in uncontrolled bacterial growth and dissemination (68).

Mechanistically, PPAR-γ maintains pulmonary lipid homeostasis via alveolar macrophage liver X receptor-alpha (LXR-α) and ABCG1 (217), which, alongside ABCA1, is critical for macrophage efflux of cholesterol and phospholipids (218). Alveolar macrophages from sarcoidosis patients and mice following MWCNT instillation show diminished expression of ABCG1 and ABCA1, and the deficiency of these transporters in MWCNT-instilled mice correlates with increased alveolar macrophage lipid accumulation (210). In accordance with these findings, activation of the PPARγ-ABCG1 pathway by the PPARγ agonist rosiglitazone tempers MWCNT-induced granulomatous inflammation by significantly attenuating alveolar macrophage activation, pulmonary granuloma formation and pulmonary lipid dysregulation (212). Thus, the consequence of PPAR-γ deficiency in sarcoid granuloma macrophages appears to be manifold, resulting in both an uncontrolled inflammatory response and dysregulation of macrophage lipid metabolism.

Indeed, a number of proteins involved in lipid metabolism have been identified as contributing factors in the pathogenesis of sarcoidosis, including apoA1, fatty acid-binding protein 4/perilipin 2, 8-isoprostane, zinc-α2 glycoprotein and serum amyloid A (SAA) (219). In particular, SAA, a highly inducible acute-phase reactant and amyloid precursor protein (220), is a well-known modulator of the innate immune response, inflammation and apolipoprotein metabolism (221), and it has been suggested as a potential biomarker for sarcoidosis due to its significantly higher serum concentrations in patients vs. healthy subjects (222). However, SAA is not a diagnostic gold standard as it is also elevated in a number of other inflammatory conditions, namely arthritis and systemic lupus erythematosus (223). In tissue samples from sarcoidosis patients, SAA was shown to localize to macrophages and giant cells within granulomas, but it also correlated with the number of $CD3^+$ cells and a local Th1 response (224). In this study, SAA was shown to promote chronic inflammation in sarcoid granulomas via TLR2 signaling and activation of NF-κB, as well as cytokine production (224). Contrastingly, recent evidence suggests that during inflammation, SAA acts synergistically with secretory phospholipase-A to remove cell membrane debris, which suggests a partial involvement in anti-inflammatory repair processes (225). Interestingly, SAA has also been suggested to be responsible for the low HDL cholesterol and apoA1 levels observed in patients with active sarcoidosis (226, 227), which has been linked to an increased risk of atherosclerosis in sarcoidosis patients (219). This is in contrast to schistosomiasis, which has been reported to counteract atherosclerosis by altering global host lipid metabolism and reducing serum cholesterol levels (154).

Metabolic Signaling as a Driver of Sarcoidosis Disease Progression

As mentioned previously in this review, Linke et al. (15) identified an involvement of macrophage-specific mTORC1 signaling in the initiation and maintenance of granulomas. In this study, constitutive activation of mTORC1 in murine macrophages was achieved via myeloid-specific deletion of the gene encoding its upstream inhibitor tuberous sclerosis complex 2 (Tsc2) (termed $Tsc2^{fl/fl}$, *Lyz2*-Cre mice). This genotype induced hypertrophy and enhanced cell proliferation in macrophages while reducing their apoptotic capacity. As a result, the $Tsc2^{fl/fl}$, *Lyz2*-Cre mice exhibited spontaneous formation of non-caseating (non-necrotic), epithelioid granulomas that were observed in multiple organs. In the lung specifically, the granuloma macrophages were shown to consist of M2-like alveolar macrophages. Thus, the disease pathology seen in the $Tsc2^{fl/fl}$,*Lyz2*-Cre mice strongly resembles the histological phenotype of sarcoidosis. Mechanistically, genes involved in both glycolysis and OXPHOS were enriched in transcriptomic datasets from $Tsc2^{fl/fl}$,*Lyz2*-Cre BMDMs compared with *Tsc2* floxed control (termed $Tsc2^{fl/fl}$) BMDMs, and the enhancement of both metabolic pathways was confirmed by extracellular flux analysis. Furthermore, the uptake of glucose was higher in $Tsc2^{fl/fl}$,*Lyz2*-Cre BMDMs and absolute glucose levels were decreased in both Tsc2-deficient macrophages as well as in the lungs of Tsc2-deficient animals, while mitochondrial mass and mitochondrial spare respiratory capacity were elevated (15). These findings are in accordance with the identification of increased levels of pyruvate in sarcoidosis patient sera, which is indicative of enhanced glycolytic activity (228). This study also reported an increase in serum metabolites that indicate enhanced mitochondrial FAO, as well as a decrease in serum levels of the key TCA cycle intermediate succinate (228). RNA-Seq data from sarcoidosis patient monocytes further revealed a dysregulation of OXPHOS and FAO pathways (229). Interestingly, the metabolic alterations described by Linke et al. were shown to be CDK4-dependent and crucial for the enhanced proliferation and reduced apoptosis of Tsc2-deficient macrophages. This study additionally identified mTORC1 activation, macrophage proliferation and glycolysis as hallmarks of disease progression in human sarcoidosis patients (15). Clinical involvement of mTORC1 has since been substantiated by its recent identification in RNA-Seq gene set enrichment data from cutaneous sarcoidosis patients (230), as well as whole exome sequencing and pathogenicity network analysis of familial cases of sarcoidosis (231). It should also be noted that successful treatment of a sarcoidosis patient with the mTOR inhibitor rapamycin has previously been reported (232). However, the molecular mechanisms underpinning mTOR involvement in human sarcoidosis remain to be elucidated.

Among other pathways, the involvement of mTORC1 points to a role of glycolytic metabolism in sarcoidosis pathogenesis. Importantly, the PI3K/Akt/mTOR axis, and mTORC1 specifically, have been shown to promote the production of the key glycolytic regulator HIF-1α irrespective of oxygen concentrations (233–235). In accordance with this, a recent study demonstrated elevated protein levels of HIF-1α,

as well as HIF-1α signaling pathway components such as HIF-1β, HIF-2α, and p300, in alveolar macrophages from sarcoidosis patients cultured under normoxic conditions (236). HIF-1α protein expression was further confirmed in granulomatous lung tissue from sarcoidosis patients, and was found to be localized to alveolar macrophages and multinucleated giant cells (236). As described previously, HIF-1α promotes pro-inflammatory macrophage functions by activating glycolytic enzymes and stimulating the production of IL-1β (36). Indeed, Talreja et al. (236) demonstrated that in alveolar macrophages from sarcoidosis patients, increased levels of HIF-1α correlated with augmented Glut1 protein expression and elevated levels of IL-1β. Furthermore, siRNA inhibition of HIF-1α in patient PBMCs significantly diminished the production of IL-1β, IL-17 and IL-6 (236). Thus, HIF-1α signaling appears to play a critical role in the pro-inflammatory milieu that defines sarcoidosis pathology, and provides an explanation for the enhanced glycolysis reported in the aforementioned studies.

These findings highlight the complexity of the metabolic landscape of granuloma macrophages both *in vivo* and in human disease. In particular, they emphasize the oversimplification of Warburg metabolism in M1 macrophages, which is classically defined by a simultaneous increase in glycolysis and decrease in OXPHOS. This accentuates the importance of future work that examines more closely the complexity of macrophage metabolism *in vivo* and in the context of individual human diseases. This is, however, in contrast to Mtb infection, during which the Warburg-like immune response has been documented and supported by *in vivo* data (84). However, this difference may simply be due to the infectious nature of Mtb and a typical pro-inflammatory reaction to initial infection, which is absent in sarcoidosis. It is also important to note that some of the sarcoidosis studies mentioned in this section utilized either patient PBMCs or patient sera, and therefore describe the peripheral/global metabolic alterations associated with sarcoidosis. The role of each of the identified metabolic pathways within the sarcoid granuloma environment, and their contribution to sarcoid granuloma macrophage function specifically, still has to be assessed. Nevertheless, the observations that glycolysis, OXPHOS and FAO may all occur simultaneously during sarcoidosis disease progression, and that these pathways are associated with both pro- and anti-inflammatory functions, may somewhat explain the paradoxical immunoregulation and anergy that are frequently associated with sarcoidosis.

