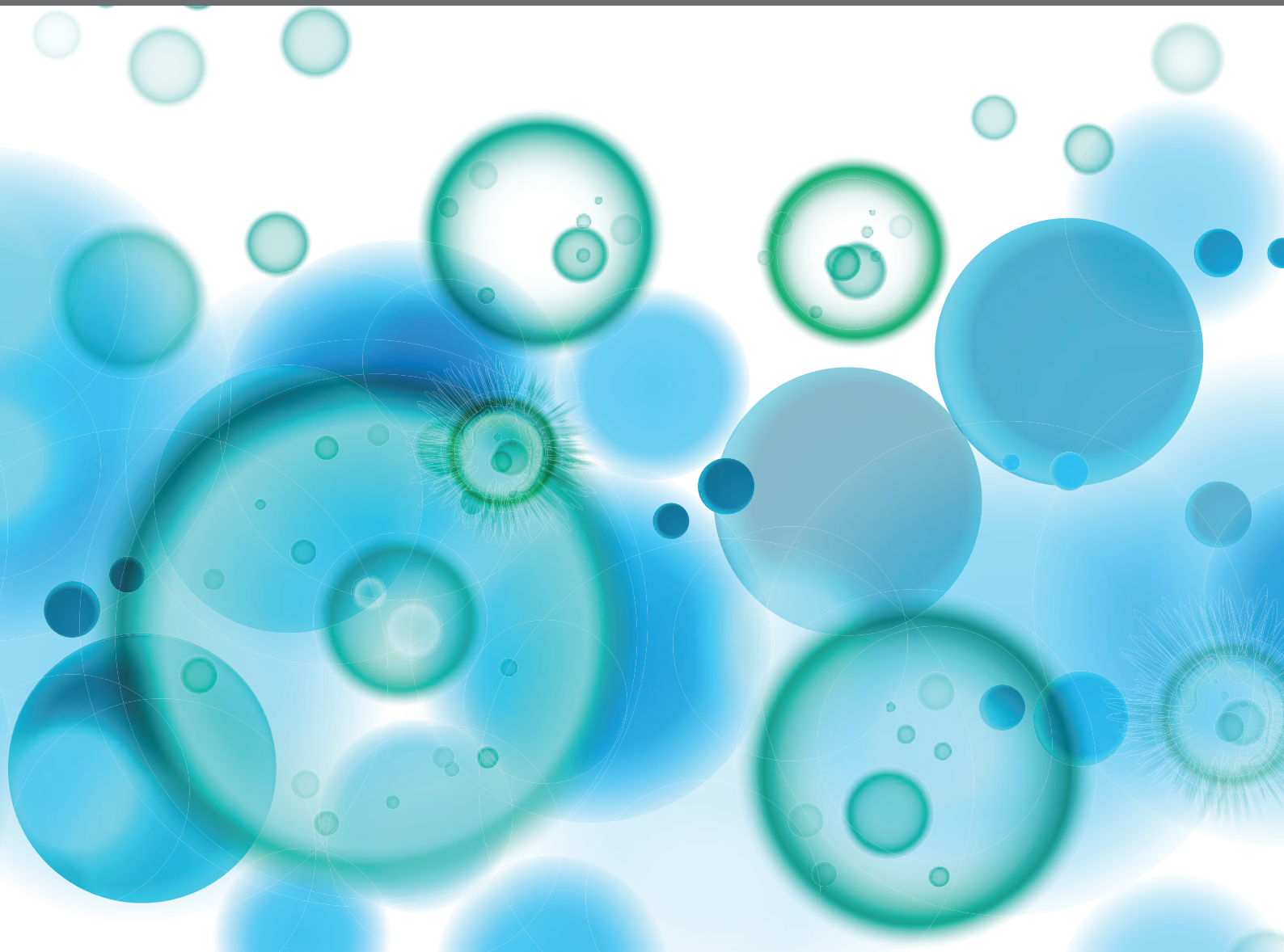


IMMUNOPHYSICS AND IMMUNOENGINEERING

EDITED BY: Jorge Bernardino De La Serna, Mario Mellado, Maria Garcia-Parajo,
Michael Loran Dustin and Dimitrios Morikis
PUBLISHED IN: Frontiers in Immunology and Frontiers in Physics





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ISSN 1664-8714

ISBN 978-2-88963-570-2

DOI 10.3389/978-2-88963-570-2

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IMMUNOPHYSICS AND IMMUNOENGINEERING

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Citation: De La Serna, J. B., Mellado, M., Garcia-Parajo, M., Dustin, M. L., Morikis, D., eds. (2020). ImmunoPhysics and ImmunoEngineering. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-570-2

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Editorial: ImmunoPhysics and ImmunoEngineering

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Keywords: immunophysics, immunoengineering, T cell biology, biophysics, cell membrane, cell sensing and remodeling, super resolution (SR), quantitative imaging

Editorial on the Research Topic

ImmunoPhysics and ImmunoEngineering

The immune system comprises a collection of specialized cells, tissues, and organs that protect the organisms against pathogens and can survey cancer cells. Immune responses are precisely coordinated events that take place in complex, specialized tissue microenvironments. For an integrated view of innate and adaptive immune responses at the molecular level, we ideally need a better understanding of how immune cells communicate and fulfill their tasks *in vivo*, following events spatially and temporally. Conventional biochemical and genetic methods consider the cell as an individual entity and ligand/receptor pairs as isolated systems. Often, the data obtained refers to the average behavior of a pool of cells and/or receptors removed from their real-life context. The use of new technologies, particularly real-time imaging approaches, is showing us that biological responses are very dynamic and extremely dependent on the context in which they take place and are therefore much more diverse than we initially thought. The combination of these new approaches is radically transforming and enriching immunology, as demonstrated by the increasing number of publications in which physical and/or engineering tools are applied to study the immune response. Whilst scientists are often questioned for the discipline their research is best framed in, we rather think that one scientific discipline cannot be reduced to the terms of another. However, defining and naming cross-disciplinary fields sets our minds on common ground and helps establish a fluent communication to eventually produce groundbreaking, beautiful pieces of science. For instance, ImmunoPhysics was probably first coined by Prof. Morikis a couple of decades ago (<https://www.biophysics.org/profiles/dimitrios-morikis>); nevertheless, ImmunoPhysics has not become widely regarded as a discipline, despite the continuously growing body of research that requires physical approaches to resolving immunological questions. Hence, with this special issue, we wanted to open a scientific platform compiling ImmunoPhysics and ImmunoEngineering research breakthroughs and future perspectives. Sadly, towards the end of this fascinating journey Prof. Morikis passed away; thus now, with this special issue, we would also like to pay tribute to his fundamental contributions to the field.

The qualitative and quantitative knowledge-advancement resulting from the application of physical and engineering methods and techniques in immunology is unarguable. Coordinated advances in physics and engineering technologies are revolutionizing our research strategies, contributing qualitatively, and quantitatively to breakthroughs in the understanding of immune system function and its regulation in health and disease. Probably, the overarching goals

OPEN ACCESS

Edited and reviewed by:

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†Deceased: May 27, 2019

Specialty section:

This article was submitted to
Medical Physics and Imaging,
a section of the journal
Frontiers in Physics

Received: 03 January 2020

Accepted: 30 January 2020

Published: 21 February 2020

Citation:

Bernardino de la Serna J, Mellado M,
Dustin ML, Garcia-Parajo MF and
Morikis D (2020) Editorial:
ImmunoPhysics and
ImmunoEngineering.
Front. Phys. 8:28.
doi: 10.3389/fphy.2020.00028

of ImmunoPhysics and ImmunoEngineering are to facilitate the development of therapeutic interventions to more precisely modulate and control the compromised immune response during diseases; but whereas ImmunoPhysics study and assess the physical basis of the immune response, ImmunoEngineering pursues its control and prediction. Possibly the greatest challenge for the growth of these fields is the establishment of fluent communication between physicists or engineers and immunologists. For this purpose, in this special issue, we aimed to provide a broad and interdisciplinary forum for researchers to present their personal views on the field, point to future challenges, and show their latest empirical or theoretical observations or method developments, with biomedical implications *in vivo*, *in vitro*, and *in silico*. Initially, we eagerly sought contributions employing highly advanced static or dynamic quantitative techniques spanning from conventional to super- or ultra-resolved microscopy in space and time to unravel protein clustering [1–8] or to disentangle the involvement of actin cortex dynamics and its mechanics in T-cell activation and synapse formation [9–15]; we also welcomed contributions applying steady and non-equilibrium quantitative fluorescence spectroscopic approaches to resolve receptor protein oligomerization and lipid-protein interactions and dynamics [16, 17] that can be applied to resolve immune-relevant events [18, 19]. Investigations employing systems immunology approaches, including quantitative and high-throughput assessment of the immune status and mathematical modeling of immune interactions [20–25] were also sought after; finally, we were also keen to include breakthroughs in the design or utilization of engineered tools and methods with applications to T-cell biology or immunotherapy [26–31].

Overall, we were gratefully surprised by the broad scope of approaches we were exposed to. For instance, in relation to the application of biophysical tools, including advanced light microscopy and electron microscopy methods, to better understand the molecular mechanisms of immuno-biological processes, we received two reviews: one describing the most used fluorescent microscopy approaches dealing with the polarized secretory intracellular traffic during the immune synapse (Calvo and Izquierdo), and another describing how techniques able to resolve elements at the nanoscale have contributed to the fundamental understanding of the immune synapse (Shannon and Owen). We also received three original articles, one showing a new developed method to track soluble mediators in the B-follicle at the single-molecule level (Miller et al.), another about possible artifacts occurring during chemical fixation in T cells (Pereira et al.), and another revealing novel structures in the podosomes formed at the dendritic cells employing correlative light and electron super-resolution microscopy (Joosten et al.). Regarding the use of *in silico* simulations and mathematical modeling of immune interactions and quantitative approaches to resolving lymphocyte dynamic immune-related events, we received four contributions: one resolving a structural model for the transmembrane domain of the B-cell receptor with molecular simulation (Friess et al.), another integrating experiments and theory to better understand the dynamics of the T-cell receptor and the cognate peptide

of the major histocompatibility complex (Buckle and Borg), a third employing molecular dynamics and electrostatic studies to evaluate the importance of two proteins of the membrane attack complex in the initial steps of the formation of the complement system (Zewde et al.), and a fourth employing mathematical models to predict the fate of a naïve T cell during migration and recirculation (de la Higuera et al.). We also received articles dealing with the study of the cognate interaction between antigen-presenting cells and T cells, where the roles of different proteins were investigated, such as the protein kinase C during polarized exosome secretion (Herranz et al.), TCR interplay with CD3/CD6 (Meddens et al.), and with CD28 (Xia et al.) during the immune synapse. We also include reviews dealing with the plastic cellular morphological changes during early activation, upon triggering, and during the immune synapse, involving perspectives spanning from the highly dynamic, very sophisticated, and complex reorganization of receptor kinases and phosphatases at the plasma membrane (Pérez-Ferreros et al.; Glatzová and Cebecauer; Junghans et al.), and its associated lipids (Baumgart et al.; Pettmann et al.; Gawden-Bone and Griffiths) to the full cytoskeletal reassembly (Martín-Cófreces and Sánchez-Madrid.) The current increasing interest in the role of mechanotransduction in T-cell biology also received attention, with contributions dealing with the application of different biophysical tools to reveal cellular forces or molecular sensing to understand the correlation between the highly coordinated forces exerted by cells with their chemical sensing and activation processes (Kolawole et al.; Harrison et al.; Brockman and Salaita; Rossy et al.). Also, some contributions showed how the application of systems immunology and protein and lipid engineering could help predict lymphocyte receptor recognition patterns (Hammerschmidt et al.; Gorby et al.; Nieto-Garai et al.; Hörner et al.). Finally, contributions are also included on how building sophisticated mimetic *in vitro* models, for instance by means of microfluidics, novel substrates, and hydrogels, can help to unravel the complexity of immune molecular events at the single-cell level (Benard et al.; Sinha et al.; Weiden et al.).

Overall, the greatest reward was to continuously receive contribution requests, even after the closure of the submission period and to observe that the number of manuscript views and downloads is continuously increasing. We truly believe that, thanks to the 30 contributions comprising this Research Topic, we have fulfilled our aim of editing an article collection that stimulates and facilitates a scientific forum for physicists, engineers, and immunologists, filling the cross-disciplinary gap, increasing awareness, and maximizing discussion and dissemination of ideas and methodologies in this nascent field. The next challenges in the ImmunoPhysics and ImmunoEngineering fields will probably be related to mechanobiology, immuno-oncotherapy, and the engineering of the tumor immunological niche. Correlating the highly coordinated forces exerted by cells with their chemical sensing and activation process is seemingly very challenging but eventually scientifically rewarding. Furthermore, immunotherapy is now widely considered to be one of the best approaches to treatments for certain types

of advanced cancer, and immune evasion is now recognized as a hallmark of cancer. The physical understanding of the immunological synapse underpins checkpoint therapies, and immunoengineering of T-cell specificity is the basis of CAR-T therapies. However, there is still much to learn about the physical mechanisms that differentiate responders and non-responders to these powerful therapies. We still have much to learn about engineering the tumor immunological microenvironment. One future goal of ImmunoPhysics could be to construct an accurate *in vitro* model tissue environment, where advanced microscopy and spectroscopy, force microscopy, and eventually optical and magnetic tweezers would enable measurement and manipulation of key physical parameters to improve

fundamental understanding, ultimately leading to applications in improved immunotherapies.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We would like to thank the reviewers for their thoughtful comments and efforts toward improving the manuscripts of this special issue.