An overview of the findings described in this section is provided in **Figure 3**.

COMMON AND DISTINCT METABOLIC FEATURES OF TUBERCULOSIS, SCHISTOSOMIASIS, AND SARCOIDOSIS

PPAR-γ signaling and dysregulated lipid metabolism are the overriding common features of each disease discussed in this review. However, these pathways are distinctly modulated depending on the disease, as well as disease severity. Such differences may be due to the granuloma-inciting agent and, in particular, the ability of mycobacteria and *Schistosoma* species to manipulate host macrophage responses to promote their own survival. For instance, both tuberculosis and schistosomiasis are defined by an acute inflammatory phase followed by a pathogen-induced shift toward M2-like macrophage polarization, marked by the activation of PPAR-γ. In tuberculosis, PPAR-γ activation appears to be virulence-dependent, leading to lipid droplet accumulation and consequent foam cell formation that are associated with the development of necrosis, which is characteristic of tuberculosis pathogenesis. However, in schistosomiasis there appears to be only minor CD14/CD36-driven lipid accumulation that is confined to certain areas of the granuloma, with a potential efflux of cholesterol from macrophages and no reports of foam cell formation or a correlation with pathogenicity for the major *Schistosoma* species. This is further compounded by the up-regulation of FAO in Mtb-infected macrophages compared to its down-regulation in *Schistosoma*-infected livers, which may, in part, constitute a protective mechanism in tuberculosis foamy macrophages to counteract some of the lipid accumulation that occurs during infection. In contrast to tuberculosis and schistosomiasis, sarcoidosis exhibits defective or deficient PPAR-γ signaling that is associated with disease severity. However, this is not surprising considering the critical role of PPAR-γ in the M1-M2 metabolic switch in macrophages, and the chronic inflammatory nature of sarcoid granulomas in comparison to tuberculosis and schistosomiasis. Furthermore, the expression of SAA, which contributes to chronic inflammation, is greater in sarcoidosis than in other granulomatous diseases (224). SAA has also been associated with the increased prevalence of atherosclerosis in sarcoidosis patients by lowering serum levels of HDL and apoA1. This is again in contrast to schistosomiasis, which has been shown to reduce atherogenesis potentially due to the expulsion of HDL cholesterol from schistosome granuloma macrophages. This may be linked to enhanced PPAR-γ expression during *Schistosoma* infection that will, in turn, lead to increased expression of the cholesterol transporters ABCA1 and ABCG1, thus enabling HDL cholesterol efflux. Whereas, down-regulation of PPAR-γ, and accordingly ABCA1 and ABCG1, in macrophages from sarcoidosis patients and mice imply a decrease in the efflux of HDL cholesterol from these macrophages. Interestingly, FAO is also up-regulated in sarcoidosis (228), which may again constitute a homeostatic response of sarcoid granuloma macrophages to the increased lipid accumulation that occurs due to PPAR-γ deficiency. These findings highlight the importance of PPAR-γ in the control of lipid homeostasis, and how its dysregulation in a disease scenario can have immunometabolic consequences that directly impact macrophage effector functions and disease outcome.

In further support of the involvement of macrophage metabolic signaling in granulomatous disease progression, macrophage mTORC1 has recently been implicated in tuberculosis and sarcoidosis pathogenesis. However, the metabolic consequences of mTORC1 activation in each case appears to be distinct. During Mtb infection, this key metabolic sensor plays a role in foamy macrophage formation, whereas in sarcoidosis mTORC1 promotes OXPHOS and glycolysis. As

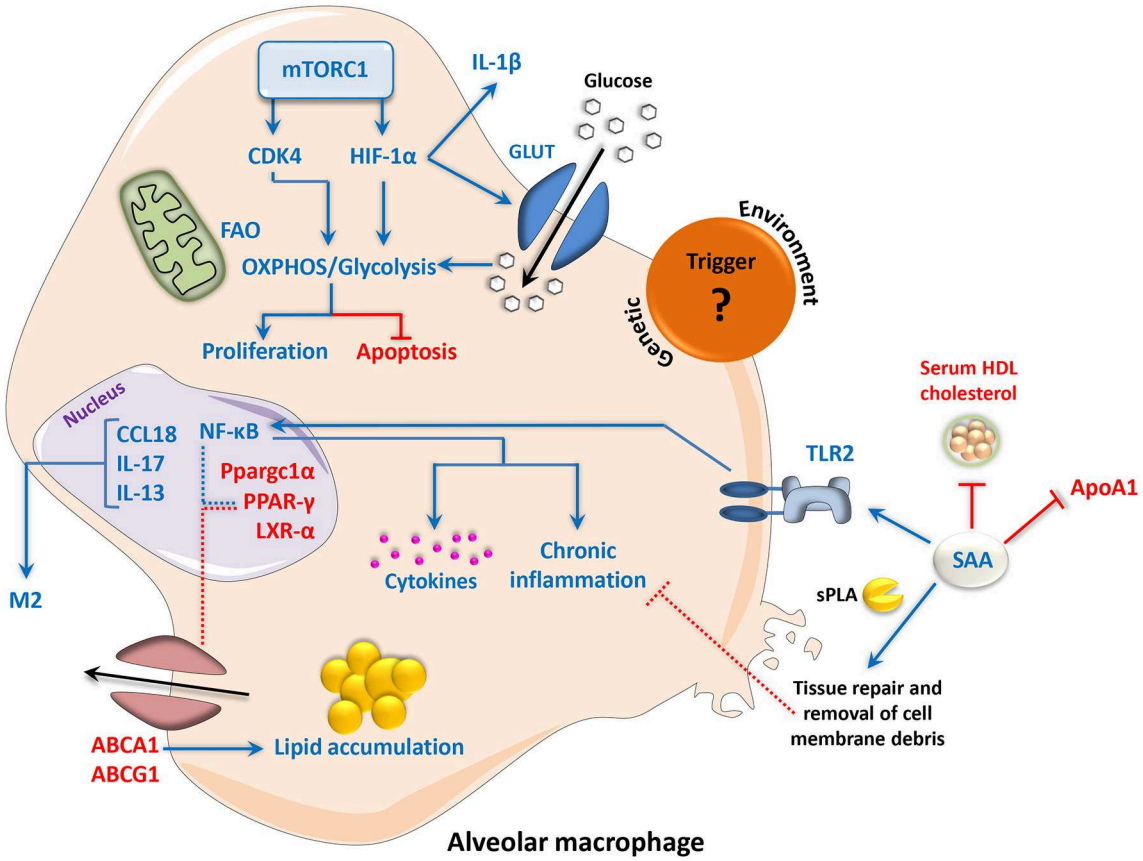

FIGURE 3 | Metabolic signaling and chronic inflammation in sarcoidosis disease progression. The etiological trigger of sarcoidosis remains unknown; however, environmental and genetic factors have been proposed. Deficiency of PPAR-γ and its transcriptional coactivator Ppargc1α in alveolar macrophages has been implicated in disease severity, resulting in the alleviation of NF-κB transrepression and enhancement of a pro-inflammatory phenotype. Reduced expression of the cholesterol and lipid transporters ABCG1 and ABCA1 and disruption of LXR-α signaling, which are involved in the maintenance of lipid homeostasis by PPAR-γ, leads to lipid accumulation in macrophages. NF-κB can also be activated via TLR2 signaling in response to increased levels of SAA, leading to the generation of pro-inflammatory molecules. SAA can reduce serum HDL cholesterol and ApoA1, increasing the risk of atherosclerosis in sarcoidosis patients. However, SAA can also work with sPLA to promote the enzymatic digestion and removal of cell debris, which contributes to the anti-inflammatory processes of wound healing and tissue repair. In sarcoid-like granuloma macrophages, mTORC1 promotes cell proliferation and inhibits apoptosis via CDK4-dependent enhancement of glycolysis and OXPHOS. mTORC1 also directly promotes the function of HIF-1α, which in turn contributes to glycolysis and inflammation. An M2-like phenotype in sarcoid granulomas has additionally been reported, involving CCL18, IL-13, and IL-17. Up- and downregulation are indicated by blue and red, respectively. Dotted lines represent interactions inferred from the literature. ABC, adenosine triphosphate-binding cassette transporter; Apo, apolipoprotein; CCL18, chemokine (C-C motif) ligand 18; CDK, cyclin-dependent kinase; HDL, high-density lipoprotein; HIF-1α, hypoxia-inducible factor 1-alpha; IL, interleukin; GLUT, glucose transporter; LXR, liver X receptor; mTORC1, mammalian/mechanistic target of rapamycin complex 1; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; OXPHOS, oxidative phosphorylation; PPAR, peroxisome proliferator-activated receptor; SAA, serum amyloid A; sPLA, secretory phospholipase A; TLR, toll-like receptor.

mTOR is a key intracellular nutrient sensor, these differences suggest that macrophages in tubercle and sarcoid granulomas may have a distinct nutritional content, which may again be due to the ability of Mtb to manipulate host cells to promote its own survival. On that note, Mtb-induced dysregulation of autophagy in macrophages is an established mechanism by which Mtb evades the host immune response. Interestingly, a link between mTOR and autophagy was recently proposed in familial cases of sarcoidosis (231). Therefore, the role of mTOR in these diseases may in fact be manifold, and the connection between mTOR and autophagy in both tuberculosis and sarcoidosis suggests that this could be a key molecular pathway in the pathogenesis of granulomatous diseases. In schistosomiasis, however, very little is known about the function of mTOR. In dendritic cells, which play a major role in Th2 priming, mTOR inhibition by both rapamycin and torin-1 has been shown to increase IL-4 expression (237). However, while schistosome infection leads to an M2/Th2 profile shift in dendritic cells, the parasite antigens appear to do so in an mTOR-independent manner (237). One study reports that 90 % of mTOR pathway genes were downregulated in liver biopsies from human schistosomiasis *japonica* patients (151), even though *in vitro* work suggests that IL-13 induced by parasitic infections may enhance the expression of the mTORC2 protein Rictor (238). mTOR expression is also enhanced in *S. haematobium*-associated bladder cancer, although this may be a

general feature of malignancy rather than schistosomiasis (239). Recently, mTOR signaling in myeloid cells has also emerged as an attractive target for the treatment of hepatic fibrosis (240, 241).

FUTURE PERSPECTIVES

While research on the immunometabolic features of the pathogenesis of granulomatous diseases is developing, particularly in the case of tuberculosis, much more work is required to further our understanding of the exact roles of the different metabolic pathways in each disease. As this review highlights, the metabolic plasticity of macrophages allows for distinct metabolic phenotypes of individual granulomas that are highly dependent on the inciting agent. Identifying the key common and distinct features will not only be critical in defining potential therapeutic targets in the future, but may also provide an insight into how we could control the more enigmatic forms of granulomatous disease, such as sarcoidosis and Crohn's disease, for which the etiological triggers remain elusive. In tuberculosis and schistosomiasis, for example, the up-regulation of PPAR-γ results in a controlled immune response that prevents against exacerbated tissue damage by inducing an M2-like macrophage polarization state. While this role of PPAR-γ can be detrimental for pathogen clearance by the host in the case of infectious granulomas, it points toward a potential mechanism by which the chronic inflammation in sarcoidosis could be alleviated. Evidence for a role of the other PPAR isoforms (PPAR-α and PPAR-β) in granulomas of schistosomiasis and sarcoidosis is sparse in the literature and needs to be assessed in more detail. This is especially relevant considering that in tuberculosis the observed antimycobacterial function of PPAR-α is in direct contrast to the implicated function of PPAR-γ in mycobacterial disease progression, as well as the role of PPAR-α in promoting autophagy in Mtb-infected cells. While the specific significance of PPAR-α has not yet been studied in schistosomiasis, Sj16, an *S. japonicum* secretory protein, has been shown to inhibit PPAR-α (152). This lends weight to the notion that PPAR-α may play a role in schistosomiasis, and one that may also contrast with PPAR-γ. Future research should aim to examine the precise role(s) of the different PPARs in granulomatous disease, which may further our understanding of the complex lipid metabolism that is observed in each of the diseases discussed in this review. Because of the emerging significance of PPAR-γ in particular, it is also important to reiterate its well-established link with mTOR [e.g., (242, 243)]. This connection suggests that research directed at determining the precise role of mTOR in schistosomiasis should prove fruitful in the future and could highlight pathways of potential significance to tuberculosis and sarcoidosis.

AMPK could, unfortunately, not be assessed for a comparison between the granulomatous diseases discussed in this review, as it has been described almost exclusively for tuberculosis. However, while there is no research to date on the role of AMPK in sarcoidosis, IL-7-induced AMPK signaling in schistosomiasis *japonicum* has been shown to counteract macrophage autophagy and potentiate liver damage (244). This is in direct contrast with the multiple reports of AMPK promoting macrophage autophagy during mycobacterial infection (20). Nevertheless, AMPK could prove a valuable component of granuloma immunometabolism as it has been linked to both PPAR-γ (245) and mTOR (118, 246). In cancer cell lines, for instance, AMPK and AMPK activators such as metformin have an inhibitory effect on PPAR-γ activity (247). In rat liver samples, AMPK activation was shown not only to suppress PPAR-γ activity, but also to decrease fatty acid synthesis while enhancing the beta-oxidation enzyme carnitine palmitoyltransferase (248). While it remains to be seen whether these findings are translatable into granuloma research, AMPK should prove an interesting target for future research.

The comparison between schistosomiasis and tuberculosis highlights an additional key difference relating to the structure of their respective granulomas and the ontogeny of the participating macrophages, which are likely influenced by the respective pathogens. For instance, tuberculous granulomas differ greatly based on the stage of disease, whereas the composition of schistosome granulomas depends on where the eggs are deposited. Additionally, in tuberculosis, granulomas appear to involve more tissue-resident pulmonary macrophages, while schistosome granulomas consist almost exclusively of monocyte-derived macrophages. Therefore, it will be interesting to investigate the activation and metabolic programming of macrophages dependent on the local environment of the granuloma vs. macrophage ontogeny. Metabolic differences have also been shown within the layers of individual granulomas, so the metabolic profiles of macrophages and/or the additional immune cells of the granuloma dependent on their spatial orientation within the granulomatous structure will also require characterization. This can also be applied to the different pathological structures of granulomas, particularly fibrotic, foam cell-containing and necrotic granulomas. Deciphering the metabolic pathways underlying the formation of these distinct structures will allow us to determine why some granulomas are more effective than others, and perhaps how to redirect granulomas toward a less harmful phenotype.