REFERENCES

- Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat Immunol.* (2012) **14**:82–9. doi: 10.1038/ni.2488
- Williamson DJ, Owen DM, Rossy J, Magenau A, Wehrmann M, Gooding JJ, et al. Pre-existing clusters of the adaptor Lat do not participate in early T cell signaling events. *Nat Immunol.* (2011) **12**:655–62. doi: 10.1038/ni.2049
- Santos AM, Ponjavic A, Fritzsche M, Fernandes RA, De La Serna JB, Wilcock MJ, et al. Capturing resting T cells: the perils of PLL. *Nat Immunol.* (2018) **19**:203–5. doi: 10.1038/s41590-018-0048-8
- Bakker GJ, Eich C, Torreno-Pina JA, Diez-Ahedo R, Perez-Samper G, Van Zanten TS, et al. Lateral mobility of individual integrin nanoclusters orchestrates the onset for leukocyte adhesion. *Proc Natl Acad Sci USA.* (2012) **109**:4869–74. doi: 10.1073/pnas.1116425109
- Calebiro D, Rieken F, Wagner J, Sungkaworn T, Zabel U, Borzi A. Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proc Natl Acad Sci USA.* (2013) **110**:743–8. doi: 10.1073/pnas.1205798110
- Fujiwara TK, Iwasawa K, Kalay Z, Tsunoyama TA, Watanabe Y, Umemura YM, et al. Confined diffusion of transmembrane proteins and lipids induced by the same actin meshwork lining the plasma membrane. *Mol Biol Cell.* (2016) **27**:1101–19. doi: 10.1091/mbc.E15-04-0186
- Martínez-Muñoz L, Rodríguez-Frade JM, Barroso R, Sorzano CÓS, Torreño-Pina JA, Santiago CA, et al. Separating actin-dependent chemokine receptor nanoclustering from dimerization indicates a role for clustering in CXCR4 signaling and function. *Mol Cell.* (2018) **71**:873. doi: 10.1016/j.molcel.2018.08.012
- Sungkaworn T, Rieken F, Lohse MJ, Calebiro D. High-resolution spatiotemporal analysis of receptor dynamics by single-molecule fluorescence microscopy. *J Vis Exp.* (2014) **89**:e51784. doi: 10.3791/51784
- Ashdown GW, Burn GL, Williamson DJ, Pandžić E, Peters R, Holden M, et al. Live-cell super-resolution reveals F-actin and plasma membrane dynamics at the T cell synapse. *Biophys J.* (2017) **112**:1703–13. doi: 10.1016/j.bpj.2017.01.038
- Colin-York H, Kumari S, Barbieri L, Cords L, Fritzsche M. Distinct actin cytoskeleton behaviour in primary and immortalised T-cells. *J Cell Sci.* (2020) **133**:jcs232322. doi: 10.1242/jcs.232322
- Fritzsche M, Fernandes RA, Chang VT, Colin-York H, Clausen MP, Felce JH, et al. Cytoskeletal actin dynamics shape a ramifying actin network underpinning immunological synapse formation. *Sci Adv.* (2017) **3**:e1603032. doi: 10.1126/sciadv.1603032
- Gawden-Bone CM, Frazer GL, Richard AC, Ma CY, Strege K, Griffiths GM. PIP5 kinases regulate membrane phosphoinositide and actin composition for targeted granule secretion by cytotoxic lymphocytes. *immunity.* (2018) **49**:427–37.e4. doi: 10.1016/j.immuni.2018.08.017
- Hons M, Kopf A, Hauschild R, Leithner A, Gaertner F, Abe J, et al. Chemokines and integrins independently tune actin flow and substrate friction during intranodal migration of T cells. *Nat Immunol.* (2018) **19**:606–16. doi: 10.1038/s41590-018-0109-z
- Ritter AT, Kapnick SM, Murugesan S, Schwartzberg PL, Griffiths GM, Lippincott-Schwartz J. Cortical actin recovery at the immunological synapse leads to termination of lytic granule secretion in cytotoxic T lymphocytes. *Proc Natl Acad Sci USA.* (2017) **114**:E6585–94. doi: 10.1073/pnas.1710751114
- Valitutti S, Dessing M, Aktories K, Gallati H, Lanzavecchia A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med.* (1995) **181**:577–84. doi: 10.1084/jem.181.2.577
- García E, Bernardino De La Serna J. Dissecting single-cell molecular spatiotemporal mobility and clustering at focal adhesions in polarised cells by fluorescence fluctuation spectroscopy methods. *Methods.* (2018) **140**:41:85–96. doi: 10.1016/j.ymeth.2018.03.008
- Schneider F, Waithe D, Galiani S, Bernardino De La Serna J, Sezgin E, Eggeling C. Nanoscale spatiotemporal diffusion modes measured by simultaneous confocal and stimulated emission depletion nanoscopy imaging. *Nano Lett.* (2018) **18**:4233–40. doi: 10.1021/acs.nanolett.8b01190
- Hedde PN, Staaf E, Singh SB, Johansson S, Gratton E. Pair correlation analysis maps the dynamic two-dimensional organization of natural killer cell receptors at the synapse. *ACS Nano.* (2019) **13**:14274–82. doi: 10.1021/acsnano.9b07486
- McCloskey MA, Poo MM. Contact-induced redistribution of specific membrane components: local accumulation and development of adhesion. *J Cell Biol.* (1986) **102**:2185–96. doi: 10.1083/jcb.102.6.2185
- Dritschel H, Waters SL, Roller A, Byrne HM. A mathematical model of cytotoxic and helper T cell interactions in a tumour microenvironment. *Lett Biomath.* (2018) **5**:1–33. doi: 10.1080/23737867.2018.1465863
- Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) **192**:1027–34. doi: 10.1084/jem.192.7.1027
- Mahlbacher GE, Reihmer KC, Frieboes HB. Mathematical modeling of tumor-immune cell interactions. *J Theoret Biol.* (2019) **469**:47–60. doi: 10.1016/j.jtbi.2019.03.002
- Polley A, Orłowski A, Danne R, Gurtovenko AA, Bernardino De La Serna J, Eggeling C, et al. Glycosylation and lipids working in concert direct CD2 ectodomain orientation and presentation. *J Phys Chem Lett.* (2017) **8**:1060–66. doi: 10.1021/acs.jpcclett.6b02824
- Qi SY, Groves JT, Chakraborty AK. Synaptic pattern formation during cellular recognition. *Proc Natl Acad Sci USA.* (2001) **98**:6548–53. doi: 10.1073/pnas.111536798
- Rosenberg EM, Harrison RES, Tsou LK, Drucker N, Humphries B, Rajasekaran D, et al. Characterization, dynamics, and mechanism of CXCR4 antagonists on a constitutively active mutant. *Cell Chem Biol.* (2019) **26**:662–73.e7. doi: 10.1016/j.chembiol.2019.01.012
- Caliendo F, Dukhinova M, Siciliano V. Engineered cell-based therapeutics: synthetic biology meets immunology. *Front Bioeng Biotechnol.* (2019) **7**:43. doi: 10.3389/fbioe.2019.00043

27. Compère M, Harwood SL, Muñoz IG, Navarro R, Zonca M, Perez-Chacon G, et al. A tumor-targeted trimeric 4-1BB-agonistic antibody induces potent anti-tumor immunity without systemic toxicity. *Nat Commun.* (2018) **9**:4809. doi: 10.1038/s41467-018-07195-w
28. Krummel MF, Allison JP. Cd28 and Ctla-4 have opposing effects on the response of t-cells to stimulation. *J Exp Med.* (1995) **182**:459–65. doi: 10.1084/jem.182.2.459
29. Roh KH. Artificial methods for T cell activation: critical tools in T cell biology and t cell immunotherapy. *Adv Exp Med Biol.* (2018) **1064**:207–19. doi: 10.1007/978-981-13-0445-3_13
30. Wu C-Y, Rupp LJ, Roybal KT, Lim WA. Synthetic biology approaches to engineer T cells. *Curr Opin Immunol.* (2015) **35**:123–30. doi: 10.1016/j.coi.2015.06.015
31. Li D, Li X, Zhou W-L, Huang Y, Liang X, Jiang L, et al. Genetically engineered T cells for cancer immunotherapy. *Signal Transduct Target Ther.* (2019) **4**:35. doi: 10.1038/s41392-019-0070-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Imaging Polarized Secretory Traffic at the Immune Synapse in Living T Lymphocytes

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Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 20 December 2017

Accepted: 20 March 2018

Published: 06 April 2018

Citation:

Calvo V and Izquierdo M (2018)
Imaging Polarized Secretory
Traffic at the Immune Synapse in
Living T Lymphocytes.
Front. Immunol. 9:684.
doi: 10.3389/fimmu.2018.00684

Immune synapse (IS) formation by T lymphocytes constitutes a crucial event involved in antigen-specific, cellular and humoral immune responses. After IS formation by T lymphocytes and antigen-presenting cells, the convergence of secretory vesicles toward the microtubule-organizing center (MTOC) and MTOC polarization to the IS are involved in polarized secretion at the synaptic cleft. This specialized mechanism appears to specifically provide the immune system with a fine strategy to increase the efficiency of crucial secretory effector functions of T lymphocytes, while minimizing non-specific, cytokine-mediated stimulation of bystander cells, target cell killing and activation-induced cell death. The molecular bases involved in the polarized secretory traffic toward the IS in T lymphocytes have been the focus of interest, thus different models and several imaging strategies have been developed to gain insights into the mechanisms governing directional secretory traffic. In this review, we deal with the most widely used, state-of-the-art approaches to address the molecular mechanisms underlying this crucial, immune secretory response.

Keywords: T lymphocytes, immune synapse, secretory granules, multivesicular bodies, exosomes, cytotoxic activity, cell death

INTRODUCTION

Immune Synapse and Polarized Secretory Traffic

T cells can initially and transiently interact with antigen-presenting cell (APC) *via* accessory, adhesion molecules such as LFA-1 (1). This allows the lymphocyte to remain in close, but also labile, contact with APC and to scan the APC's surface for specific antigen-major histocompatibility complex (MHC) complexes (2, 3). If the APC does not carry a specific antigen, then the T cell does not remain attached to the APC and can interact and examine other potential APCs for specific

Abbreviations: AICD, activation-induced cell death; ADAP, adhesion and degranulation promoting adapter protein; APC, antigen-presenting cell; CCD, charged coupled device; cSMAC, central supramolecular activation cluster; CTL, cytotoxic T lymphocyte; DHPSF, double-helix point spread function; F-actin, filamentous actin; ILV, intraluminal vesicles; IS, immune synapse; MHC, major histocompatibility complex; LAT, linker activation T cells; LLSFM, lattice light sheet fluorescence microscopy; LSF, light sheet fluorescence microscopy; LSCM, laser scanning confocal microscopy; NA, numeric aperture; MVB, multivesicular body; MTOC, microtubule-organizing center; NK, natural killer; OVA, ovalbumin; PSF, point spread function; pSMAC, peripheral supramolecular activation cluster; PALM, photoactivated localization microscopy; sCMOS, scientific complementary metal-oxide semiconductor; SEE, Staphylococcal enterotoxin E; SEB, Staphylococcal enterotoxin B; SIM, structured illumination microscopy; SMLM, single-molecule localization microscopy; SNR, signal-to-noise ratio; SR, super resolution; STED, stimulated emission depletion microscopy; STORM, stochastic optical reconstruction microscopy; TCR, T-cell receptor for antigen; Th, helper T; TIRFM, total internal reflection microscopy; TPFM, two-photon fluorescence microscopy; VCP, vertical cell pairing; WFM, wide-field fluorescence microscopy.

antigen (**Figure 1**) (3, 4). This trial-and-error characteristic is considered an important mechanism to assure the specific interaction of the T cell receptor (TCR) with specific antigen-bearing APCs (1, 3). When TCR encounters specific antigen on APC, a productive TCR stimulation by antigen presented on APC induces the formation of the immune synapse (IS) (5, 6). The formation of the IS constitutes an essential component of the immune system (6). IS comprises a highly ordered and plastic, signaling platform that integrates signals and coordinates molecular interactions leading to an appropriate and specific

immune response (6). In T lymphocytes, once TCR encounters a specific antigen bound to MHC, one early consequence of IS formation constitutes the convergence of the secretory granules toward the microtubule-organizing center (MTOC) and, almost simultaneously, the polarization of the MTOC to the IS (7, 8) (**Figure 1**). Acting coordinately, these two trafficking events finely ensure the specificity of T cell effector responses, by enabling polarized secretory traffic toward the APC (7, 8), spatially and temporally focusing the secretion at the synaptic cleft (9). However, it should be pointed out that not always MTOC

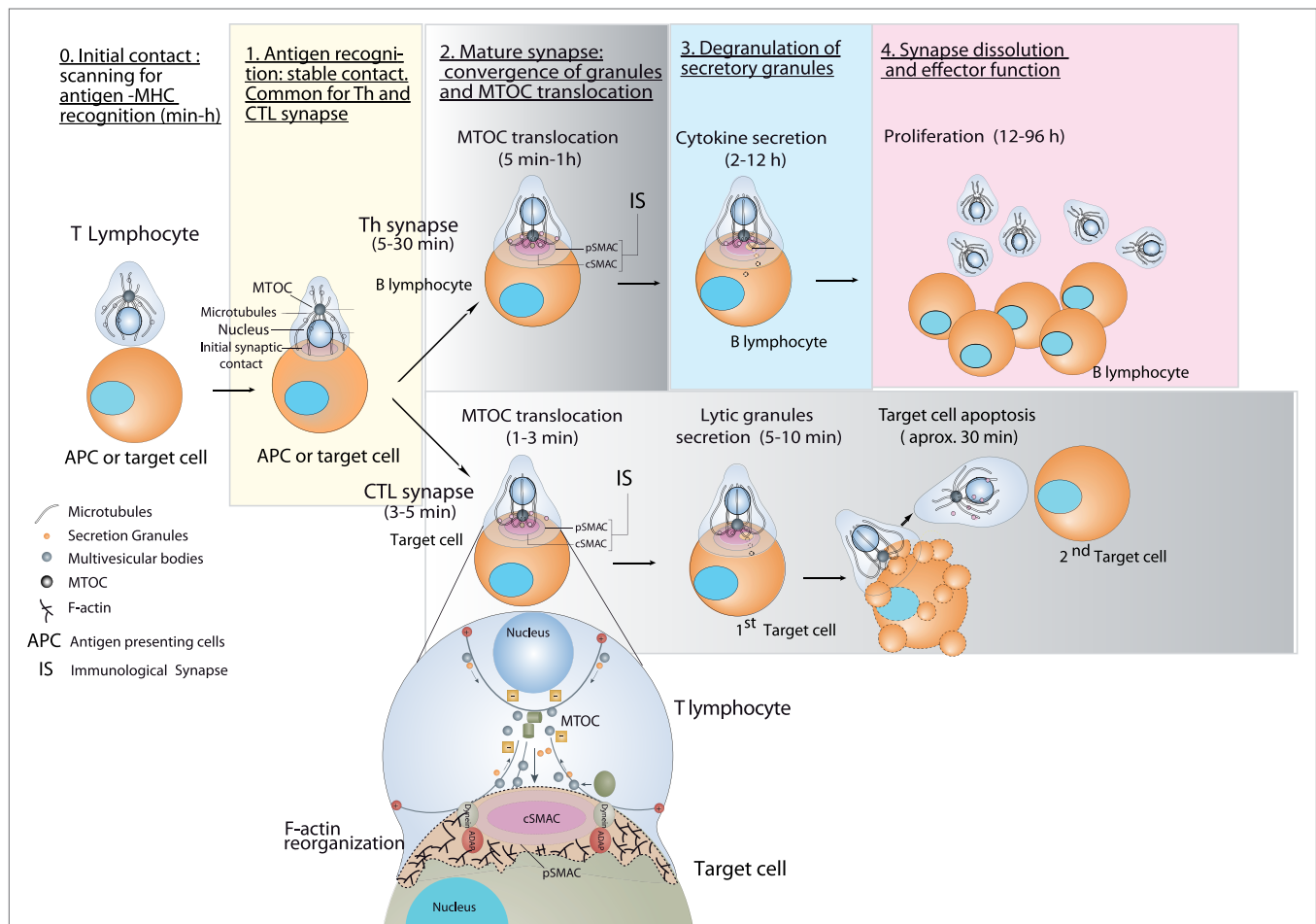


FIGURE 1 | Stages of helper T (Th) and cytotoxic synapses and polarized secretion toward the IS. Stages 0 and 1 are common for both Th and cytotoxic T lymphocyte (CTL) synapses. After the initial scanning contact for specific antigen–major histocompatibility complex (MHC) complexes, Th effector T lymphocytes (upper chain of events) form mature synapses with antigen-presenting B lymphocytes within several minutes. This IS lasts many hours during which *de novo* cytokine (i.e., IL-2, IFN- γ) production (involving *de novo* gene transcription) and secretion occurs, that requires continuous T cell receptor (TCR) signaling. After IS formation, Th lymphocytes may also undergo non-polarized (multidirectional) secretory traffic of certain cytokines (TNF- α , IL-4) (13). This fact has not been depicted for clarity reasons. The cell conjugates split after several hours, and then the lymphocytes eventually proliferate. Primed effector CTLs (lower chain of events) establish more transient, mature synapses after scanning their target cells (i.e., a cell infected with a virus), and deliver their lethal hits within a few minutes. Secretory lysosomes (lytic granules) are very rapidly transported (within very few minutes) toward the microtubule-organizing center (MTOC) (in the minus “–” direction) and, almost simultaneously, the MTOC polarizes toward the central supramolecular activation cluster (cSMAC) of the IS, a filamentous actin (F-actin)-depleted area that constitutes a secretory domain (14). Non-polarized, multidirectional exocytosis of lytic granules from naïve CTLs has been shown also to be induced by resting human B cells (12). This fact has not been depicted for clarity reasons. MTOC translocation to the IS appears to be dependent of dynein motors anchored to adhesion and degranulation promoting adapter protein (ADAP) at the peripheral supramolecular activation cluster (pSMAC), that pull MTOC in the minus direction (15, 16). In both types of synapses (lower zoom panel), the initial F-actin reorganization in the cell–cell contact area, followed by a decrease in F-actin at cSMAC and an accumulation at the pSMAC appears to be involved in granule secretion (17, 18). Multivesicular bodies (MVBs) are also secretion granules involved in the polarized secretion of exosomes at the IS upon their degranulation (19, 20).

polarization is necessary or sufficient for lytic granule transport to the IS and cytotoxic hit delivery. In this context, it has been shown that an initial and rapid step of lytic granule secretion constitutively located nearby the IS precedes MTOC polarization at the cytotoxic T lymphocyte (CTL)/target cell synapse (10). In addition, it has been shown that PKC δ -deficient CTL efficiently reoriented MTOC in response to target cell recognition but were not able to polarize their lytic granules (11). These results broaden current views of CTL biology by revealing an extremely rapid step of lytic granule secretion and by showing that MTOC polarization is dispensable for efficient lethal hit delivery. Moreover, there is evidence that resting human B cells escape killing by CTLs by inducing non-polarized exocytosis of their lytic granules, although MTOC translocated normally toward the IS (12). Non-polarized degranulation was associated with an altered formation of the IS and may represent a mechanism that allows B cell malignancies to evade CTLs (12). These examples of segregation between MTOC movement and lytic granules traffic point out that the analyses of both MTOC repositioning and traffic of secretory vesicles should be considered to obtain the full picture of the secretion process.