Finally, it is important to note that there is a significant epidemiological overlap between tuberculosis, HIV, malaria and schistosomiasis (249). It would therefore be interesting for future research to consider a schistosomaiasis-tuberculosis co-infection approach. Even in urban settings of the southern hemisphere, studies report that over 60% of Mtb-exposed children also suffer from a helminth infection, the vast majority of which are caused by *Schistosoma* species (250). Furthermore, it was recently shown that infection with *S. haematobium* induces long-term, persistent epigenetic alterations resulting in a weakened inflammatory response and increased susceptibility to tuberculosis (251). Hence, research on tuberculosis therapies and vaccine candidates has made a point to assess the effect of *Schistosoma* species on tuberculosis disease progression (252). As both pathogens form granulomas but affect macrophage metabolism in a vastly different manner, it would be interesting to analyze granuloma formation in co-infection settings, particularly with regard to lipid metabolism.

Overall, the findings highlighted in this review demonstrate the importance and complexity of immunometabolic signaling in granulomatous diseases of different etiology. This emphasizes the need for future research that deciphers more closely the contribution of different metabolic pathways to each individual disease, as we all as the regulation of these pathways by PPARs, mTOR, and AMPK. Furthermore, it is paramount that the role of key metabolic pathways and regulators are then verified *in vivo*, and where possible, by human studies. Building comprehensive metabolic networks that define granuloma macrophage function will enable us to identify novel therapeutic targets in the future.

AUTHOR CONTRIBUTIONS

JW conceived of, and with HM wrote, reviewed and edited the manuscript. TW reviewed and edited the manuscript.

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Metabolic Control of Epigenetics and Its Role in CD8$^+$ T Cell Differentiation and Function

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Epigenetic programs that control posttranslational modifications of histone proteins and DNA itself tightly regulate transcriptional networks determining the identity and function of CD8$^+$ T cells. Chromatin-modifying enzymes such as histone acetyltransferases and deacetylases, represent key molecular determinants of the epigenetic imprinting of CD8$^+$ T cells. The functions of these enzymes highly depend on the availability of key products of cellular metabolism pathways such as acetyl-CoA, NAD (Nicotinamide adenine dinucleotide) and SEM (S-adenosylmethionine), suggesting that there is a close crosstalk between the metabolic and the epigenetic regulation of CD8$^+$ T cells. In this review, we will discuss the metabolic regulation of CD8$^+$ T cell epigenetics during activation and differentiation. We will furthermore summarize how metabolic signals from the tumor microenvironment (TME) shape the epigenetic landscape of CD8$^+$ T cells to better understand the mechanism underlying CD8$^+$ T cell exhaustion in anti-tumor and anti-viral immunity, which might help to overcome limitations of current CD8$^+$ T cell-based therapies.

Keywords: epigenetics, metabolism, CD8 T cell, exhaustion, anti-tumor immunity, anti-viral immunity

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CROSSTALK BETWEEN EPIGENETICS AND METABOLISM

In order to adapt to shifting environments, CD8$^+$ T cells dynamically modulate their transcriptional programs, which not only influence their differentiation but also alter their function and metabolic setup (1). Epigenetic changes are heritable and consist of post-translational modifications of DNA and surrounding histone proteins rather than alterations of primary DNA sequences. In changing external conditions, external stimuli like growth hormones and cytokines activate classical pathways such as mitogen activated protein kinase (MAPK) and nuclear factor of activated T cell (NFAT) signaling resulting in the recruitment, activation or induction of epigenetic modifying enzymes that promote epigenetic alterations in CD8$^+$ T cells (2). Similarly, nutrient levels and the metabolic status of CD8$^+$ T cells also interfere with the epigenetic programming and subsequently with the function of CD8$^+$ T cells (3). Given the fact that epigenetic modifiers harness intermediates or products of key cellular metabolic processes as their cofactors/substrates, regulation of epigenetics by cellular metabolism represents a common biological process (**Figure 1**) (3), which can disrupt adequate immune responses by CD8$^+$ T cells during anti-viral and anti-tumor immune responses (3).

While a wide range of epigenetic mechanisms exists that interfere with the accessibility of the genome by specific transcriptional programs, we will here recapitulate key epigenetic

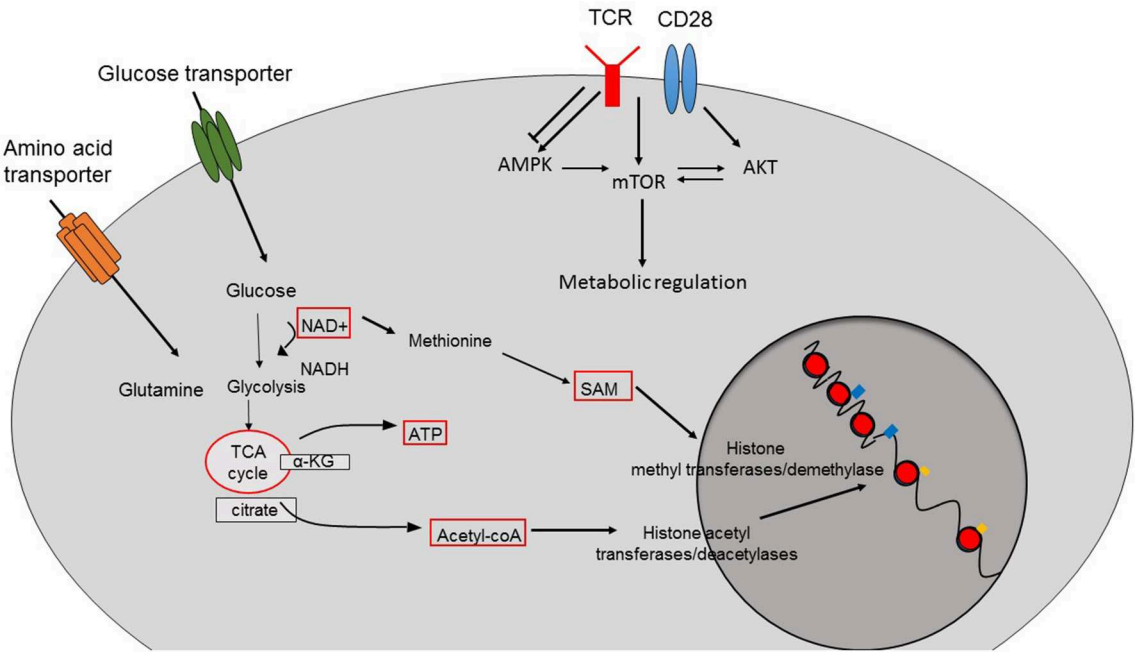

FIGURE 1 | Crosstalk between cellular metabolism and epigenetic changes. The function of epigenetic modifier enzymes depends on the intermediates or the products of cellular metabolism pathways resulting in epigenetic changes and therefore the transcriptional programs of the cells. Acetyl-CoA is the main source for histone acetylation leading to open and permissive chromatin structure. SAM is used by histone methyltransferases and demethylases. The function of sirtuin deacetylases depend on the availability of NAD. TCR-induced activation of pathways such as AMPK, mTOR, and AKT also contributes to the metabolic reprogramming of $CD8^+$ T cells.

mechanisms and their modifiers as well as their dependency on specific metabolic substrates. Subsequently, we will summarize recent insights into $CD8^+$ T cell specific aspects of metabolism-regulated epigenetics in anti-viral and anti-tumor immunity and discuss possible implications of T cell epigenetics for the development of better immunotherapies of cancer.