Two Kinds of Secretory Synapses

The outcome of the IS depends on the type of T lymphocyte and APC. The interaction of helper T (Th) cells (usually CD4⁺ cells) with the antigen-presenting, MHC-II-bearing APC results in the activation of the T cell (cytokine secretion, proliferation, etc.). In the case of CTLs (CD8⁺ cells) interacting with APC displaying antigen-associated MHC-I the response depends on the pre-stimulation of the CTL with the antigen. Thus, naïve CTLs recognizing antigen–MHC-I complexes on APC are “primed” to kill target cells and proliferate. Primed CTL also form IS with target cells (tumor cells or cells infected by viruses) resulting in specific killing (**Figure 1**). The functional changes produced by the establishment of a productive, mature IS include activation (for Th cells), activation (naïve CTL) or killing (primed CTL), apart from functional anergy or apoptosis (3). Thus, there are two major groups of secretory immune synapses made by T lymphocytes that lead to very different, but crucial immune effector functions (5, 6, 21). On one hand, IS made by primed CTL triggers the rapid polarization (from seconds to very few minutes) of secretory granules (called “lytic granules” or “secretory lysosomes”) toward the synapse (**Figure 1**). Degranulation of these lytic granules induces the secretion of perforin and granzymes to the synaptic cleft (22). In addition, relocalization of pro-apoptotic Fas ligand from the limiting membrane of the secretory lysosome to the cell surface occurs (23). Fas ligand on the CTL cell surface subsequently triggers apoptosis by binding to Fas-expressing target cells (24). Acting together, all these death-inducing factors trigger the death of the target cells (25). CTLs form somewhat transient synapses, lasting only few minutes, as the target cells are killed (8, 26). This is probably due to the fact that the optimal CTL function requires a rapid and transient contact to deliver as many lethal hits as possible to several target cells (8, 26) (**Figure 1**). On the other hand, Th lymphocytes make stable, lengthy synapses (>20–30 min up to several hours) that are necessary for both directional and

continuous secretion of stimulatory cytokines (8, 26). The fact these Th synapses are more stable than the synapses formed by CTLs does not exclude the possibility that Th may establish sequential contacts with several APCs, as has been shown for B cells and dendritic cells (5, 27). These cytokines are contained in secretory granules, and some of them (IL-2, IFN- γ) also undergo polarized traffic to the IS (8). Accordingly, in long-lived synapses made by Th lymphocytes the MTOC takes from several minutes up to hours to move and dock to the IS, whereas in primed CTLs the directional movement of MTOC toward the synapse lasts very few minutes (6, 8, 26) (**Figure 1**). Considering these points, it becomes clear that a major difference between the IS established by primed CTL and that established by Th cells is the overall duration of the process and its immediate repeatability. To eliminate target cells rapidly, CTL contacts with target cells are quick, and CTLs can establish multiple IS with different target cells over short periods of time (8). Conversely, Th cells establish prolonged IS and do not rapidly form consecutive IS once activated properly (**Figure 1**) (4, 6). In any case, both the convergence of secretory granules toward the MTOC and MTOC polarization to the IS appear to be necessary for optimal polarized and focused secretion in many cell types of the immune system, including innate natural killer (NK) cells (28, 29), CTLs (17, 30), primary CD4⁺ T cells (31), and Jurkat cells (30), although some exceptions to this situation have been described in the first paragraph. This specialized mechanism appears to specifically provide the immune system with a finely tuned strategy to increase the efficiency of crucial secretory effector functions, while minimizing non-specific, cytokine-mediated stimulation of bystander cells target cell killing and activation-induced cell death (AICD).

Polarized Secretion of Exosomes

Polarized secretion of exosomes at the IS constitutes an emerging and challenging field involved in relevant immune responses (32). In this context, it has been shown that late endosomes with multivesicular body (MVB) structure carrying intraluminal vesicles (ILVs) undergo polarized traffic toward the IS (**Figure 1**, lower panels) (19). The degranulation of MVBs at the IS induces the release of ILVs as exosomes to the synaptic cleft. This occurs in synapses formed by Jurkat cells, TCR-stimulated CD4⁺ lymphoblasts, and primed CTLs (19, 20, 33–37). While exosomes are constitutively secreted by various cell lineages and tumor cells, in T and B lymphocytes exosome secretion is triggered upon activation of cell surface receptors, which in turn regulates antigen-specific immune responses (25, 38). The exosomes participate in important processes related to TCR-triggered immune responses, including T lymphocyte-mediated cytotoxicity, AICD of CD4⁺ lymphocytes, antigen presentation, intercellular miRNA exchange (20, 35, 39–42), and thymus development (43). However, the mechanisms underlying MVB traffic and exosome secretion are poorly understood when compared with those corresponding to other secretory granules in T lymphocytes (21, 44, 45). Thus, the study of this partially unknown polarized secretion pathway using the proposed imaging techniques will provide information regarding an important, specialized function of the immune system.

METHODS FOR IMMUNE SYNAPSE FORMATION AND IMAGING

The formation of the IS consists of a highly rapid and plastic process that may conduct to several different polarized traffic outcomes, and some of these may differ in time. For instance, polarization of lytic granules of CTLs takes very few minutes, whereas certain cytokine-containing granules take from several minutes up to several hours to complete their polarization. These time differences need to be considered in advance to design the experiment and to choose the right experimental approach, since for some imaging strategies time is a limiting factor.

One important feature of the IS consists of the establishment of exploratory, labile contacts between the T cell and the APC, that may lead or not to a stronger contact and the formation of a mature synapse, providing that TCR recognizes the antigen–MHC complexes and the establishment of proper costimulatory interactions (3). Both the onset of the initial contacts and the establishment of a mature, fully productive IS, are intrinsically stochastic, rapid and asynchronous processes (3, 46). In addition, there is a low frequency in the formation of cell–cell conjugates (47). Another major challenge in studying polarization of secretory machinery in T lymphocytes, particularly in CTLs, is that the whole process is quite fast (a few minutes). For all these reasons, most early studies have used an end-point approach in which T lymphocytes and target cells are mixed together in suspension, concentrated by low speed centrifugation to favor conjugate formation, incubated for several minutes, fixed and scored for the repositioning of MTOC and/or secretory granules toward the IS (48). This approach has two important drawbacks: no dynamic trafficking information was obtained, and high levels of background MTOC/secretory granules polarization due to the stochastic nature of IS formation were observed (46). In addition, any correlation between TCR-derived early signaling events (i.e., rises in intracellular calcium, actin reorganization) and secretory granule polarization is difficult to analyze. Therefore, important pre-requisites for proper imaging of the IS in living cells include to increase the conjugate formation efficiency, to synchronize the formation of the IS and, if possible, to guarantee the formation of conjugates at certain microscope fields (XY) and focal plane (Z) positions. Several approaches have been designed to guarantee this synchronicity and to circumvent all these caveats. We will describe below some examples for these approaches, pointing out some of their advantages and weaknesses.

Planar Lipid Bilayers and Coverslip/ Beads-Coated With Surface Proteins or Agonistic Antibodies

This approach reduces a three-dimensional (3D) complex structure such as an IS to two dimensions (XY) and enables high-resolution imaging (4). Using this strategy several relevant issues can be solved. On one hand, the synchronicity issue can be solved since cells will eventually sediment and will get into the microscope focus and stimulated at the coverslip interface with a similar time course. On the other hand, stimulation will occur

at a homogenous, defined Z position; this will facilitate image capture. It is well known that conventional epifluorescence and confocal microscopes, but also super-resolution microscopes, have poor resolution in the Z-axis (49). Thus, by excluding Z dimension, specific high-resolution techniques such as total internal reflection microscopy (TIRFM) (see below) will benefit from this approach. Movement of secretory granules at the focus plane will consist of centripetal convergence toward the central supramolecular activation cluster (cSMAC) area (5, 50) and this can be recorded and analyzed (**Figure 1**). Unfortunately, this technique can be somewhat reductionist since it is not possible to guarantee that all the molecular interactions occurring in a cell–cell synapse will occur also upon interaction with the bilayer (5). In fact, supported lipid bilayers do not mimic the complex surface of an APC or target cell, giving rise to non-physiological interactions in the IS (51). In addition, capture in a defined Z in the very initial moments of the interaction with the coverslip will exclude intracellular structures (i.e., secretory vesicles) located beyond a certain Z distance from the coverslip (>200–300 nm). However, for some structures contained and reorganizing within the IS (i.e., actin reorganization), the features of some images obtained by using anti-TCR-coated coverslips and lipid bilayers and imaged by TIRF and TIRF–structured illumination microscopy (SIM) combination (see below) probably exhibit by far the highest quality obtained to date (50, 52). The possibility to define and change the composition of the lipid bilayer by loading antigens, accessory molecules, lipids, etc., allows for “reconstitution approaches” and offers flexibility to this approach (4).

However, the information obtained from *in vitro* bilayers systems must be considered with caution when extrapolated to *in vivo* T cell activation and synapse formation. In this context, the antigen or antigenic peptide dose affects the size of the cSMAC, and the levels of antigen–MHC on endogenous APCs may be lower than the levels used in the *in vitro* models (5). The rigid and flat nature of the glass-supported bilayer may also drive kinetic size segregation, and *in vitro* cell–cell conjugates tend to have multifocal and quite heterogeneous synapses (5). For instance, kinetic segregation of CD45 has been shown to be crucial for TCR triggering (53) and this may differently occur in T cells interacting with lipid bilayers with respect to cell–cell conjugates. Finally, indeed the APC develops mechanical and functional contributions to IS surfaces but also modulates synapse structure and function (5, 12). Thus, it is important to take into account that studies with supported planar bilayers are powerful in terms of resolution and sensitivity, but it is always important to test the predictions of these model systems in *in vitro* cell–cell or *in vivo* systems (54).

Another variant of this technique consists in the use of anti-TCR- (or anti-BCR-) coated latex beads to induce the formation of cell-bead conjugates in suspension (18, 55, 56). These beads, as lipid bilayers, may allow the use of costimulatory agonists (i.e., anti-CD28 for T lymphocytes, anti-CD40 for B lymphocytes) and recombinant proteins such as ICAM-1. Indeed, the use of beads allows lymphocytes to establish a stimulatory contact with a surface more similar to the ellipsoid geometry of an immune cell than the planar lipid bilayer (56).

A more sophisticated variant included in this group of approaches allows to enhance the spatial and temporal control of MTOC repositioning (57) and circumvents the background MTOC polarization issue (see above). This technique consists of the focused activation of a photoactivatable peptide–MHC bound to glass surfaces and has been successfully used to analyze the contribution of the gradient of the second messenger diacylglycerol (DAG) and its negative regulator DAG kinase α to the polarized traffic of lytic granules in CTLs (57, 58). However, this approach suffers, as all the coverslip-based protocols, from the fact that the engaging entity is a lipid laying on a glass-surface and not a “real” cell.

T Cell-APC Model Systems

Conjugates of Jurkat T Cells With Superantigen-Coated Raji B Cells and Human Primary T Lymphoblasts With APCs

For the first approach, Jurkat T cells are challenged with Raji B cells coated with Staphylococcal enterotoxin E (SEE) superantigens (59). This approach can be done in cultured cells in suspension but also using Raji B cells attached to fibronectin-coated coverslips to image the early stages of IS formation [i.e., Supplementary Video 1 in Ref. (36)]. A good number of advantages include: easy handling, well-established human cell lines, feasibility of multiple transfection, availability of Jurkat mutants (60), appropriate kinetics of polarization, valuable model for T cell signaling (60), and the fact that real cell–cell conjugates are imaged. In addition, early signals but also T cell-derived late responses such as IL-2 secretion, and activation-induced apoptosis (19, 36) can be easily measured and/or imaged [Supplementary Material in Ref. (19)] and correlated with trafficking events. Despite of a good number of publications using this model, there are drawbacks related to the use of transformed cells that may lack or overexpress adhesion and accessory molecules typical of primary cells. A certainly more physiological alternative consists in the use of polyclonal, mitogen-activated human T lymphoblasts, or alloreactive human T cell clones of known specificity. Synapses formed by T lymphoblasts with SEE or SEB, Staphylococcal enterotoxin B (SEB) superantigens-coated Raji cells may be used for imaging although a low proportion (20–30%) of human T lymphoblasts harbor the superantigens-interacting V β TCR (61). Another option is to polyclonally stimulate T lymphoblasts with anti-TCR bound to lipid bilayers. However, several drawbacks inherent to the use of human-derived samples (such as the presence of potential pathogens), availability of healthy donors, variability in activation responses, requirement for re-stimulation of clones, and low transfection efficiency—among other facts—preclude the extensive use of these models for imaging.

Primary (*In Vitro* Stimulated or *In Vivo* Primed) T Cells From TCR-Transgenic Mice

This approach circumvents the problems derived from the use of tumor cells, thus constitutes a fair approach to a more physiological scenario. With the appearance of lentiviral and retroviral gene transduction, the expression of fluorescent chimeras is feasible and these models gained in flexibility (17). However,

there is often certain degree of variability (kinetics of activation, expression levels of surface receptors, etc.) that makes this approach somewhat more difficult than the Jurkat–Raji model. Several models can be used with primary T lymphocytes in mice. These models have the additional advantage that the stimulatory peptides or superantigens can be injected *in vivo* to prime immune responses (62). In addition, since all the transgenic lymphocytes express the same TCR with known specificity in quite homogeneous levels, an increase in the efficiency of conjugate formation should be expected when challenged with the specific antigen. A good number of papers on IS imaging have been published by using transgenic murine lymphocytes expressing the ovalbumin (OVA) peptide-specific OT-I TCR (63), which recognizes an OVA peptide. This transgenic TCR is positively selected during thymocyte development in CD8+ CTLs. Thus, OT-I constitutes a good model in the context of CTL polarization and there is a good number of publications arising from this model (17, 64). In addition to OT-I, transgenic murine lymphocytes expressing the influenza NP68 peptide-specific F5 TCR (65), have been successfully used to study traffic in CD8+ CTLs (55). This model also allows for *in vivo* priming of mice with the antigen (62). When the secretory traffic of cytokines in Th synapses needs to be studied, TCR-transgenic lymphocytes positively selected to CD4+ cells (i.e., expressing the transgenic cytochrome C peptide-specific c5.c7 TCR) (2, 13, 31) have been successfully used for imaging. In all these models, potential effects of transgenic expression of the TCR and positive selection during T cell development should be taken into account.

In addition, superantigens models comparable to the described Jurkat–Raji (SEE) human model may also be used in non-TCR-transgenic mice. For instance, SEB superantigens bound to syngeneic APC (i.e., mouse lymphoma EL-4 cells) can be used in mice harboring the appropriate MHC background to challenge a considerable proportion of T lymphoblasts (around 30%, those lymphocytes bearing V β 3, V β 7, or V β 8 TCRs) (36, 66). Caution needs to be taken since synapses made by either CTLs or Th can be simultaneously formed and imaged [Supplementary Video 8 in Ref. (36)] unless a previous separation of CD4+ and CD8+ lymphoblasts is achieved. These superantigens models also allow *in vivo* priming of mice (62). One caveat to consider: although trial-and-error scanning of superantigens-pulsed APCs by T lymphoblasts will indeed be imaged, not all lymphocytes will render mature synapses and this will diminish the frequency of productive, mature synapses.

Vertical Cell Pairing (VCP) Device

Observation of lateral cell–cell synaptic conjugates made using the cell models described earlier faces important imaging limitations. Since most conjugates obtained in the classical end-point studies using fixed T lymphocytes and APCs lie parallel to the focus plane, the IS interface lies perpendicular to the plane of focus along the Z-axis (Figure 1). Thus, sequential Z-stacks need to be imaged to 3D-reconstitute the entire synapse. This is an important caveat for *in vivo* imaging of IS, since conventional confocal microscopes are too slow for 4D imaging (X, Y, Z, T) and, in addition have, as already mentioned earlier, a poor resolution in the Z-axis. To circumvent

all these issues, clever “parking and pairing” devices have been developed by some researchers to study NK synapse (47). The microfluidics contained in this device enhanced the frequency of conjugate formation and induced the positioning of the IS on a horizontal imaging plane, which was the ideal focal plane for IS imaging. Thus, the inherent poor Z resolution of microscopes, lack of synchronicity and low frequency of IS interactions can be all together solved by alignment cell devices as VCP. Using these devices, the authors were able to register high-resolution images of lytic granules converging on the IS, simultaneously to actin reorganization (47), in living NK cells forming synapses with target cells. No reports on super-resolution microscopy of T lymphocytes using these devices have been published to date. Drawbacks to consider include that these microfluidic devices are not easy to produce and have not been commercialized to date and therefore are not easily accessible to researchers.

Optical Tweezers

For this clever approach, which constitutes an interesting alternative to a physical “parking and pairing device” described earlier, an optical trap was used to place the APC (i.e., Raji cells pulsed with SEE) such that the initial point of contact with the Jurkat cell is opposite from the location of its MTOC, visualized by the expression of GFP-tagged centrin (46). This approach allowed to dynamically image the complete process of MTOC repositioning, avoiding background MTOC polarization. As a drawback, this is a somewhat technically difficult approach and for some unclear reason the speed of MTOC repositioning obtained in Th lymphocytes and Jurkat–Raji conjugates (46) is quite high (less than 1 min to complete) when compared with that obtained using the same cell models without optical trap (several minutes up to few hours) (67, 68). Similarly, by using optical tweezers to manipulate live-cell conjugates and orientate the synapse in the imaging plane of a laser scanning confocal microscope, some authors have imaged protein organization at living NK cell–target cell and T cell–APC immune synapses with high speed and high resolution (69). Optical tweezers have been used in combination with 3D-SIM (see below) to study polarization of lytic granules in NK IS (70).

Acoustic Trap

An interesting alternative to physical and optic pairing devices that was used to facilitate, synchronize, and image the NK–target cell synaptic interactions into different wells from multiple well plates, using laser scanning confocal microscopy (LSCM), and also allowed to analyze the heterogeneity in the NK cytolytic response toward tumor target cells (29, 71). The technique allowed en face projections of the intercellular contact (cell interface), but since the formation of conjugates was somewhat forced, this probably may interfere with the establishment of both exploratory/labile and specific/stable contacts that occur between the effector and the target cells. To the best of our knowledge, this application has not yet been used in T lymphocyte synapses, probably because of this caveat.

IMAGE CAPTURE AND PROCESSING

To properly image an IS (as in any fluorescence microscopy technique) a compromise among spatial resolution, temporal resolution, and improved signal-to-noise ratio (SNR) is required (72). Apart from these factors, it is necessary to circumvent the photobleaching and cytotoxicity issues inherent to any living cell imaging (72). Exhaustive reviews have been recently published explaining and comparing the more relevant fluorescence microscopy techniques in cell biology (49, 72), as well as their main features (temporal and spatial resolution) and advantages (72, 73). Please refer to these excellent reviews for additional details, since it is out of the scope of this review to deal with these technical issues. Therefore, we will focus in the most relevant details of these techniques that have been used specifically to image the IS and some representative examples [please note that Table 2.1.2 in Ref. (72) has been modified accordingly in **Table 1**].

Conventional Wide-Field Fluorescence Microscopy (WFM)

WFM combined with image deconvolution after acquisition (**Table 1**) is still a powerful approach followed by a considerable number of researchers. Not only economic reasons substantiate this choice; the poor resolution in Z-axis (the most important drawback of this technique) can be solved in part by using post-acquisition image deconvolution. Deconvolution is a computational image processing technique that can improve image resolution and contrast (72) up to two times, down to 150–100 nm in XY and 500 nm in Z-axis (<https://svi.nl/Deconvolution>) (**Table 1**). Deconvolution requires the knowledge of the idealized or measured point spread function (PSF) of the microscope and the imaging technique used. Deconvolution requires the acquisition of specific images of the sample, whose number and characteristics are usually determined by the method used for imaging. User has to image the sample following these conditions for a correct deconvolution process and this requires certain expertise. Often the metadata [lens numeric aperture (NA), λ] necessary to generate an idealized PSF are contained in the image file and directly loaded by the deconvolution software. Pros: flexibility in a wide number of fluorochromes that can be simultaneously recorded, rapid and sensitive image capture using last generation, highly sensitive, wide field, and rapid scientific complementary metal-oxide semiconductor cameras, improved SNR after image deconvolution and easy training of users. Cons: blurred raw images (bleed-through of out of focus light) before deconvolution, but significantly improved SNR ratio in de-blurred images after deconvolution, enhancing resolution and maintaining the benefits of WFM. Almost all the imaging software available contain deconvolution algorithms, but the state-of-the-art, choice software is provided by SVI (Huygens) that includes several optical options [WFM, LSCM, and stimulated emission depletion (STED) microscopy] specific for the different techniques. Among all these techniques WFM is, by far, the microscopy technique that improves most after deconvolution. One example of the power of deconvolution applied to epifluorescence videos on the polarized traffic of MVBs at the IS (19) is provided:

TABLE 1 | Comparison of microscopy techniques used for IS imaging.

Technique	XY resolution ^a	Z resolution ^a	Temporal resolution	Imaging depth	Usability	Cost ^b	SNR ^c	Photobleaching/phototoxicity ^e	Reference
Wide-field (WFM)	Diffraction limited (≈ 200 nm)	Poor (usually < 1 μ m)	Best (ms/frame, signal limited) Good for 3D imaging	Typically < 30 μ m	Simple versatile	1	High	Best (usually μ W distributed over large imaging field)	(19) (36)
+Deconvolution	(≈ 100 nm) ^d	(≈ 500 nm) ^d					Better (deconvolution)		
Total internal reflection microscopy (TIRFM) TIRFM-structured illumination microscopy (SIM)	Diffraction limited but low background	Best but only first 200–300 nm	Good (ms/frame, signal limited)	Worst < 300 nm	Good	2	High	Better	(74) (75, 50, 52)
Laser scanning confocal (LSCM)	Diffraction limited to nearly 2 $\times$ diffraction limit (airy scan)	Good (< 700 nm)	Varies with scanner type (typically 1–30 fps) medium	Better (< 100 μ m)	Complex but most versatile	2–7	Moderate	Can be bad (μ W of power focused to spot)	(67) (47)
Multi-point/slit confocal spinning disk	Diffraction limited	Good (slightly worse than LSCM)	Good (ms/frame, signal limited)	Typically < 50 μ m	Better	2–4	Moderate	Better (usually lower excitation flux density than LSCM)	(68) (17) (46)
Two-photon fluorescence microscopy (TPFM)	Diffraction limited	Good (slightly less than LSCM)	Varies with scanner type (typically 1–30 fps) medium-slow	Best (hundreds of μ m)	Complex	3–7 with 1 pulsed laser	Moderate	Can be bad (μ W power focused to spot but only eliciting fluorescence from the focal plane)	(76) (27)
(SIM)	Diffraction limited	Good—usually worse than LSCM	Typically 1–10 fps slow	Typically < 30 μ m	Complex	1.5	Moderate	Good (varies with number of images needed)	(70) (77)
Super-resolution-SIM (SR-SIM) (3D-SIM)	Super-resolution to at least 2 $\times$ diffraction limit with deconvolution	To 2 $\times$ diffraction limit with deconvolution	Good (can be ms/frame with iSIM, signal limited)	Typically < 10 μ m, iSIM < 50 μ m	Better (if deconvolved)	4–9	Moderate	Typically good	(52)
Stimulated emission depletion (STED) microscopy	Super-resolution (< 70 nm)	Same as LSCM or < 100 nm with axial phase plate	Varies with scanner type (typically 1–30 fps) medium	Typically < 50 μ m	Complex	6–10	Low	Worst (second beam with many μ W of power)	(78) (79)
Single molecule localization microscopy (SMLM) Photoactivated localization microscopy (PALM) Stochastic optical reconstruction microscopy (STORM) SMLM-3D	Best super-resolution (< 30 nm)	Can be ~ 100 nm or less	Worst requires thousands of images Very slow	Typically $< a$ few μ m or < 200 nm	Complex and requires pos acquisition processing	3–4	Low (noisy if marker density too low)	Varies with technique, can be harsh, typically requires thousands of images	(74) (80) (81) (82) (83)
Light sheet fluorescence microscopy (LSFM)	Diffraction limited but typically low-mid level numeric aperture (NA) lenses are used	Good depends on light sheet thickness and objective NA	Best for 3D imaging	Best (hundreds of μ m)	Better but requires calibration	2–6	High	Best for 3D (Z-stack) or 4D (Z-stack over time) imaging	(84)
Lattice light sheet fluorescence microscopy (LLSFM) with SIM	Super-resolution to 2 $\times$ diffraction limit with deconvolution	Super-resolution to 2 $\times$ diffraction limit with deconvolution	Best for 3D imaging	Typically < 20 μ m	Complex	2–6	Moderate	Best for 3D (Z-stack) or 4D (Z-stack over time) imaging	(85) (17) (78)

Green boxes are best in category, red are worse, modified from reference (72). SR indicates the Super-resolution techniques, based in the axial, Z resolution as the main criteria.

^aFor a superb diagram of 3D resolution data obtained from cell interiors for all the microscopy techniques please refer to in Ref. (49).

^bRelative cost is shown, 1 equals to 100,000\$ approximately.

^cSignal-to-noise ratio (SNR) is the intensity of the signal of interest divided by the variance in the signal due to noise.

^dDeconvolution can be applied to the different techniques. Data for WFM was obtained from SVI web: <https://svi.nl/ResolutionImprovement>.

^eThis is a general assumption; the photobleaching/phototoxicity issue has a large dependence on the sample preparation, not only on the technique used. Reproduced with permission from reference (72).

(video before deconvolution) <https://www.youtube.com/watch?v=mID0m3usQOQ>

(video after deconvolution) <https://www.youtube.com/watch?v=Aj0vPj6WAI1>

Total Internal Reflection Microscopy (TIRFM)

This technique is particularly useful to study the degranulation processes occurring on a coverslip or a functionalized surface/lipid bilayer (50, 52) due to its intrinsic low background and high SNR (Table 1). However, the technique is somewhat limited since only an illuminated homogeneous surface can be used to stimulate the cells. Another major disadvantage is that TIRFM excludes intracellular structures (i.e., secretory vesicles) or molecules located beyond a small Z distance from the coverslip (>200–300 nm). Thus, their initial movement from distal subcellular locations cannot be imaged, although their final movement approaching the IS membrane can be properly imaged. One possibility to avoid these last limitations is to simultaneously combine TIRFM and WFM (57), although “real” cell–cell synapses cannot be imaged. Another recent and interesting option has been developed by using TIRFM-SIM in combination with STED (see below), to show that local dynamism of filamentous actin rearrangements at the nanometer scale is required for cytolytic function of CTLs and NK cells through facilitating degranulation (75). The quality of the imaging obtained in this context is remarkable.

Laser Scanning Confocal Microscopy (LSCM)

Still one of the techniques of choice due to its versatility (availability of fluorochromes, etc.), although its low temporal resolution can be a problem when ongoing synapses and derived early signaling need to be imaged, in particular with CTL synapses. However, a considerable number of publications use this particular technique since confocal microscopes are standard core equipment widely used in biology institutes. In addition the quality of certain synapse imaging, both for end-point and time-lapse analyzes is unbeatable (67).

Spinning-Disk Confocal Microscopy

Conventional LSCM uses a laser to illuminate a single point on the sample and scans across to generate the image. Because it takes several seconds to generate each optical section, events that occur in milliseconds or seconds/minutes range (i.e., intracellular calcium rises, MTOC translocation to the IS) cannot be visualized *via* LSCM. In addition, while the image is being assembled point by point, the laser hits the complete thickness of the specimen frequently but only detects a single focal plane, leading to cytotoxicity and bleaching of the fluorophore (Table 1). Thus, the “efficiency” of excitation and the emission detection is quite low. However, in spinning-disk confocal microscopy, the excitation light is split through a disk containing multiple micropinhole, allowing for several points on the sample to be imaged at the same time. This allows for high-speed confocal imaging and reduced toxicity because of

decreased repeated illumination, but also increases the temporal resolution by a factor of 10- to 100-fold when compared with LSCM. This technique has been successfully used for rapid 4D imaging of MTOC translocation to Jurkat–Raji synapses (46, 68). For rapid CTL synapses, the even faster lattice light sheet fluorescence microscopy (LLSFM) (see below) appears to be even better. Pros: rapid acquisition, good image quality, and reduced photobleaching.

Two-Photon Fluorescence Microscopy (TPFM)

By far, this is the most appropriate technique for *in vivo* imaging since the high penetration of the excitation light allows to deeply penetrate into specimens such as whole lymphoid organs and organoids (76). However, some other features are not so appealing when compared with other techniques (Table 1), thus few publications analyzing intracellular traffic use this technique. However, it has allowed to track migrating T cells, to image synapse formation by CD4+ lymphocytes and to study subcellular dynamics of T cell immune synapses in lymph nodes (27, 76).

Light Sheet Fluorescence Microscopy (LSFM)

A faster method to generate optically sectioned images when compared with SIM and LSCM, while cytotoxicity and bleaching are reduced. A thin sheet of light produced by a cylindrical lens excites a single plane of the sample, and the emission from the whole plane is collected with a CCD camera that is orthogonal to the excitation light. Because the image is not assembled as points (as in LSCM), imaging is fast and there is a good correlation between illumination and detection of fluorescence, thus decreasing phototoxicity and bleaching (72). Strictly, in its original basic version LSFM is not able to achieve super-resolution images, but depending of light sheet thickness and NA of the lenses, allows better XYZ resolution than LSCM and spinning-disk microscopes. LSFM has been used to image immunologically relevant tissues such as lymph nodes (84), but the implementation of LLSFM (see below), which provides further improvement, has overtaken LSFM as a choice technique. LSFM allows rapid 3D time-lapse with reduced photobleaching.

Super-Resolution (SR) Techniques

Images from the microscopes described earlier are limited by the diffraction of light to a maximal XY resolution of about 200 nm (blue light). Although indeed coarse and valuable information can be extracted from these images, they are unsuitable for the study, when required, of nanoscale molecular changes and submicron organization (i.e., detailed interactions of myosin with actin and TCR microclusters, etc.). To circumvent this, a family of super-resolution fluorescence microscopy techniques that achieve resolution beyond this diffraction limit (labeled with a vertical line as “SR” in Table 1) has been developed during the last years. It is important to remark that super-resolution techniques have an important cost: acquisition times are often

too long for fast biological processes such as those occurring at the IS, fluorescence probes are limited and 3D imaging is still challenging for single-molecule localization microscopy (SMLM) techniques. This fact, together with the possibility of artifacts (see below), causes that SR techniques should be preferred only when diffraction-limited methods cannot provide the information required (73).

SIM and Super-Resolution SIM (SR/3D-SIM)

This technique encompasses a range of techniques based on sample being illuminated with spatially structured light, generating images with high spatial frequency information, which are mathematically reconstructed into a super-resolution image that has twofold better resolution than a conventional WFM image. This technique uses potentially less light, achieves higher temporal resolution, costs less and works well with many more fluorophores than, for instance, STED and SMLM techniques (see **Table 1**). Part of the improvements of this technique involves also super-resolution (SR-SIM), also called 3D-SIM. This enhances 2-fold the SIM spatial resolution and improves also the temporal resolution up to 100-fold. Superb images of cortical actin reorganization and lytic granules at the IS in NK cells have been published (70). Cons of conventional SIM include low speed and the computational time necessary to obtain the final image as well as the susceptibility to artifact generation during the image reconstitution processes (73).

Stimulated Emission Depletion (STED) Microscopy

This super-resolution technique enhances resolution by approximately one order of magnitude when referred to diffraction-limited approaches such as conventional LSCM (**Table 1**). Sample is scanned with two overlapping lasers: the first one excites and the second depletes the fluorophores, minimizing the volume of detection. Thus, PSF undergoes shrinking by STED leading to an enhancement in XYZ resolution. The level of resolution is only surpassed by SMLM (**Table 1**), but since STED is based on optical design it does not need complex post-acquisition processing as compared with SMLM. STED does not obligatorily entail image processing so it is praised as less susceptible to artifacts than other SR techniques (SR-SIM and SMLM) (73). Since STED acquisition is faster than SMLM, it can be more versatile for live-cell and 3D imaging, although it is significantly slower than spinning-disk microscopy or LLSFM. However, there is a limitation in the number and various fluorochromes that can be used, the power of the lasers induces photobleaching and toxicity and the equipment has a high cost. This technique has been used to image actin cytoskeleton rearrangements and lytic granules in NK synapses (79) and T lymphocytes stimulated on modified glass surfaces (78).

SMLM, Photoactivated Localization Microscopy (PALM), and Stochastic Optical Reconstitution Microscopy (STORM)

In practice, SMLM techniques provide the highest spatial resolution available (less than 20 nm in *X/Y* and less than 100 nm in *Z*) (**Table 1**). PALM and STORM use confined (to a higher precision

than the diffraction limit), single fluorophore molecule imaging of many cycles of photoactivation and photoconversion to reconstruct a pattern of fluorophores too crowded for single fluorophore imaging. This is achieved by using a limited number of fluorescent probes (photo-switchable fluorescent protein for PALM and conventional synthetic dyes for STORM), which constitutes a caveat. Another important drawback is the large number of images that are required to capture to fully define a single super-resolution image. In addition, complex reconstitution post-acquisition algorithms are necessary to create the final image. Although there are emerging, fast algorithms, processing time and big data storage remain as important concerns, limiting the application of these techniques to fixed samples. Thus acquisition time is a limiting factor and therefore living image applications and 3D/4D experiments are still technically challenging. In addition, because of topological constraints and the difficulty of imaging the irregular surface between two cells, SMLM imaging has been performed using various model systems in which the activating surface can be visualized in a single plane. For this reason these studies have been performed in immobilized surfaces and glass-supported lipid bilayers, and have not been extended to cell-cell conjugates yet. This limitation has motivated the development of a number of three-dimensional (3D), single-molecule imaging techniques, which allow for complete sampling of the protein distribution with high precision in all dimensions. In this context, the development of the double-helix point spread function is an example of a rotating PSF whose intensity distributions rotate as they propagate along the optical axis, allowing access to a significantly larger depth of field (~4 μm). This technique allowed to accurately visualize large-scale receptor reorganization of the membrane of whole T cells and single-particle tracking analyzes in living cells (83).