HISTONE MODIFICATIONS

Eukaryotic DNA is packed in the form of chromatin. Thereby, nucleosomes, the basic unit of the chromatin, consist of 147 bp of DNA, which wraps around the histone octamer composed of two H2A and H2B dimers as well as a tetramer of H3 and H4 proteins. N- and C-terminus of histone tails, which protrude from the nucleosome, represent the main sites for posttranscriptional modifications (PTMs) including acetylation, methylation, phosphorylation and ubiquitination (4). PTMs of histone tails can either directly regulate the chromatin structure, resulting in altered DNA accessibility (5), or can act as platforms for the binding or recruitment of non-histone proteins, known as writers (histone-modifying), readers (histone-modification-recognizing) or erasers (histone modification-erasing) (6). This combination of PTMs on histone tails constitutes the "histone code" that regulates the eukaryotic transcription (5). Histone chaperons are also critical regulators of DNA accessibility since the association of histones with specific chaperons regulates their folding, oligomerization, PTMs or stabilities (5). Therefore, different histone variants contribute to the regulation of DNA accessibility and epigenetic memory (7).

Histone Acetylation and Deacetylation

Acetylation and deacetylation of histones are among the best-studied epigenetic modifications. Acetylation of lysine residues is catalyzed by histone acetyltransferases (HATs) and reduces their positive charge, therefore the strength of electrostatic interaction between negatively charged DNA, resulting in relaxation of histone-DNA interactions, which leads to an increased accessibility of the DNA for transcription or transcription factors (TFs), respectively (8). Deacetylation reverses this permissive state via condensation of the chromatin structure (9). HATs are classified according to their localization. Cytoplasmic B-type HATs for example participate in the transport of newly synthesized histones from the cytoplasm to the nucleus, while nuclear A-type HATs take control of acetylation events related to the transcription (10). HATs can be further grouped according to their functional motifs consisting of Gcn5-related N-acetyltransferase (GNAT), Moz, Ybf2/Sas3, Sas2, Tip60 (MYST), Creb-binding protein/P300 (CBP/P300) and Rtt109 HAT families (10, 11).

HATs use acetyl-CoA as their primary source for histone acetylation. Acetyl-CoA is a central metabolite and the only source of acetyl groups in the cell (12). Most commonly, acetyl-CoA is produced in the mitochondrial matrix through glycolysis, β-oxidation or the catabolism of branched amino acids (12). As a

central metabolite and important signal transducer, acetyl-CoA regulates several cellular processes by controlling the balance between anabolic and catabolic reactions. Therefore, fluctuations in cellular acetyl-CoA levels can also affect the acetylation patterns of histones resulting in varying gene expression and function as well as distinct differentiation programs of cells (12).

Histone deacetylases (HDACs) are responsible for removing acetyl groups from acetylated histones resulting in chromatin condensation and a repressive chromatin structure. Depending on their homology and functions, HDACs are grouped into four different classes: class-I (HDAC1, HDAC2, HDAC3, HDAC8), class-IIa (HDAC4, HDAC5, HDAC7, HDAC9), class-IIb (HDAC6, HDAC10), class-III (Sirt1-Sirt7) as well as class-IV (HDAC11) (13). Although the diversity of the HDAC classes challenge the design of HDAC-inhibitors, several HDAC inhibitors are in clinical use or are under clinical investigation (14). While Vorinostat (SAHA) and Romidepsin (FK288) have been approved for the treatment of cutaneous T-cell lymphoma, Panobinostat (LBH589) and Belinostat (PXD101) are currently used for treating peripheral T-cell lymphoma and multiple myeloma, respectively (14). However, these inhibitors are pan-HDAC inhibitors, therefore studies attempting to design specific HDAC-inhibitors are active areas of research. According to clinical and experimental studies, inhibition of HDACs results in anti-neoplastic effects mostly via cytotoxic and pro-apoptotic mechanisms (15) [e.g., via stabilization of acetylated p53 (16)]. There are also accumulating data proving that inhibition of HDACs in non-oncological settings has important anti-inflammatory effects depending on the cell, tissue and context (15, 17, 18). For example, the gut microbiota-derived short-chain fatty acid butyrate modulates the transcriptional program of CTLs resulting in increased expression of IFNγ and granzyme B (19). However, the effects of butyrate are not mediated by the interaction with its receptors GPR41 and GPR43, but rather through HDAC inhibition resulting in a differential gene expression of CTL effector molecules, which was further validated by pan-HDAC inhibitor treatments (19). HDAC7, which is a Class-IIa HDAC, plays a pivotal role in the regulation of positive and negative selection of thymocytes and immune tolerance as well as their survival (20–22). Serine-threonine phosphoproteome analysis of CTLs by high resolution mass spectrometry revealed that HDAC7 is phosphorylated independently of T-cell receptor (TCR) activation and signaling, leading to its constitutive cytosolic localization (23). The exclusion of HDAC7 from the nucleus is critical for maintaining normal CTL function since the ectopic expression of the nuclear-trapped mutant phosphorylation-defective HDAC7 resulted in lower CD25 expression and subsequently reduced proliferation of CTLs in response to IL-2 (23). However, the role of HDAC7 in adult $CD4^+$ and $CD8^+$ T cells is still poorly understood. HDAC5, another class-IIa HDAC, has been described as a modulator of the inhibitory functions of $Foxp3^+$ regulatory $CD4^+$ T cells (Treg) (24), but inoculation of *Hdac5* knockout mice with congenic TC61 lung adeno-carcinoma cells did not result in decreased tumor growth compared to wild type littermates despite a defective immune suppressive capacity of *Hdac5*-deficient Treg, which can be explained by a simultaneous impairment of IFNγ production in *Hdac5*-deficient $CD8^+$ T cells (24). The inhibition of HDAC6 (Class-IIb) with its specific inhibitor (ACY-1215) results in impaired proliferation and activation as well as impaired pro-inflammatory cytokine production of $CD8^+$ T cells during mouse models of skin inflammation, suggesting that HDAC6 represents a key regulator of TCR-signaling and function, therefore might serve as a new drug target for the treatment of $CD8^+$ T cell-related skin disorders (25). On the other hand, the inhibition of HDAC6 in T cells of melanoma patients results in improved anti-tumor capacities of T cells (26). HDAC6 also takes role in the dynamics, transport and secretion of lytic granules to the immune synapse in $CD8^+$ T cells, further proving its significance for $CD8^+$ T cell function (27). In addition, HDAC3 (Class-I) is required for the proper T cell development in the thymus since its lymphocyte specific deletion resulted in reduced immature CD8 single-positive as well as CD4/CD8 double positive populations (28). Similarly, Class-I HDACs, HDAC1, and HDAC2, also participate in the proper thymic development of T cells (29, 30). Tschismarov et al. further confirm the critical role of HDAC1 during the development of T cells in the thymus. Additionally, they prove that T-cell specific deletion of HDAC1 results in impaired anti-viral responses upon LCMV infection and impaired expansion of LCMV-specific $CD8^+$ T cells (31).

Among other HDAC isoforms, Sirtuins (HDAC class-III) were intensively studied in terms of their metabolic functions. They participate in different cellular processes including the regulation of metabolism, DNA repair and mitochondrial function (32). For their deacetylation functions, sirtuins require NAD^+, which is an essential coenzyme and participating in many redox reactions as in glycolysis, TCA cycle and fatty acid oxidation. Thereby, the provision of NAD^+ depends on its intracellular compartmentalization, synthesis as well as on metabolic and other pathways that use NAD^+. For instance, SIRTs were found to be an indirect target of the compound resveratrol leading to histone deacetylation due to increased NAD^+ availability suggesting that the level of NAD^+ is critical for the regulation of the epigenome of $CD4^+$ T cells through sirtuins (33, 34). Interestingly, human $CD8^+CD28^-$ T cells, which represent a highly cytotoxic population of terminally differentiated memory T cells (Tmem), display an increased glycolytic capacity, which could be linked to a decreased expression of SIRT1 through a forkhead box protein (FOXO1)-dependent manner suggesting that the evolutionary conserved FOXO1-SIRT1 axis is critical for the metabolic reprogramming of human $CD8^+$ Tmem (35).