Using STORM/PALM, it has been shown that early TCR stimulation on glass coverslips recruits *lck* into clusters (80); using PALM it has been shown that TCR and linker activation T cells (LAT) exist in separate membrane domains (protein islands) in resting T lymphocytes and that these domains concatenated after T cell activation (81). Recently, SMLM has been extended to 3D (82) but is still far from being implemented as a standard technique. In this 3D-SMLM example, the authors explore LAT clustering at the T cell IS in 3D using iPALM and quantitative Bayesian cluster analysis. They show that the increase in LAT clustering observed in 2D results, at least in part, from the recruitment of LAT vesicles to the IS from a deep intracellular pool, distinct from the membrane population (82). More generally, with 3D-SMLM becoming a regularly used tool to address biological questions, the development of an accurate and robust 3D cluster analysis method, as presented by these authors, is an important and necessary advance.

Lattice Light Sheet Fluorescence Microscopy (LLSFM)

This state-of-the-art technique is an improvement of rapid LSFM and allows for very rapid 4D imaging of IS (85), in particular rapid signaling and trafficking events in CTL and NK synapses (17, 64). Since the capture in *Z* dimension is extremely

fast (10- to 15-fold improvement in temporal resolution when compared with rapid spinning-disk confocal microscopy) and also achieves super-resolution, a 3D full volume information can be imaged each second. In addition, the resolution achieved (threefold finer optical sectioning) allowed to gain a deep insight into actin cytoskeleton reorganization and to establish spatiotemporal relationships between actin reorganization and secretory traffic in CTLs synapse (17). Pros: very rapid acquisition, good image quality, very good 3D sectioning-reconstitution, optimized for live-cell imaging. Cons: complex and expensive equipment.

POTENTIAL FUTURE DEVELOPMENTS IN THE FIELD

The particular limitations of some of the described techniques can be avoided by applying several of the techniques in the same synapse model [i.e., WFM and LSCM (19), 3D-SIM and LSCM (70), STED and LSCM (79), STED and LLSFM (78), TIRFM-SIM and SMLM-STORM (74), STED, SIM, and TIRF-SIM (75)]. This is an immediate possibility that indeed allows to circumvent the limitations of the current techniques and to synergize in solving nascent biological issues. Alternatively, but not exclusively, combining several techniques when possible in the same experimental setup (i.e., photoactivation, TIRFM and WFM) (15, 57), and/or developing new “smart,” user-friendly instrumental designs (86) and new analytical approaches [i.e., super-resolution radial fluctuations (87)] will allow to tackle the present and future biological problems related to IS imaging. One important challenge occurring mainly in SR techniques is the huge amount of data generated; storing and analyzing the data can be a challenge in itself, since some of these microscopes (SMLM, SIM) do not allow visualizing the images during the acquisition and require post-acquisition complex data processing. Thus, the possibility of secondary artifacts at this stage is a reality (73). One possibility to detect artifacts before or after image processing and calibrate the SR instruments consists of the use of appropriate external image standards (73). Unfortunately, they are not available yet and will not likely be commercially available in the near future. Another interesting option to avoid potential SMLM-derived artifacts is to contrast SMLM results with those obtaining by imaging in parallel the same biological sample with a different technique such as SIM (77). In addition, and during the pre-acquisition stage, the generation of new fluorochromes with more photostability, emission colors, and brighter fluorochromes such as silicon-rhodamine SiR dyes (88) will indeed help in the development of super-resolution techniques. Another biologically relevant and immediate area of application of SR techniques includes the study of maturation (formation of ILVs inside MVBs) and secretory traffic of MVBs. Currently, the nano size of MVBs (up to 500 nm) and ILVs (50–100 nm) requires the use of electron microscopy techniques (32) or correlative microscopy techniques (89, 90), which do not allow studies of living

cells. MVBs and ILVs thus are ideal candidates to be imaged by SR techniques, particularly 3D-SMLM, in living cells. The nanoimmunology field will indeed benefit in the immediate future of the application of these techniques.

CONCLUSION

Some of the strategies we describe here have been shown to be very useful to obtain high-quality, high-resolution and super-resolution imaging of the IS in living cells, including simultaneous imaging of cytoskeleton changes and trafficking events occurring in living cells forming IS. This has allowed establishing correlations among certain early signaling events, cytoskeleton changes and secretory vesicle trafficking. However, some of these tactics require the use of reductionist approaches (at least from the biological point of view) that need to be considered in advance. Other experimental approaches, by avoiding these caveats, have proved to be very useful since they allow 4D, improved SNR imaging of “real” cell–cell synapses (17, 64). However, in some of these approaches the low frequency of conjugate formation and the low probability to image synapses in formation from the beginning (in particular in the rapid synapses formed by CTL) precludes obtaining valuable data without a previously designed, high-throughput cell “parking and pairing” strategy. The drawbacks inherent to certain SR techniques cause that, in general, these techniques should be preferred only when diffraction-limited microscopy methods cannot provide the information required.

AUTHOR CONTRIBUTIONS

VC and MI conceived the manuscript and the writing of the manuscript and approved its final content.

ACKNOWLEDGMENTS

The authors apologize for not including some relevant references due to space limitations. We acknowledge Dr. Combs CA, and Shroff H to allow us to use the modified table 2.1.2. from Fluorescence Microscopy: A Concise Guide to Current Imaging Methods. Curr Protoc Neurosci (2017) 79:2.1–2.1.25. doi: 10.1002/cpns.29. PubMed PMID: 28398640 and John Wiley and Sons Inc. (owner of the Copyright) for their permission. We acknowledge Dr. Jose A. García-Sanz (CIB, CSIC) for his superb review of the manuscript and useful comments. We acknowledge all the past and present members of the lab for their generous contribution.

FUNDING

This work was supported by grants from the Spanish Ministerio de Economía y Competitividad (MINECO), Plan Nacional de Investigación Científica (SAF2016-77561-R) to MI, which was in part granted with FEDER funding (EC). We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

REFERENCES

- Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, et al. The immunological synapse. *Annu Rev Immunol* (2001) 19:375–96. doi:10.1146/annurev.immunol.19.1.375
- Ueda H, Morphew MK, McIntosh JR, Davis MM. CD4+ T-cell synapses involve multiple distinct stages. *Proc Natl Acad Sci U S A* (2011) 108(41):17099–104. doi:10.1073/pnas.1113703108
- Friedl P, den Boer AT, Gunzer M. Tuning immune responses: diversity and adaptation of the immunological synapse. *Nat Rev Immunol* (2005) 5(7):532–45. doi:10.1038/nri1647
- Huppa JB, Davis MM. T-cell-antigen recognition and the immunological synapse. *Nat Rev Immunol* (2003) 3(12):973–83. doi:10.1038/nri1245
- Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, et al. Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol* (2010) 28:79–105. doi:10.1146/annurev-immunol-030409-101308
- Xie J, Tato CM, Davis MM. How the immune system talks to itself: the varied role of synapses. *Immunol Rev* (2013) 251(1):65–79. doi:10.1111/immr.12017
- de la Roche M, Asano Y, Griffiths GM. Origins of the cytolytic synapse. *Nat Rev Immunol* (2016) 16(7):421–32. doi:10.1038/nri.2016.54
- Huse M. Microtubule-organizing center polarity and the immunological synapse: protein kinase C and beyond. *Front Immunol* (2012) 3:235. doi:10.3389/fimmu.2012.00235
- Billadeau DD, Nolz JC, Gomez TS. Regulation of T-cell activation by the cytoskeleton. *Nat Rev Immunol* (2007) 7(2):131–43. doi:10.1038/nri2021
- Bertrand F, Muller S, Roh KH, Laurent C, Dupre L, Valitutti S. An initial and rapid step of lytic granule secretion precedes microtubule organizing center polarization at the cytotoxic T lymphocyte/target cell synapse. *Proc Natl Acad Sci U S A* (2013) 110(15):6073–8. doi:10.1073/pnas.1218640110
- Ma JS, Monu N, Shen DT, Mecklenbrauker I, Radoja N, Haydar TF, et al. Protein kinase Cdelta regulates antigen receptor-induced lytic granule polarization in mouse CD8+ CTL. *J Immunol* (2007) 178(12):7814–21. doi:10.4049/jimmunol.178.12.7814
- Kabanova A, Sanseviero F, Candi V, Gamberucci A, Gozzetti A, Campoccia G, et al. Human cytotoxic T lymphocytes form dysfunctional immune synapses with B cells characterized by non-polarized lytic granule release. *Cell Rep* (2016) 15(1):9–18. doi:10.1016/j.celrep.2016.02.084
- Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* (2006) 7(3):247–55. doi:10.1038/ni1304
- Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* (2006) 443(7110):462–5. doi:10.1038/nature05071
- Liu X, Kapoor TM, Chen JK, Huse M. Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin II. *Proc Natl Acad Sci U S A* (2013) 110(29):11976–81. doi:10.1073/pnas.1306180110
- Combs J, Kim SJ, Tan S, Ligon LA, Holzbaue EL, Kuhn J, et al. Recruitment of dynein to the Jurkat immunological synapse. *Proc Natl Acad Sci U S A* (2006) 103(40):14883–8. doi:10.1073/pnas.0600914103
- Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen BC, Gawden-Bone C, et al. Actin depletion initiates events leading to granule secretion at the immunological synapse. *Immunity* (2015) 42(5):864–76. doi:10.1016/j.immuni.2015.04.013
- Chemin K, Bohineust A, Dogniaux S, Tourret M, Guegan S, Miro F, et al. Cytokine secretion by CD4+ T cells at the immunological synapse requires Cdc42-dependent local actin remodeling but not microtubule organizing center polarity. *J Immunol* (2012) 189(5):2159–68. doi:10.4049/jimmunol.1200156
- Alonso R, Mazzeo C, Rodriguez MC, Marsh M, Fraile-Ramos A, Calvo V, et al. Diacylglycerol kinase alpha regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of Fas ligand-containing exosomes in T lymphocytes. *Cell Death Differ* (2011) 18(7):1161–73. doi:10.1038/cdd.2010.184
- Mittelbrunn M, Gutierrez-Vazquez C, Villarroja-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun* (2011) 2:282. doi:10.1038/ncomms1285
- Huse M, Quann EJ, Davis MM. Shouts, whispers and the kiss of death: directional secretion in T cells. *Nat Immunol* (2008) 9(10):1105–11. doi:10.1038/ni.f.215
- Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, et al. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med* (1991) 173(5):1099–109. doi:10.1084/jem.173.5.1099
- Bossi G, Griffiths GM. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat Med* (1999) 5(1):90–6. doi:10.1038/4779
- Vignaux F, Vivier E, Malissen B, Depraetere V, Nagata S, Golstein P. TCR/CD3 coupling to Fas-based cytotoxicity. *J Exp Med* (1995) 181(2):781–6. doi:10.1084/jem.181.2.781
- de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol* (2010) 10(8):568–79. doi:10.1038/nri2803
- Griffiths GM, Tsun A, Stinchcombe JC. The immunological synapse: a focal point for endocytosis and exocytosis. *J Cell Biol* (2010) 189(3):399–406. doi:10.1083/jcb.201002027
- Azar GA, Lemaitre F, Robey EA, Bousso P. Subcellular dynamics of T cell immunological synapses and kinapses in lymph nodes. *Proc Natl Acad Sci U S A* (2010) 107(8):3675–80. doi:10.1073/pnas.0905901107
- Mentlik AN, Sanborn KB, Holzbaue EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell* (2010) 21(13):2241–56. doi:10.1091/mbc.E09-11-0930
- Hsu H-T, Mace EM, Carisey AF, Viswanath DI, Christakou AE, Wiklund M, et al. NK cells converge lytic granules to promote cytotoxicity and prevent bystanderkilling. *J Cell Biol* (2016) 215(6):875–89. doi:10.1083/jcb.201604136
- Nath S, Christian L, Tan SY, Ki S, Ehrlich LIR, Poenie M. Dynein separately partners with NDE1 and dynactin to orchestrate T cell focused secretion. *J Immunol* (2016) 197(6):2090–101. doi:10.4049/jimmunol.1600180
- Ueda H, Zhou J, Xie J, Davis MM. Distinct roles of cytoskeletal components in immunological synapse formation and directed secretion. *J Immunol* (2015) 195(9):4117–25. doi:10.4049/jimmunol.1402175
- Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Ann Rev Cell Dev Biol* (2014) 30(1):255–89. doi:10.1146/annurev-cellbio-101512-122326
- Martinez-Lorenzo MJ, Anel A, Gamen S, Monle NI, Lasierra P, Larrad L, et al. Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. *J Immunol* (1999) 163(3):1274–81.
- Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol* (2002) 168(7):3235–41. doi:10.4049/jimmunol.168.7.3235
- Alonso R, Rodriguez MC, Pindado J, Merino E, Merida I, Izquierdo M. Diacylglycerol kinase alpha regulates the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes. *J Biol Chem* (2005) 280(31):28439–50. doi:10.1074/jbc.M50112200
- Mazzeo C, Calvo V, Alonso R, Merida I, Izquierdo M. Protein kinase D1/2 is involved in the maturation of multivesicular bodies and secretion of exosomes in T and B lymphocytes. *Cell Death Differ* (2016) 23(1):99–109. doi:10.1038/cdd.2015.72
- Li L, Jay SM, Wang Y, Wu SW, Xiao Z. IL-12 stimulates CTLs to secrete exosomes capable of activating bystander CD8(+) T cells. *Sci Rep* (2017) 7(1):13365. doi:10.1038/s41598-017-14000-z
- Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* (2009) 9(8):581–93. doi:10.1038/nri2567
- Peters PJ, Geuze HJ, Van der Donk HA, Slot JW, Griffith JM, Stam NJ, et al. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *Eur J Immunol* (1989) 19(8):1469–75. doi:10.1002/eji.1830190819
- Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G. The biogenesis and functions of exosomes. *Traffic* (2002) 3(5):321–30. doi:10.1034/j.1600-0854.2002.30502.x
- Zuccato E, Blott EJ, Holt O, Sigismund S, Shaw M, Bossi G, et al. Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. *J Cell Sci* (2007) 120(Pt 1):191–9. doi:10.1242/jcs.03315
- Monleon I, Martinez-Lorenzo MJ, Montegudo L, Lasierra P, Taules M, Iturralde M, et al. Differential secretion of Fas ligand- or APO2 ligand/

- TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. *J Immunol* (2001) 167(12):6736–44. doi:10.4049/jimmunol.167.12.6736
43. Lundberg V, Berglund M, Skogberg G, Lindgren S, Lundqvist C, Gudmundsdottir J, et al. Thymic exosomes promote the final maturation of thymocytes. *Sci Rep* (2016) 6:36479. doi:10.1038/srep36479
 44. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* (2014) 14(3):195–208. doi:10.1038/nri3622
 45. Ventimiglia LN, Alonso MA. Biogenesis and function of T cell-derived exosomes. *Front Cell Dev Biol* (2016) 4:84. doi:10.3389/fcell.2016.00084
 46. Yi J, Wu X, Chung AH, Chen JK, Kapoor TM, Hammer JA. Centrosome repositioning in T cells is biphasic and driven by microtubule end-on capture-shrinkage. *J Cell Biol* (2013) 202(5):779–92. doi:10.1083/jcb.201301004
 47. Jang JH, Huang Y, Zheng P, Jo MC, Bertolet G, Zhu MX, et al. Imaging of cell-cell communication in a vertical orientation reveals high-resolution structure of immunological synapse and novel PD-1 dynamics. *J Immunol* (2015) 195(3):1320–30. doi:10.4049/jimmunol.1403143
 48. Kupfer A, Singer SJ. Cell biology of cytotoxic and helper T cell functions: immunofluorescence microscopic studies of single cells and cell couples. *Annu Rev Immunol* (1989) 7:309–37. doi:10.1146/annurev.iy.07.040189.001521
 49. Sahl SJ, Hell SW, Jakobs S. Fluorescence nanoscopy in cell biology. *Nat Rev Mol Cell Biol* (2017) 18(11):685–701. doi:10.1038/nrm.2017.71
 50. Sinha S, Hoshino D, Hong NH, Kirkbride KC, Grega-Larson NE, Seiki M, et al. Cortactin promotes exosome secretion by controlling branched actin dynamics. *J Cell Biol* (2016) 214(2):197–213. doi:10.1083/jcb.201601025
 51. Bertolet G, Liu D. The planar lipid bilayer system serves as a reductionist approach for studying NK cell immunological synapses and their functions. *Methods Mol Biol* (2016) 1441:151–65. doi:10.1007/978-1-4939-3684-7_13
 52. Murugesan S, Hong J, Yi J, Li D, Beach JR, Shao L, et al. Formin-generated actomyosin arcs propel T cell receptor microcluster movement at the immune synapse. *J Cell Biol* (2016) 215(3):383–99. doi:10.1083/jcb.201603080
 53. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol* (2006) 7(8):803–9. doi:10.1038/ni1369
 54. Dustin ML. Supported bilayers at the vanguard of immune cell activation studies. *J Struct Biol* (2009) 168(1):152–60. doi:10.1016/j.jsb.2009.05.007
 55. Tsun A, Qureshi I, Stinchcombe JC, Jenkins MR, de la Roche M, Kleczkowska J, et al. Centrosome docking at the immunological synapse is controlled by Lck signaling. *J Cell Biol* (2011) 192(4):663–74. doi:10.1083/jcb.201008140
 56. Obino D, Farina F, Malbec O, Saez PJ, Maurin M, Gaillard J, et al. Actin nucleation at the centrosome controls lymphocyte polarity. *Nat Commun* (2016) 7:10969. doi:10.1038/ncomms10969
 57. Quann EJ, Merino E, Furuta T, Huse M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol* (2009) 10(6):627–35. doi:10.1038/ni1734
 58. Chauveau A, Le Floch A, Bantilan NS, Koretzky GA, Huse M. Diacylglycerol kinase alpha establishes T cell polarity by shaping diacylglycerol accumulation at the immunological synapse. *Sci Signal* (2014) 7(340):ra82. doi:10.1126/scisignal.2005287
 59. Montoya MC, Sancho D, Bonello G, Collette Y, Langlet C, He HT, et al. Role of ICAM-3 in the initial interaction of T lymphocytes and APCs. *Nat Immunol* (2002) 3(2):159–68. doi:10.1038/ni753
 60. Abraham RT, Weiss A. Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol* (2004) 4(4):301–8. doi:10.1038/nri1330
 61. Herman A, Kappler JW, Marrack P, Pullen AM. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu Rev Immunol* (1991) 9:745–72. doi:10.1146/annurev.iy.09.040191.003525
 62. Izquierdo M, Grandien A, Criado LM, Robles S, Leonardo E, Albar JP, et al. Blocked negative selection of developing T cells in mice expressing the baculovirus p35 caspase inhibitor. *EMBO J* (1999) 18(1):156–66. doi:10.1093/emboj/18.1.156
 63. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell* (1994) 76(1):17–27. doi:10.1016/0092-8674(94)90169-4
 64. Ritter AT, Kapnick SM, Murugesan S, Schwartzberg PL, Griffiths GM, Lippincott-Schwartz J. Cortical actin recovery at the immunological synapse leads to termination of lytic granule secretion in cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*. (2017). doi:10.1073/pnas.1710751114
 65. Mamalaki C, Elliott J, Norton T, Yannoutsos N, Townsend AR, Chandler P, et al. Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigens. *Dev Immunol* (1993) 3(3):159–74. doi:10.1155/1993/98015
 66. White J, Herman A, Pullen AM, Kubo R, Kappler JW, Marrack P. The V beta-specific superantigens staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* (1989) 56(1):27–35. doi:10.1016/0092-8674(89)90980-X
 67. Martin-Cofreces NB, Robles-Valero J, Cabrero JR, Mittelbrunn M, Gordon-Alonso M, Sung CH, et al. MTOC translocation modulates IS formation and controls sustained T cell signaling. *J Cell Biol* (2008) 182(5):951–62. doi:10.1083/jcb.200801014
 68. Zyss D, Ebrahimi H, Gergely F. Casein kinase I delta controls centrosome positioning during T cell activation. *J Cell Biol* (2011) 195(5):781–97. doi:10.1083/jcb.201106025
 69. Oddos S, Dunsby C, Purbhoo MA, Chauveau A, Owen DM, Neil MA, et al. High-speed high-resolution imaging of intercellular immune synapses using optical tweezers. *Biophys J* (2008) 95(10):L66–8. doi:10.1529/biophysj.108.143198
 70. Brown AC, Oddos S, Dobbie IM, Alakoskela JM, Parton RM, Eissmann P, et al. Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol* (2011) 9(9):e1001152. doi:10.1371/journal.pbio.1001152
 71. Christakou AE, Ohlin M, Vanherberghen B, Khorshidi MA, Kadri N, Frisk T, et al. Live cell imaging in a micro-array of acoustic traps facilitates quantification of natural killer cell heterogeneity. *Integr Biol (Camb)* (2013) 5(4):712–9. doi:10.1039/c3ib20253d
 72. Combs CA, Shroff H. Fluorescence microscopy: a concise guide to current imaging methods. *Curr Protoc Neurosci* (2017) 79:21–2125. doi:10.1002/cpns.29
 73. Lambert TJ, Waters JC. Navigating challenges in the application of superresolution microscopy. *J Cell Biol* (2017) 216(1):53–63. doi:10.1083/jcb.201610011
 74. Ashdown GW, Burn GL, Williamson DJ, Pandzic E, Peters R, Holden M, et al. Live-cell super-resolution reveals F-actin and plasma membrane dynamics at the T cell synapse. *Biophys J* (2017) 112(8):1703–13. doi:10.1016/j.bpj.2017.01.038
 75. Carisey AF, Mace EM, Saeed MB, Davis DM, Orange JS. Nanoscale dynamism of actin enables secretory function in cytolytic cells. *Curr Biol* (2018) 28(4):489–502e9. doi:10.1016/j.cub.2017.12.044
 76. Cahalan MD, Parker I. Choreography of cell motility and interaction dynamics imaged by two-photon microscopy in lymphoid organs. *Annu Rev Immunol* (2008) 26:585–626. doi:10.1146/annurev.immunol.24.021605.090620
 77. Hu YS, Cang H, Lillemeier BF. Superresolution imaging reveals nanometer- and micrometer-scale spatial distributions of T-cell receptors in lymph nodes. *Proc Natl Acad Sci U S A* (2016) 113(26):7201–6. doi:10.1073/pnas.1512331113
 78. Fritzsche M, Fernandes RA, Chang VT, Colin-York H, Clausen MP, Felce JH, et al. Cytoskeletal actin dynamics shape a ramifying actin network underpinning immunological synapse formation. *Sci Adv* (2017) 3(6):e1603032. doi:10.1126/sciadv.1603032
 79. Rak GD, Mace EM, Banerjee PP, Svitkina T, Orange JS. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* (2011) 9(9):e1001151. doi:10.1371/journal.pbio.1001151
 80. Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat Immunol* (2013) 14(1):82–9. doi:10.1038/ni.2488
 81. Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol* (2010) 11(1):90–6. doi:10.1038/ni.1832
 82. Griffie J, Shlomovich L, Williamson DJ, Shannon M, Aaron J, Khuon S, et al. 3D Bayesian cluster analysis of super-resolution data reveals LAT recruitment to the T cell synapse. *Sci Rep* (2017) 7(1):4077. doi:10.1038/s41598-017-04450-w
 83. Carr AR, Ponjavic A, Basu S, McColl J, Santos AM, Davis S, et al. Three-dimensional super-resolution in eukaryotic cells using the double-helix point spread function. *Biophys J* (2017) 112(7):1444–54. doi:10.1016/j.bpj.2017.02.023

84. Abe J, Ozga AJ, Swoger J, Sharpe J, Ripoll J, Stein JV. Light sheet fluorescence microscopy for in situ cell interaction analysis in mouse lymph nodes. *J Immunol Methods* (2016) 431:1–10. doi:10.1016/j.jim.2016.01.015
85. Chen BC, Legant WR, Wang K, Shao L, Milkie DE, Davidson MW, et al. Lattice light-sheet microscopy: imaging molecules to embryos at high spatio-temporal resolution. *Science* (2014) 346(6208):1257998. doi:10.1126/science.1257998
86. Scherf N, Huysken J. The smart and gentle microscope. *Nat Biotechnol* (2015) 33(8):815–8. doi:10.1038/nbt.3310
87. Gustafsson N, Culley S, Ashdown G, Owen DM, Pereira PM, Henriques R. Fast live-cell conventional fluorophore nanoscopy with ImageJ through super-resolution radial fluctuations. *Nat Commun* (2016) 7:12471. doi:10.1038/ncomms12471
88. Lukinavicius G, Umezawa K, Olivier N, Honigsmann A, Yang G, Plass T, et al. A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nat Chem* (2013) 5(2):132–9. doi:10.1038/nchem.1546
89. Sepulveda FE, Burgess A, Heiligenstein X, Goudin N, Menager MM, Romao M, et al. LYST controls the biogenesis of the endosomal compartment required for secretory lysosome function. *Traffic* (2015) 16(2):191–203. doi:10.1111/tra.12244
90. Ventimiglia LN, Fernandez-Martin L, Martinez-Alonso E, Anton OM, Guerra M, Martinez-Menarguez JA, et al. Cutting edge: regulation of exosome secretion by the integral MAL protein in T cells. *J Immunol* (2015) 195(3):810–4. doi:10.4049/jimmunol.1500891

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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High-Speed Single-Molecule Tracking of CXCL13 in the B-Follicle

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
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Council, United Kingdom

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Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 26 January 2018

Accepted: 30 April 2018

Published: 22 May 2018

Citation:

Miller H, Cosgrove J, Wollman AJM,
Taylor E, Zhou Z, O'Toole PJ,
Coles MC and Leake MC (2018)
High-Speed Single-Molecule
Tracking of CXCL13 in the B-Follicle.
Front. Immunol. 9:1073.
doi: 10.3389/fimmu.2018.01073

Soluble factors are an essential means of communication between cells and their environment. However, many molecules readily interact with extracellular matrix components, giving rise to multiple modes of diffusion. The molecular quantification of diffusion *in situ* is thus a challenging imaging frontier, requiring very high spatial and temporal resolution. Overcoming this methodological barrier is key to understanding the precise spatial patterning of the extracellular factors that regulate immune function. To address this, we have developed a high-speed light microscopy system capable of millisecond sampling in *ex vivo* tissue samples and submillisecond sampling in controlled *in vitro* samples to characterize molecular diffusion in a range of complex microenvironments. We demonstrate that this method outperforms competing tools for determining molecular mobility of fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP) for evaluation of diffusion. We then apply this approach to study the chemokine CXCL13, a key determinant of lymphoid tissue architecture, and B-cell-mediated immunity. Super-resolution single-molecule tracking of fluorescently labeled CXCL13 in collagen matrix was used to assess the heterogeneity of chemokine mobility behaviors, with results indicating an immobile fraction and a mobile fraction for both molecules, with distinct diffusion rates of 8.4 ± 0.2 and $6.2 \pm 0.3 \mu\text{m}^2\text{s}^{-1}$, respectively. To better understand mobility behaviors *in situ*, we analyzed CXCL13-AF647 diffusion in murine lymph node tissue sections and observed both an immobile fraction and a mobile fraction with an example diffusion coefficient of $6.6 \pm 0.4 \mu\text{m}^2\text{s}^{-1}$, suggesting that mobility within the follicle is also multimodal. In quantitatively studying mobility behaviors at the molecular level, we have obtained an increased understanding of CXCL13 bioavailability within the follicle. Our high-speed single-molecule tracking approach affords a novel perspective from which to understand the mobility of soluble factors relevant to the immune system.

Keywords: single-molecule imaging, single-molecule tracking, chemokines, biophysics, lymphoid tissues

INTRODUCTION

Within the immune system, soluble factors such as chemokines, cytokines, and growth factors drive graded responses to extracellular signals, regulating processes including immune cell recruitment at sites of infection (1), lymphoid tissue formation (2, 3), and the maturation of adaptive immune responses (4). Despite their fundamental importance, the precise spatial distribution of soluble factors within tissues remains unclear, due in part to a dearth of experimental techniques capable of measuring diffusion *in situ*.

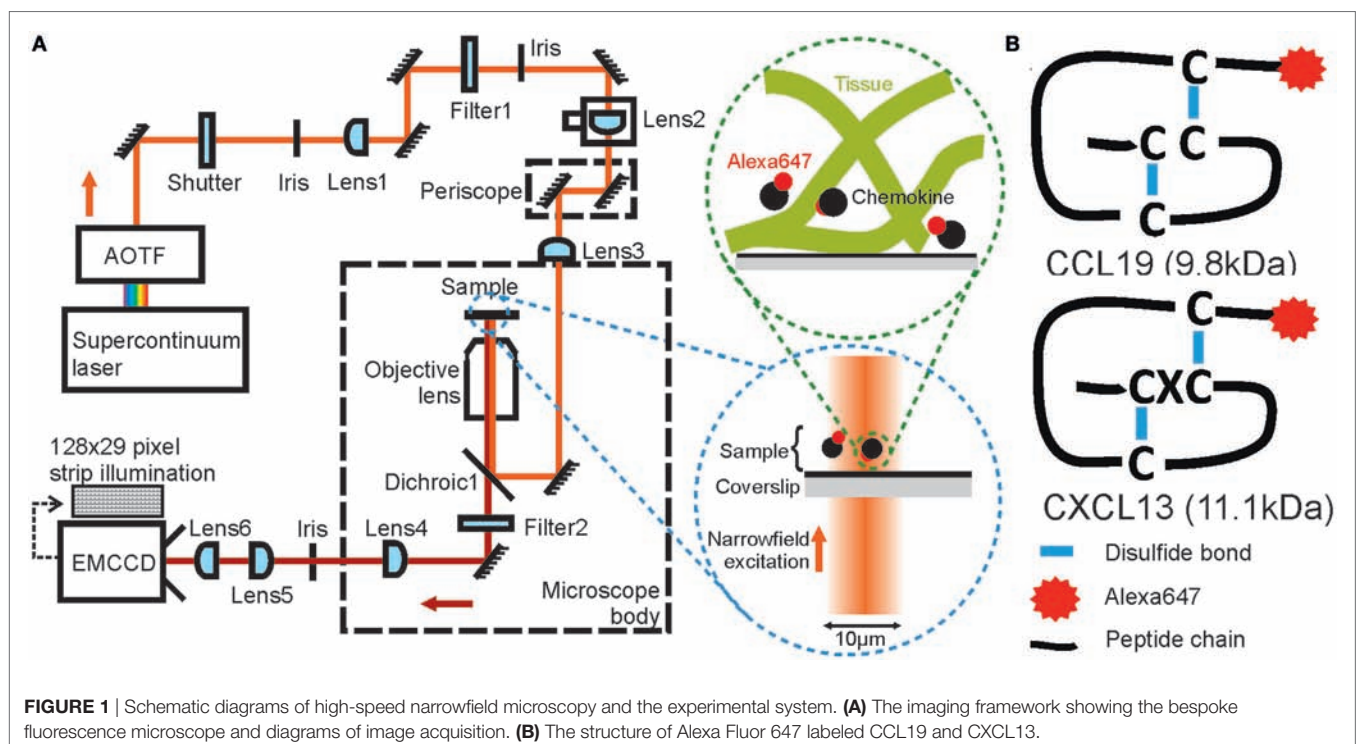
The emergence of super-resolution imaging in light microscopy has yielded significant insights into the structure and dynamics of the immune synapse (5), with the potential to elucidate the precise spatial localization of soluble factors within the context of a complex tissue. These methods enable spatial localization of single fluorescent probes more than an order of magnitude better than the standard optical resolution limit of ~ 250 nm, facilitating direct visualization of dynamic molecular processes. Barriers to using super-resolution for quantifying rapid molecular diffusion in biological processes in the aqueous inter- and intra-cellular regions in tissues include poor temporal resolution, due to constraints imposed from limited photon emission, and challenges in data interpretation due to heterogeneous mobility behaviors such as complex underlying diffusion modes or the presence of mixed populations of molecules in multimeric forms.