Histone Methylation

Unlike histone acetylation and deacetylation, which mark the chromatin for either transcriptional activation or repression, the effects of histone methylation on transcription are context dependent. For instance, while tri-methylation of lysine 4 on histone 3 (H3K4me3) triggers transcription, tri-methylation of histone 3 on lysine 27 (H3K27me3) is a sign for condensed chromatin and repressed gene transcription. Depending on the degree of methylation, different groups of histone methyltransferases catalyze the methylation of lysine residues. For example, H3K4 methylation is catalyzed by Set1

methyltransferases, whereas H3K9 methylation is catalyzed by KMT1 methyltransferases as well as H3K27 methylation by enhancer-of-zest homolog-2 (EZH2) (6).

Histone methyltransferases use S-adenosyl-methionine (SAM) as their source for methyl groups (36). Thereby, SAM is produced from methionine via one-carbon metabolism. In immune cells, one-carbon metabolism plays important roles especially in the regulation of proliferation. For instance, Ma et al. showed that once $CD4^+$ or $CD8^+$ T cells are activated, the expression of genes coding for one-carbon metabolism-related enzymes such as *Shtm1* and *Shmt2*, that are essential regulators of the entry of serine-dependent carbon into the cytosolic and mitochondrial tetrahydrofolate cycle, are up-regulated. Serine that is metabolized through this pathway is required for proper T cell proliferation both *in vitro* and *in vivo* (37).

ATP-Dependent Chromatin Remodeling Complexes

The formation of higher order chromatin structures is pivotal for the transcriptional programming by regulating or limiting the access of TFs to their binding sites. This structure can be modulated by either PTMs of histone tails or via nucleosome- and chromatin-remodeling complexes. These complexes are capable of removing histones, changing the path of DNA around the nucleosome and hence altering their position. Nucleosome remodeling complexes use the energy generated from ATP hydrolysis (38). Since the activity of these complexes is ATP-dependent, it is expected that fluctuations in cellular ATP levels affect their function, therefore the remodeling of nucleosomes and chromatin structure. However, cellular ATP levels are saturating for their catalytic sites and the activities of chromatin remodeling complexes are not influenced by changes in ATP in the cell. Nevertheless, gene expression states can still be regulated by AMPK signaling which can sense ADP/ATP ratios and induce transcriptional regulation (39). Previously, Blagih et al. showed that both $CD4^+$ and $CD8^+$ T cells are metabolically adapting in response to limited nutrient levels mediated by AMPK regulated mRNA translation as well as glutamine dependent mitochondrial metabolism. This is a key mechanism for the maintenance of T cell bioenergetics and survival. Their data equally indicated that AMPK signaling is mandatory for primary T cell responses to both, bacterial and viral infections, thus driving adaptive immunity (40). Interestingly, T cell specific deletion of AMPK in mice resulted in increased tumor growth, caused by an impaired tumor killing of $CD8^+$ T cells. Deletion of AMPK in T cells resulted in a decreased production of IFNγ and granzyme B as well as an elevated serine/protein phosphatase activity upon activation, resulting in decreased survival rates and anti-tumor functions of $CD8^+$ T cells, which could be reversed by inhibition of phosphatase activity (41).

METABOLIC REPROGRAMMING OF $CD8^+$ T CELL DIFFERENTIATION AND FUNCTION

In order to adapt to dynamic environments and to meet the demands of cells for their different functions, cellular metabolism is tightly controlled. Cells are capable of performing catabolic and anabolic processes to break down or synthesize macromolecules, which supply either energy in the form of ATP to meet their energy demands, or metabolic intermediate products that are essential for cellular growth (**Figure 2A**). Via the glycolysis pathway, two molecules of ATP per glucose molecule and pyruvate are produced. In oxygen-rich conditions, pyruvate can enter into tricarboxylic acid (TCA) cycle where it is further processed to generate 38 ATP (maximal number) molecules via oxidative phosphorylation (OXPHOS) (42). Catabolism of pyruvate is not the only mechanism providing substrates for TCA. While fatty acids are converted into acetyl-CoA through fatty acid oxidation (FAO), amino acids are catabolized into 3-, 4-, and 5- carbon substrates that are fed into the TCA cycle (42).

Different metabolic requirements for different cell states are also valid for $CD8^+$ T cells. $CD8^+$ T cells mainly have three phases as naïve, effector (Teff) and memory T cells. When naïve T cells encounter their antigens, this results in their activation leading to rapid proliferation, growth and differentiation (43). $CD8^+$ T cells mostly differentiate into CTLs, producing cytotoxic molecules such as granzyme B, perforin, and pro-inflammatory cytokines including IFNγ and TNFα. Following this effector phase, the effector cell population contracts and a small population of memory T cells (Tmem) persists, which will turn again into CTLs in case of antigen re-challenge and which can persist in the body for years (43). While naïve T cells are metabolically quiescent and depend on OXPHOS, their activation results in a switch into glycolysis pathway to meet the demand for anabolic intermediates necessary for their rapid growth, proliferation and effector functions (**Figure 2A**). Recently, Store-Operated Calcium Entry (SOCE) signaling, which is the main calcium influx pathway in T cells in response to TCR activation and is mediated by stromal interaction molecule (STIM) 1 and STIM2 as well as ORAI proteins, was shown to control clonal expansion of both, $CD4^+$ and $CD8^+$ T cells, via controlling glycolysis and OXPHOS through the transcriptional regulation of glycolysis related gene expression (44). It was also shown that these effects are mediated by calcineurin and NFAT, which are the downstream regulators of SOCE (44). Metabolically, Tmem depend mostly on FAO and have a higher spare respiratory capacity (SRC) which supplies the high energy levels needed for their rapid function in case of antigen re-encounter (45–48). However, during most of these studies CPT1α, the rate-limiting enzyme of long-chain FAO was targeted by the drug etomoxir (49, 50). Interestingly, T cell specific deletion of *Cpt1α in vivo* proved that CPT1α is dispensable for Teff or Tmem responses as well as $CD4^+$ Treg suppressive function, differentiation and hemostasis (50). As the use of the CPT1α inhibitor etomoxir at a concentration higher than 3 μM causes off-target effects, it appears that other metabolic pathways than long-chain FAO are involved in Teff and Tmem differentiation (49, 50).

In conditions of continuous antigen exposure like chronic infections and tumors, T cells fail to differentiate into functional memory cells, but enter a state in which they are hypo-responsive (51). This so-called exhausted cells are incapable of cytokine secretion, proliferation or lysing target cells, paralleled by a

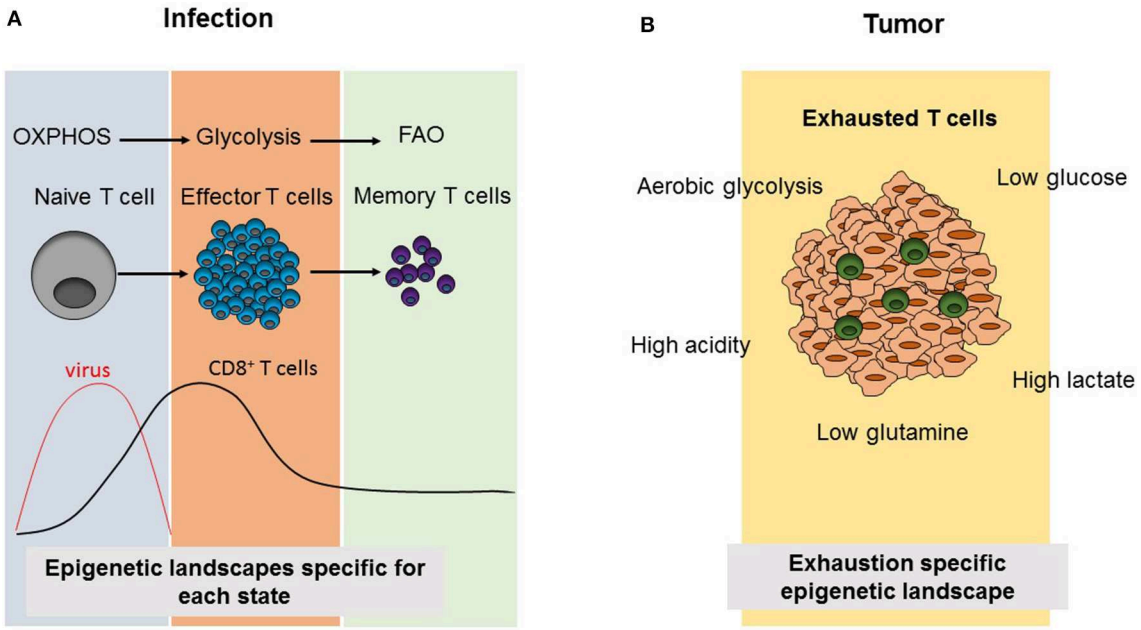

FIGURE 2 | Comparison of $CD8^+$ T cell differentiation and metabolism as well as epigenetic landscapes during infection and tumorigenesis. **(A)** Virus infection results in the activation of naïve $CD8^+$ T cells triggering the differentiation into effector cells, which induce viral clearance. Subsequently, effector T cells contract and leave behind a small population of memory $CD8^+$ T cells. During this differentiation process, $CD8^+$ T cell subsets use the indicated cellular metabolism pathways and acquire different epigenetic landscapes specific to each phase. **(B)** In tumors, the presence of immunosupressive environments due to metabolic alterations in tumor cells results in an exhausted phenotype, in which tumor infiltrating T cells are not able to compete with tumor cells for metabolic products and they become non-functional resulting in increasing tumor growth. Exhausted T cells also acquire an exhaustion-specific epigenetic landscape.

sustained expression of co-inhibitory molecules such as PD-1, LAG3 and TIM3 (51). There are several studies, which link the expression of co-inhibitory molecules in T cells with disturbances of metabolic pathways including the PI3K/Akt/mTOR pathway. Thus, Staron et al. recently demonstrated in a mouse model of chronic lymphocytic choriomeningitis mammarenavirus (LCMV) infection, that AKT and mTOR activation are impaired in virus antigen-specific CTLs resulting in a defective anabolic metabolism and enhanced activity of the TF FOXO1 due to its defective phosphorylation and subsequent nuclear trapping (52). Additionally, FOXO1 acts as a direct transcriptional activator of PD-1 as the nuclear localization of FOXO1 promotes the differentiation of terminally exhausted PD-1^{hi}Eomes^{hi} CTLs. In contrast, during chronic LCMV infection in mice blockage of PD-1 improves mTOR activity in antigen-specific CTLs while anti-PD-1 treatment was ineffective if mTOR was inhibited by rapamycin (52). Remarkably, the glycolytic metabolism of $CD8^+$ T cells is already affected during the acute phase of viral infection in LCMV-infected mice and precedes further dysfunction of antigen-specific T cells suggesting that antigen-specific $CD8^+$ T cells are unable to meet the metabolic demands needed for proper cytotoxic function (53). PD-1 is also an early regulator of genes related to glycolysis and mitochondrial function and represses peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), whose overexpression is able to improve the metabolism of exhausted T cells and hence, partially restoring their functions (53). These studies suggest that metabolic impairments, which are regulated by PD-1, are early drivers of $CD8^+$ T cell exhaustion (53).

The expression of the pro-inflammatory cytokine IFNγ by activated T cells is regulated through 3′-untranslated region (UTR)-dependent mechanisms (54). Peng et al. showed that in activated T cells the expression of lactate dehydrogenase A (LDHA) is induced in order to support high levels of aerobic glycolysis, but also regulates the expression of IFNγ through 3′-UTR-independent mechanisms. Interestingly, high LDHA levels in activated T cells result in the maintenance of increased acetyl-CoA concentrations leading to increased histone acetylation and facilitating H3K9Ac accumulation on the IFNγ locus, therefore resulting in its increased transcription (55).

Upon TCR-activation, S-2-hydroxyglutarate (S-2-HG) accumulates in murine $CD8^+$ T cells up to millimolar concentrations under physiological oxygen conditions through hypoxia inducible factor 1 alpha (HIF-1α) predominating over R-2-hydrooxyglutarate, which is an oncometabolite, produced via mutant isocitrate hydrogenase (IDH) (56). The accumulation of this metabolite results in changes in T cell differentiation, especially resulting in a central memory (Tcm) like phenotype that is stable after transfer into wild-type host mice. The accumulation of S-2-HG also resulted in higher proliferation, maintenance and anti-tumor functions of $CD8^+$ T cells *in vivo* following their adoptive transfer into mice (56). Interestingly, these effects were mediated by the modulation of histone and DNA methylation. S-2-HG is an immunometabolite, thus

further supporting a metabolism-dependent regulation of T cell epigenetics and functions (56).

EPIGENETIC LANDSCAPES DURING CD8$^+$ T CELL DIFFERENTIATION

Enabled by Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq), which allows the identification of accessible regions of chromatin, the dynamic changes of chromatin as well as accessible TF binding sites were identified in response to acute and chronic LCMV infection during naïve, activated, effector, and memory states of polyclonal or antigen-specific CD8$^+$ T cells (57, 58). In these studies, more than 70,000 regions were identified as open (accessible) at least in one of the differentiation state and half of these regions were shared among all states suggesting a CD8$^+$ T cell-specific chromatin state. Thus, it was shown that in Tmem cells many regulatory sites are in the open configuration, demonstrating that these cells keep a memory-primed gene expression program that can readily and rapidly be activated in case of a secondary infection (57, 58).

Several TFs were identified to control the fate of CD8$^+$ T cells during the differentiation of these cells. For instance, T-box expressed in T cells (Tbet), inhibitor of DNA-bing 2 (ID2), interferon regulatory factor 4 (IRF4), B lypmphocyte induced maturation protein 2 (BLIMP-2), and zinc finger E-box-binding homeobox 2 (ZEB2) are required for the differentiation of Teff cells, whereas T-cell factor 1 (TCF1), eomesodermin (EOMES), inhibitor of DNA-binding protein 3 (ID3), B- cell lymphoma protein 6 (BCL6), and FOXO1 control for memory formation in CD8$^+$ T cells (59). However, some of these TFs are not differentially expressed in Teff and Tmem subsets, suggesting that additional mechanisms are involved in controlling the fate decision of T cells. By using a model of bacterial infection in mice, Yu et al. have recently characterized the epigenetic landscapes of naïve, effector, memory precursor and memory CD8$^+$ T cells, followed by the prediction of putative TF-binding to accessible chromatin regions in each cell subset (59). In addition, the importance of TFs was ranked in each cell subset via bioinformatic analysis. With this approach, the authors identified and experimentally validated two TFs, Yin Yang 1 (YY1) as well as nuclear receptor subfamily3 group C member 1 (NR3C1) as promoters of effector and memory precursor phenotypes (59).

Other studies applying the ATAC-seq technology demonstrated that also exhausted T cells possess a unique chromatin state (60). During acute models of viral infection in mice, exhausted T cells and effector cells share common accessible chromatin sites. However, additional sites on chromatin open or close during the exhaustion of T cells leading to the expression of PD-1. The treatment of exhausted T cells with anti-PD-1 antibodies resulted in the rescue of gene expression associated with effector functions. However, this treatment failed to fully rescue or reverse the exhaustion-specific chromatin signature as well as the exhaustion specific transcriptional program of exhausted T cells (60).