To achieve the most rapid sampling possible, traditional single-molecule fluorescence tracking techniques must compromise on the image quality or on the type of probe used. Elastic and interferometric scattering can overcome poor fluorophore photophysics to enable rapid sampling; however, they either use relatively large probes that exhibit steric hindrance, or achieve poor specificity in heterogeneous sample environments unless used in conjunction with fluorescent labeling (6–10). Scanning fluorescence methods such as stimulated emission depletion microscopy (STED (11) are limited to ~ 1 Hz typical frame rates with faster imaging up to $\sim 1,000$ Hz possible by trading image quality (12); MINIFLUX imaging (13) can operate at 8,000 localizations per second in bacterial cells, but only tracks one molecule at a time, while widefield approaches such as fast variants of photoactivatable localization microscopy (PALM) (14) and stochastic optical reconstruction microscopy (STORM) (15)

have integration times of on the order of tens of milliseconds for individual image frames with full reconstructions commonly taking several seconds. Structured illumination approaches (16, 17) at best have frame rates of several hundred Hz and high-intensity illumination methods have enabled super-resolution imaging in living cells at around millisecond timescales (18, 19). Submillisecond fluorescence imaging has been reported previously using relatively large fluorescent bead probes (20), tracking a single molecule at a time (21), in plasma membranes using fluorescently labeled cholesterol analogs or Fab fragments (22, 23) and at short distances from the coverslip using TIRF and HILO imaging (24). However, these methods encounter significant challenges in data interpretation when samples and mobility are heterogeneous, as encountered in tissues. Our method is the first, to the best of our knowledge, to enable submillisecond molecular tracking using a minimally perturbative nanoscale organic dye reporter in a heterogeneous 3D aqueous environment typical of interstitial regions between cells in tissues.

Single-molecule tracking can be used to measure diffusion coefficients of proteins and molecules in localized regions and offers the opportunity to investigate the heterogeneity one molecule at a time compared with the ensemble technique of fluorescence recovery after photobleaching (FRAP) (25–27) and quasi single-molecule approach of fluorescence correlation spectroscopy (FCS) (28, 29). These three techniques have been compared using proteins present in the plasma membrane of cells (30–33), supported lipid bilayers (33, 34), and giant unilamellar vesicles (34), which are all approximated as 2D surfaces.

In this study, we use single-molecule imaging approaches to quantify the diffusion of the chemotactic cytokines (chemokines) CXCL13 and CCL19 (Figure 1B). These molecules are key



regulators of lymphocyte migration that are present in spatially distinct regions of the lymphoid tissues such as the lymph node (4). Chemokines are small proteins (~10kDa) that bind G-protein Coupled Receptors (GPCRs) leading to polarization of the actomyosin cytoskeleton and directed migration along localized concentration gradients (35). Chemokine bioavailability is regulated across broad spatiotemporal scales, making direct visualization of these molecules *in situ* challenging. Chemokines are secreted within a dense, heterogeneous microenvironment and undergo transient interactions with their cognate GPCRs and components of the extracellular matrix (ECM) before undergoing receptor-mediated scavenging or enzymatic degradation (35–37). In addition, chemokines are heterogeneous in their binding affinities and are subject to multimerization effects; characteristics that may alter their mobility (38, 39). Simplified hydrodynamic predictions (40) employing estimations for the Stokes radius of chemokines and the fluid environment viscosity suggest that chemokine diffusion in hypothetically homogeneous intracellular media in the absence of binding effects would be rapid at $\sim 150 \mu\text{m}^2 \text{s}^{-1}$, implying ~ 50 s for a single molecule to diffuse across a 200- μm diameter region of lymphoid tissue. However, this estimate is likely to be a poor predictor of diffusivity as it does not account for dynamic molecular interactions encountered in dense, heterogeneous tissues.

In the following sections, we describe a method to overcome previous technological barriers to the study of molecular mobility *in situ*. Specifically, we have adapted a standard inverted epifluorescence microscope, making important modifications to facilitate minimally perturbative submillisecond single-molecule tracking of rapidly diffusing fluorescently labeled biomolecules *via* subdiffraction limit localizations, and developed bespoke software for precise quantification of underlying molecular mobility of tracked particles. We compared FCS, FRAP, and single-molecule tracking on the well-characterized test system for molecular mobility of bovine serum albumin (BSA), labeled with Alexa Fluor 647 (AF647). We then applied our method to quantify the diffusion of CCL19 and CXCL13 (Figure 1B), in a range of environments of increasing complexity comprising (i) buffer alone and in the presence of the highly branched polysaccharide Ficoll to vary the fluid environment viscosity, (ii) the presence of either surface-immobilized heparan sulfate, or a collagen gel matrix, and further (iii) AF647-tagged CXCL13 was tracked in an *ex vivo* native mouse lymph node environment. Our data suggest that CCL19 and CXCL13 have distinct diffusion rates, and that CXCL13 exhibits both specific binding and diffusion at $6.6 \pm 0.4 \mu\text{m}^2 \text{s}^{-1}$ within example sections of the B-cell follicle.

RESULTS

Overview of the High-Speed Single-Molecule Tracking Methodology

To enable precise localization and tracking of rapidly diffusing biomolecules, we modified the optical path of a standard inverted epifluorescence microscope (Figure 1A) to implement a broadband laser whose output was selectable over wavelengths ~ 400 – $2,000$ nm (Figure S1 in Supplementary Material), spanning the excitation spectra of visible-light and near-infrared fluorophores; the beam

was de-expanded using a series of lenses to generate a narrow illumination field of $\sim 12 \mu\text{m}$ full-width at half maximum (FWHM) which could be switched from epifluorescence into total internal reflection fluorescence (TIRF) by controllable translation of a lens, although TIRF was not used in this work. High-contrast epifluorescence images magnified to 120 nm/pixel were captured by an ultrasensitive back-illuminated EMCCD detector (860 iXon+, Andor Technology Ltd.) which could be subarrayed to 29×128 pixels to enable rapid frame rates of 1,515 Hz. Images were analyzed using bespoke software (41) written in MATLAB (Mathworks), which enabled automated 2D submillisecond tracking of single fluorescent dye molecules and determination of the microscopic diffusion coefficient D from the measured mean square displacement (42). Diffusion coefficients were found by an iterative fitting procedure developed with simulated data.

Speed of Tracking and Image Analysis

A range of sample exposure times of 0.44–1.98 ms per frame were used, with most data acquired at 0.59 ms per frame (0.65 ms cycle time) as a compromise between sampling speed and localization precision. In all cases, we were able to resolve distinct diffusing fluorescent foci of measured 2D half-width at half maximum in the range of 250–300 nm, consistent with single point spread function (PSF) images. Automated foci tracking was utilized for the determination of molecular diffusivity. Foci could be tracked continuously in 2D with ~ 40 nm precision (Figure S4 in Supplementary Material).

The presence of single molecules was determined by the observation of stepwise photobleaching steps. Examples of this are shown in Figures S1C,D in Supplementary Material. From the manufacturer's specifications, BSA-AF647 was expected to be labeled with between 3 and 6 AF647 dye molecules and the chemokines were expected to be singly labeled. Only single-molecule bleaching steps were observed in the chemokine data.

The initial intensity of observed foci of the AF647 dye molecule was measured in five conditions: CCL19-AF647 and CXCL13-AF647 in collagen and under heparan sulfate immobilization, and BSA-AF647 in 10% Ficoll. The kernel density estimate was found from the intercept of a line fitted to the first three intensity values measured. Within experimental error the initial intensity for all conditions fell in the 2,000–3,000 count range. The initial intensity is expected to vary for each condition due to the different local environment of the AF647 tag on each molecule, including different allowed orientations and varying flexibility of the linker. Furthermore, the viscosity of the medium is known to affect the emission profile of AF647 and will cause slight variations in total measured intensity due to the use of a band pass filter in the emission path.

Analysis of the tracking data from CXCL13-AF647 and CCL19-AF647 using step-wise dye photobleaching showed predominantly monomeric populations for each (Figure 2; Figure S1 in Supplementary Material). Apparent stoichiometry values determined from the intensity of tracked fluorescent foci greater than one molecule per foci may be due to real multimeric complexes or potentially due to the overlap of monomeric foci images in the 2D projection that is imaged, especially in the case of high dye concentration. The maximum number of detected foci in one

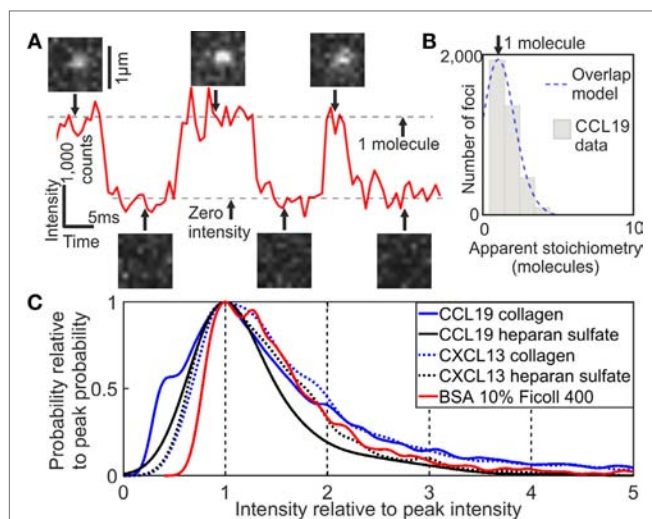


FIGURE 2 | Single-molecule stoichiometry of CCL19-AF647. **(A)** Tracking of photoblinking Alexa Fluor 647 (AF647): localizations and intensity over time with sample images from the acquisition. **(B)** Distribution of apparent CCL19 foci stoichiometry (gray) overlaid with the predicted distribution based on randomly overlapping point spread functions (blue). **(C)** Kernel density estimates of intensity of AF647 labeled CCL19 in collagen (solid blue line), and under heparan sulfate immobilization (solid black line); CXCL13 in collagen (dotted blue line), and under heparan sulfate immobilization (dotted black line); and bovine serum albumin in 10% Ficoll (solid red line). All traces are normalized to the primary peak for clarity (see Supplementary Material).

frame of 15 foci in our case was used in a random overlap model which assumes a Poisson distribution for nearest neighbor foci overlap probability (43, 44). This analysis indicates an 18% probability for random single foci overlap. The predicted intensity of foci due to random overlap could be obtained by convolving the intensity distribution of a single molecule of AF647 (width scaled by the square root of the apparent stoichiometry) with the apparent stoichiometry distribution generated by the random overlap probability prediction. The overlap model was found to be statistically identical to the experimentally observed distribution below apparent stoichiometry values of six AF647 molecules per foci ($p < 0.05$, Pearson's χ^2 test). A small proportion of less than 5% of foci we found to have a higher apparent stoichiometry than that predicted from the random overlap model; it is possible that these may be indicative of some additional factors not captured in the basic overlap model such as non-uniformity in illumination across the field of view and experimental autofluorescence.

Iterative Data Simulation and Experimental Data Fitting

Our analysis of the distribution of effective diffusion coefficients obtained from the single-molecule tracking data was corroborated through simulations of diffusing and immobilized foci using realistic signal and background noise values. Iterative cycles of simulation and experimental data fitting were used to determine initial parameters for fitting and the form of the fitting functions. All simulations were run and fitted with and without the addition of Gaussian white noise. The first simulated values were chosen by fitting the experimental data with a

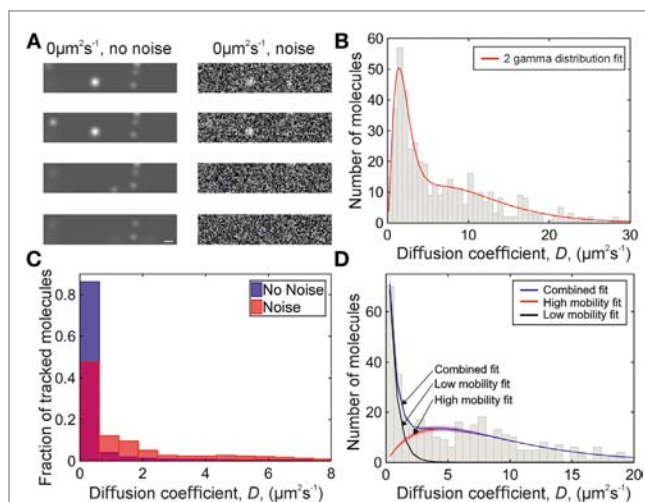


FIGURE 3 | Simulations of chemokine data. **(A)** Sample simulation images, shown with and without Gaussian white noise added. Scale bar 1 μm . **(B)** Two-gamma distribution fit (red) to diffusion coefficients found from a simulation of 1.6 and 10 $\mu\text{m}^2\text{s}^{-1}$ data with Gaussian white noise. **(C)** Histograms showing the distribution of simulated 0 $\mu\text{m}^2\text{s}^{-1}$ data with (red, overlaid) and without (blue) Gaussian white noise. **(D)** Diffusion coefficient distribution from a simulation of 0 and 9 $\mu\text{m}^2\text{s}^{-1}$ data with Gaussian white noise. Fitted populations are shown in black for the immobile, red for the mobile, and blue for the combined fit. Shaded areas indicate one SD.

two gamma distribution model (45) to account for two diffusive populations.

Initially two distributions, 1.6 $\mu\text{m}^2\text{s}^{-1}$ and a 50:50 mixed population of 1.6 and 10 $\mu\text{m}^2\text{s}^{-1}$, were simulated (sample images in **Figure 3A**). The data were plotted *via* kernel density estimation, and fitted with 1, 2, and 3 gamma distribution functions with four independent steps, all parameters constrained to be positive, each term was multiplied by a fractional prefactor to preserve the unity area of the kernel density plot, and the χ^2 goodness-of-fit parameter was evaluated (Table S1 in Supplementary Material). The χ^2 statistic accounts for the number of free parameters in the fit, and is used to determine if decreasing residuals are caused by increasing the number of free parameters. For the one component distribution, the one-gamma fit gave the lowest χ^2 , and for the two-component distribution, the two gamma fit gave the lowest χ^2 value, as expected. Applying these three models to the experimental collagen and heparan sulfate immobilized chemokine data gave the lowest χ^2 for a two-component fit, except for CCL19 in heparan sulfate, which contained a very low proportion of mobile tracks and was well fitted by a single-component distribution. From this, it was determined that a two-component distribution should be fitted to the experimental data.

The number of independent steps is usually taken to be the same as the number of steps analyzed in gamma distribution fitting analysis of microscopic diffusion coefficients, where it is a parameter governing the shape of the distribution. Strictly, steps are only independent when non-overlapping steps are used (45), but when the localization precision (in nm) of single particles is small compared with the distance moved between localizations in a track the diffusion coefficient distributions from overlapping

steps are still well-approximated by the assumption of independent steps. In this work, the temporal resolution is increased to a level where the localization precision is no longer negligible compared with the distance moved between localizations, and steps containing the same localizations are no longer well approximated as being independent. To investigate the independence of the steps in the data and determine the relevant fitting parameter, simulations of 1.6 and 10 $\mu\text{m}^2\text{s}^{-1}$ were made separately, and fitted with a single-component gamma distribution where the number

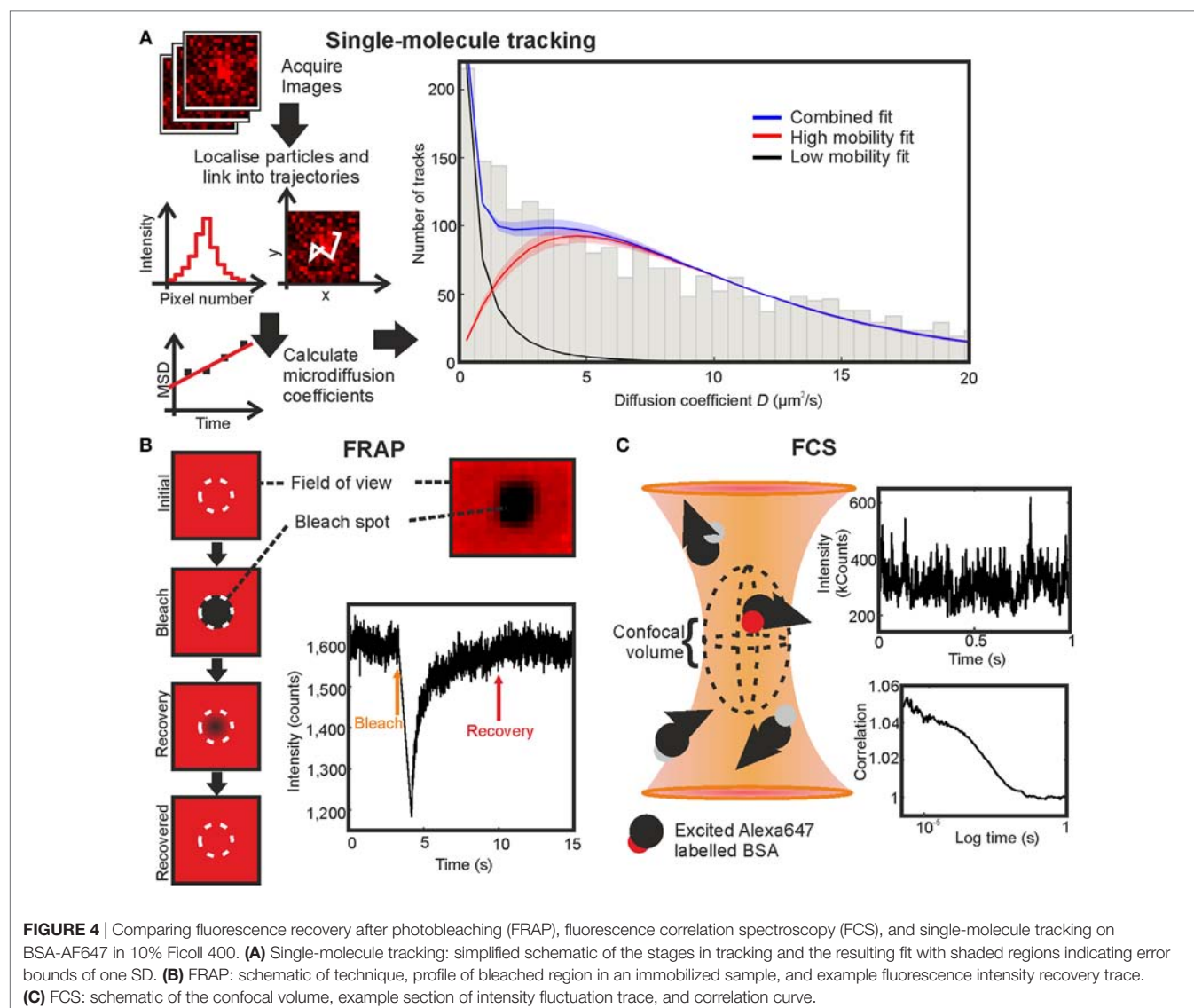
of independent steps was allowed to vary. The results (Table 1) indicate this value to be around two, in line with expectations of consecutive steps containing the same localization not being independent, reducing the number of steps by half.