Although it is now known that exhausted CD8$^+$ T cells have a unique chromatin state compared to effector or memory subsets, the mechanisms driving their transcriptional and epigenetic development are poorly understood. Recently, three studies identified the transcription factor thymocyte selection associated high mobility box (TOX) as the main factor promoting CD8$^+$ T cell exhaustion by regulating early epigenetic events (61–63). These include decreased accessibility of genes associated with effector T cell differentiation as well as increased accessibility of memory and exhausted phenotype associated genes (62). The expression of TOX is a robust hallmark of exhausted T cells. However, it is transiently expressed at low levels during acute viral infections. In addition, the expression of TOX is essential and sufficient to induce the exhausted T cell phenotype as defined by the marker proteins PD-1, TIM3, LAG3, TIGIT as well as EOMES. Interestingly, initial TOX expression requires NFATc2 and calcineurin signaling, however, the sustained expression of TOX in exhausted CD8$^+$ T cells is calcineurin-independent (62). This study suggests that among others, TOX expression-related mechanisms, can force exhausted cells into an irreversible exhaustion-specific and developmentally fixed chromatin state, which cannot be remodeled by anti-PD-1 treatment.

Further studies also provide information on the dynamic regulation of methylation patterns during virus-induced T cell differentiation (64, 65). For instance, in naïve CD8$^+$ T cells, genes associated with effector functions are marked with H3K4me3 indicating that effector function related genes are repressed in naïve CD8$^+$ T cells. Upon activation, the same genes acquire chromatin marks that are related to an active transcription (64, 65).

In addition to the regulation of chromatin marks of promoters or enhancers, which are characterized by H3K4me1 and H3K27ac, are also differentially remodeled during T cell activation and differentiation (59, 66). In studies by Kakaradov et al., around 50,000 enhancers were identified and about 50% of them were found to be shared between all stages of T cell activation and differentiation, whereas the other half of enhancers was either gained or lost depending on the state of the cells suggesting that there is a dynamic regulation of enhancers similar to the other epigenetic modifications of T cells during differentiation (66). According to single-cell sequencing studies, in which individual CD8$^+$ T cells were analyzed during an acute LCMV infection in mice, the differentiation of terminal effector cells was initiated by an early burst of transcriptional activity followed by a refinement of epigenetic silencing of transcripts related to memory lymphocytes through H3K27me3 and Ezh2, which is the catalytic subunit of polycomb repressive complex 2 (PRC2) (67).

THE EFFECTS OF TUMOR MICROENVIRONMENT ON T CELL METABOLISM AND EPIGENETICS

Chronic infections and cancer share common properties in terms of CD8$^+$ T cell functions. In both cases, antigen specific CD8$^+$ T cells progress into the so-called exhausted state due to continuous

antigen exposure resulting in increased expression of exhaustion markers. Therefore, research on chronic infection models in mice as well as patient samples contributed to the development of anti-cancer therapies, which target T cell exhaustion such as anti-PD-1 or adoptive T cell therapies (68). Although the reprogramming of cellular metabolism and changes of the epigenetic landscape of $CD8^+$ T cells have been intensively studied during chronic infections, these mechanisms are still poorly understood in tumor infiltrating lymphocytes (TILs). Although overlapping features of exhausted $CD8^+$ T cells can be observed both in chronic infection and cancer models, the specific metabolic environment in tumors represents an additional, unique factor shaping T cell activation and differentiation via the specific supply provision of metabolites and various secreted signaling molecules (**Figure 2B**).

The metabolism of tumor cells is altered compared to normal cells, which metabolize glucose through OXPHOS. Instead, highly proliferating tumor cells use glycolysis pathway to metabolize glucose in order to supply the anabolic products needed for rapid cell growth and division. This metabolic alteration of tumor cells was characterized by Otto Warburg almost a century ago and is now considered as a hallmark of cancer (69). However, this phenomenon is only a portion of the unique tumor cell metabolism. In addition to glucose metabolism, lipid, amino acid, and adenosine metabolism are also altered in tumor cells to meet their high-energy demands (70). For instance, HIF1α in tumor cells upregulates the expression of CD73, which is located on the surface of many tumor cells and which is responsible for the conversion of adenosine monophosphate to adenosine resulting in increased adenosine concentrations in TME (71). Similarly, HIF1α also regulates genes critical for the lipid metabolism such as COX2 whose overexpression is associated with poor prognosis in several solid tumor cancers (72). Glutamine metabolism is also altered in tumor cells, that are known as glutamine traps since they have higher levels of glutamine uptake (70).

TILs are mostly non-functional or exhausted due to the highly immunosuppressive TME. The depletion of glucose in TME by tumor cells represents one "exhausting" mechanism and results in a decrease of aerobic glycolysis in TILs and decreased phosphoenolpyruvic acid (PEP) production that is a crucial metabolite participating in TCR-dependent activation of calcium pathways like SOCE and NFAT signaling in T cells (73, 74), which is critical for proper anti-tumor functions (73). Additionally, due to high lactate production of tumor cells, the acidity of TME increases, resulting in the inhibition of key T cell responses such as proliferation after activation and effector cytokine production by $CD8^+$ T cells (70, 75). Similarly, due to the hypoxic environment of the tumors, HIF1α upregulates the expression of PD-1-ligand leading to inhibition of $CD8^+$ T-cell mediated cytotoxicity (76).

The epigenetic landscapes of tumor infiltrating $CD8^+$ T cells are not well understood. In a recent study, Philipp et al. defined the chromatin dynamics of tumor-specific dysfunctional cells over the course of tumorigenesis (77). They observed that naïve tumor-specific T cells that encounter their antigen firstly acquire a plastic, dysfunctional chromatin state that can be remodeled. Later, the same cells differentiate into a fixed dysfunctional chromatin state, which cannot be remodeled or rescued anymore during the progression of large established tumors. In addition, human dysfunctional tumor specific T cells with high PD-1 expression share many core elements with these mouse models (77). Interestingly, tumor-specific memory T cells also differentiate into the same fixed dysfunctional chromatin state suggesting that regardless of the initial chromatin states of the cells, continuous antigen exposure in tumors can overwrite this fixed dysfunctional chromatin state (77).

CONCLUSION

Since the manipulation of metabolic pathways *in vivo* is very challenging, so far mostly *in vitro* systems served in this field, to provide mechanistic information to reveal the regulation of T cell metabolism in a controlled environment. However, the field is still in need of experimental models that are able to better provide the physiological context of changing T cell environments such as nutrient availability, interaction between different cell types and cytokine milieu to fully investigate the role of metabolism during T cell activation and differentiation.

The link between epigenetic changes and cellular metabolism has been intensively studied in cancer cells. However, the role of metabolism on T cell function and differentiation has only recently been characterized despite growing knowledge about the connection of T cell epigenetics during differentiation and function by using genome-wide mapping of accessible chromatin sites. However, it still remains elusive how metabolites regulate the epigenome of T cells in a gene-specific manner.

Although this interplay between tumor cells and the epigenetic regulation of TILs remains elusive, a better understanding of the epigenetic regulation of exhaustion and the metabolic fitness of TILs might hold potential to improve current cancer therapies such as checkpoint blockade and adoptive T cell therapies. The relationship between the unique metabolism in TME and how it affects the epigenome of TILs might help to find ways to rescue their exhausted phenotype via epigenome-targeting pharmacological drugs to boost immune responses against tumor cells.

AUTHOR CONTRIBUTIONS

CY designed, wrote, and revised the manuscript. BS revised the manuscript. CW and RG designed and revised the manuscript.

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