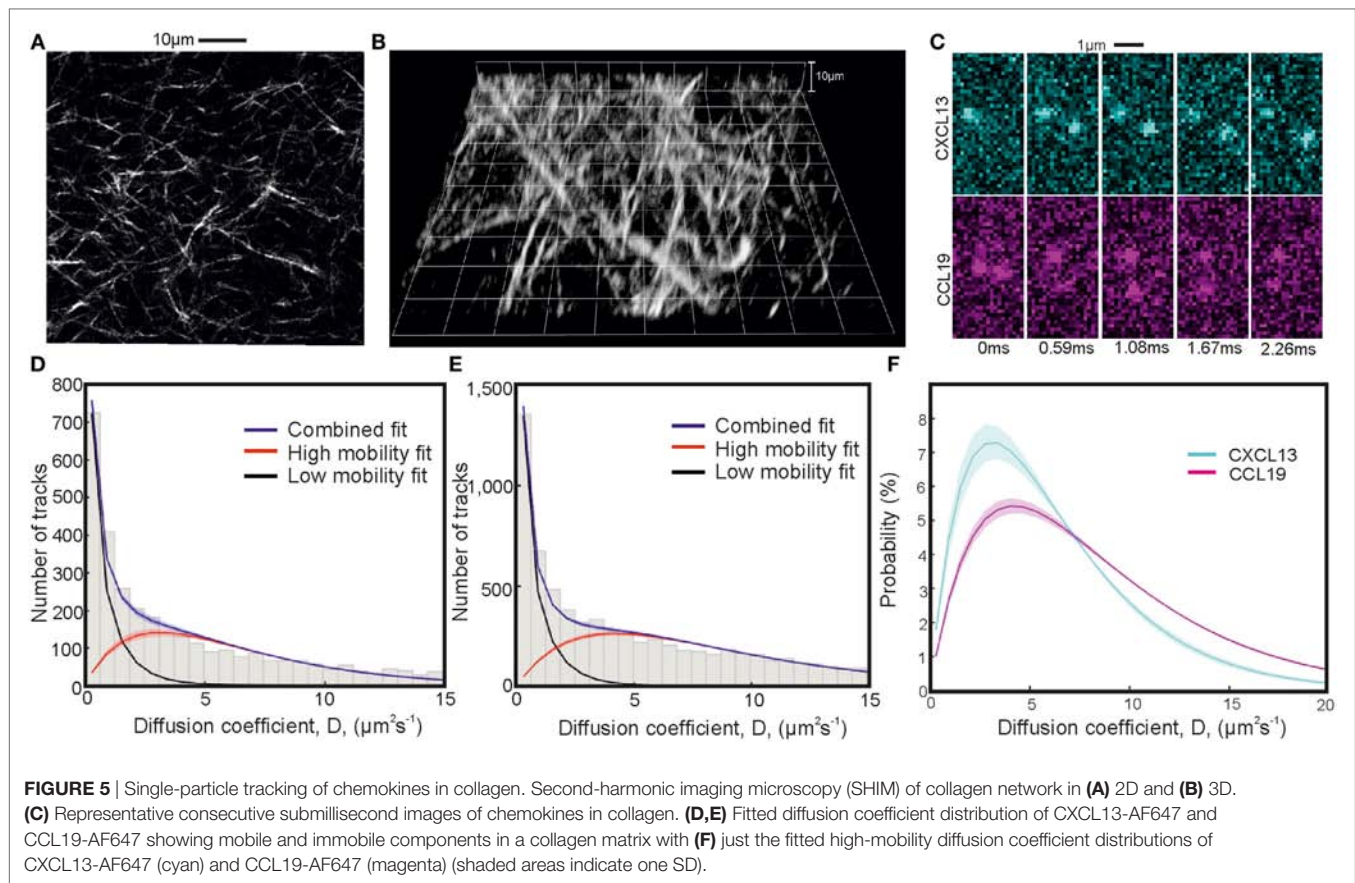
Fitting simulations of a 50:50 population of molecules with diffusion coefficients of 1.6 and 10 $\mu\text{m}^2\text{s}^{-1}$ with two gamma distributions with the same fitted value of N in each distribution, and N constrained to be in the range 0–4 (Figure 3B; Table S2 in Supplementary Material), gives a distribution which does

TABLE 1 | Results of one-gamma distribution fitting to simulated single diffusion coefficient distributions.

Simulated condition	Number of tracks	Fitted value of D ($\mu\text{m}^2\text{s}^{-1}$)	Fitted value of N ($\mu\text{m}^2\text{s}^{-1}$)	R^2 value of fit
1.6 $\mu\text{m}^2\text{s}^{-1}$, no noise	1,579	1.72 (1.68, 1.76)	2.24 (2.15, 2.33)	0.9892
1.6 $\mu\text{m}^2\text{s}^{-1}$, noise	401	2.19 (2.12, 2.25)	1.75 (1.67, 1.83)	0.9777
10 $\mu\text{m}^2\text{s}^{-1}$, no noise	1,519	10.21 (9.77, 10.6)	2.27 (2.08, 2.45)	0.9343
10 $\mu\text{m}^2\text{s}^{-1}$, noise	463	10.04 (9.53, 10.54)	2.77 (2.47, 3.08)	0.8968

Noise or no noise refers to the presence of Gaussian white noise proportional to the intensity in the simulation. 95% confidence intervals are given in brackets.





not match the experimental data (**Figure 4A** and **5D,E**): when the data are placed in a histogram based on measured diffusion coefficient the experimental data show a peak in the first bin width, which is not seen in the simulation of $1.6 \mu\text{m}^2\text{s}^{-1}$ data. The $1.6 \mu\text{m}^2\text{s}^{-1}$ data were simulated because these were found as a preliminary result of fitting to the experimental data, but a simulation of truly immobile data with a diffusion coefficient of $0 \mu\text{m}^2\text{s}^{-1}$ gave a peak in the first bin of the histogram when put into bins with the width of the localization precision (see **Figure 3C**), matching the experimental data.

Our simulated particle diffusion analysis suggests that the low-mobility population in the experimental data is immobile at the level of the localization precision. Fitting the distribution of simulated $0 \mu\text{m}^2\text{s}^{-1}$ data with a single-gamma distribution gave a value of N less than 1, and requires the fit applied to the experimental data to include a different value of N in the distribution fitted to each population with the value of N being less than 1 for the low-mobility population, and 2 for the higher mobility population.

Applying this fit, with the constraint that the diffusion coefficient of the immobile population must fall within the first bin of the histogram, gave the fitted experimental diffusion coefficients. To qualitatively compare the simulated and experimental data, a mixed simulation of 0 and $9 \mu\text{m}^2\text{s}^{-1}$ data with Gaussian white noise was performed and fitted in the same way, giving a diffusion coefficient for the mobile peak of $8.9 \pm 0.4 \mu\text{m}^2\text{s}^{-1}$. The

distribution is similar in profile to the data for CCL19-AF647 in collagen (see **Figure 3D**).

Diffusion in Buffer and Ficoll Solutions

In PBS buffer alone, the chemokine diffusion was in general too fast to track over consecutive image frames (Video S1 in Supplementary Material). While this is consistent with theoretical estimates using the Stokes–Einstein relation which gives $D \sim 150 \mu\text{m}^2\text{s}^{-1}$ for chemokines in an aqueous environment, the application of 10% Ficoll increased the fluid viscosity by a factor of 5.6 to 0.005 Pa s^{-1} , which enabled single-particle tracking; if diffusion had been well modeled by the Stokes–Einstein relation the diffusion coefficients in the higher viscosity Ficoll solution would be expected to be $\sim 30 \mu\text{m}^2\text{s}^{-1}$ and particles would still not be tracked. The ability to track single chemokines in a medium of this viscosity demonstrates that the theory is not adequate to describe chemokine diffusion and motivates our experimental measurements.

The experimental system was tested first on a non-chemokine control of AF647-tagged BSA (BSA-AF647). The results of single-particle tracking of BSA-AF647 were consistent with a proportion of immobile tracks associated with the coverslip surface and a freely diffusing mobile population with $D_{\text{mobile}} = 9.3 \pm 0.4 \mu\text{m}^2\text{s}^{-1}$ (**Figure 4**; Videos S2 and S3 in Supplementary Material). Including theoretical expectations based on hydrodynamic modeling of BSA as a Stokes sphere of radius 3.48 nm for monomeric BSA,

with a monomer to dimer ratio of 15:2 (measured using SEC-MALLS quantification, Figure S2 and Table S3 in Supplementary Material) and incorporating Faxen's law for distances of 10 nm, at which distances increased viscosity effects occur at the coverslip boundary (46), the fitted mobile value is found to be in agreement with the theoretical value of $9.4 \mu\text{m}^2\text{s}^{-1}$.

Comparison of SMT With FCS and FRAP

The diffusion coefficient of AF647 labeled BSA in a Ficoll solution was additionally measured with FCS and FRAP to generate a comparison of the three methods in a complex, non-surface environment (Figures 3A–E in Supplementary Material). FRAP and FCS gave diffusion coefficients of 7.1 ± 0.3 and $18.8 \pm 0.3 \mu\text{m}^2\text{s}^{-1}$, respectively (Figures 4B,C; Table 2). The values found for the diffusion coefficients by these methods are summarized in Table 1 with the number of traces used for each measurement. The result for FCS is higher than the theoretical value, while that for FRAP is significantly lower even considering Faxen's law, temperature fluctuation, and non-monomer content in BSA measured by SEC-MALLS (Figure S2 and Table S3 in Supplementary Material); however, the effects of using an axially thin sample and including only 2D recovery in the FRAP analysis were not accounted for.

The FRAP and FCS results differ by a factor of 2.6, in general agreement with previous results from others in which diffusion coefficients found by FCS are mostly higher than those found by FRAP (34), with FCS giving values up to an order of magnitude higher than the values found by FRAP (30, 31) and often attributed to the different spatial scales of the two measurements or the high number of assumptions required in fitting FRAP data (33), such as the profile of the bleaching laser, which are likely to be factors in the experiments performed here. The value found by SMT was the closest to the theoretical estimate of the diffusion coefficient in Ficoll of the experimental viscosity.

Fluorescence correlation spectroscopy and FRAP were also performed on the chemokines in 10% Ficoll 400. FCS produced autocorrelation curves with similar amplitude to BSA-AF647, but high variation was observed in the relative sizes and characteristic decay times of the triplet and translational diffusion populations (Figure S3F in Supplementary Material), resulting in no consensus value of the diffusion coefficient. Both FCS and FRAP measurements were hindered by the presence of large multimers

of chemokine (Figure 3G in Supplementary Material). Multimers of this type are simply avoided by visual identification in single-molecule tracking experiments.

Diffusion Coefficients of CXCL13 and CCL19 in Collagen

The values of the diffusion coefficients were determined in collagen reconstituted from rat tails to produce a simplified tissue mimic. The structure of the collagen was checked for the required formation of non-centrosymmetric structure with second-harmonic imaging microscopy (SHIM) (see Figures 5A,B). The fibril diameters observed are in agreement with those seen by Chen et al. (47), and show qualitatively similar structure.

The values of the diffusion coefficients of CXCL13 and CCL19 in collagen were found by fitting a two gamma distribution to a histogram of the single-molecule tracking data with bin width corresponding to 40nm given by peaks in the localization precision at low diffusion coefficient found from heparan sulfate immobilization of the labeled chemokines (Figures S4A,B; Videos S7 and S8 in Supplementary Material). Heparan sulfate immobilization was verified by an extremely high proportion of immobile tracks (Figures S4C,D in Supplementary Material). The results of the fitting are given in Table 3, and the distributions for each chemokine showing the mobile and immobile populations are shown in Figures 5C–E with sample images shown in Figure 5C. The relative size of the mobile and immobile populations cannot be accurately accounted for as immobile populations were photobleached to enable imaging of highly diffuse mobile populations.

Modeling the submillisecond tracking data as a mixture of immobilized and mobile tracks generated excellent agreement to the experimental data (Table 3). Our findings indicated a higher diffusion coefficient for CCL19-AF647 than for CXCL13-AF647 in the controlled environment of collagen (Figure 5F; Videos S4 and S5 in Supplementary Material), which we measured as 8.4 ± 0.2 and $6.2 \pm 0.3 \mu\text{m}^2\text{s}^{-1}$, respectively. This heterogeneity is consistent with molecular mass expectations and may contribute to the formation of distinct spatial patterning profiles *in situ*.

Binding of CXCL13 to Lymph Node Tissue Sections

The experiments with BSA-AF647, CCL19-AF647, and CXCL13-AF647 in collagen suggested a 4–6 times higher proportion of molecules in the immobile fraction than the mobile fraction for the chemokines than for BSA-AF647, even allowing

TABLE 2 | Measurements of the diffusion coefficient of Alexa-647 labeled BSA in 10% Ficoll 400.

Condition	Diffusion coefficient ($\mu\text{m}^2\text{s}^{-1}$)	Number of measurements
Theoretical with stokes radius 3.48 nm	12.3 ± 0.1	
FCS	18.8 ± 0.3	27 traces
FRAP	7.1 ± 0.3	30 repeats
Single-molecule tracking	9.3 ± 0.4	2,608 tracks (fitted 1,113 mobile tracks)

Variation on the theoretical value is due to a potential $\pm 2^\circ\text{C}$ temperature change in the laboratory.

TABLE 3 | Diffusion coefficients of CXCL13 and CCL19 in collagen.

AF647 labeled chemokine	Theoretical diffusion coefficients in water ($\mu\text{m}^2\text{s}^{-1}$)	Fitted diffusion coefficient ($\mu\text{m}^2\text{s}^{-1}$)	Error ($\mu\text{m}^2\text{s}^{-1}$)	Number of highly mobile tracks	R^2 of combined fit
CXCL13	149	6.2	0.3	1,930	0.980
CCL19	146	8.4	0.2	4,859	0.984

Optimized values were found by fitting a two gamma distribution to single-molecule tracking data.

for differences introduced by utilizing a pre-bleach to increase the fraction of mobile tracks. This is in agreement with previous results: CXCL13 is secreted in soluble form, but is known to interact with ECM components (48) and thus is likely to comprise a significant immobile fraction. To assess both fractions while also ensuring a sufficiently low concentration of CXCL13-AF647 to enable single-molecule detection, we incubated murine lymph node tissue cryosections with CXCL13-AF647 and performed a short wash step. While removing a large component of the soluble fraction of CXCL13-AF647, this preparation facilitated tracking of both mobile and immobile fractions of CXCL13-AF647 *in situ*, depending on the microscopy method employed.

To assess the specificity of binding, we used confocal microscopy to quantify the fluorescent intensity of B220⁺ regions (Figure 6A) of lymph node tissue sections that had been incubated with either CXCL13-AF647 or BSA-AF647. The fluorescent intensity values obtained were significantly higher for samples incubated with CXCL13-AF647 (Figures 6B,C), suggesting that the binding of CXCL13 to lymph node follicles was specific.

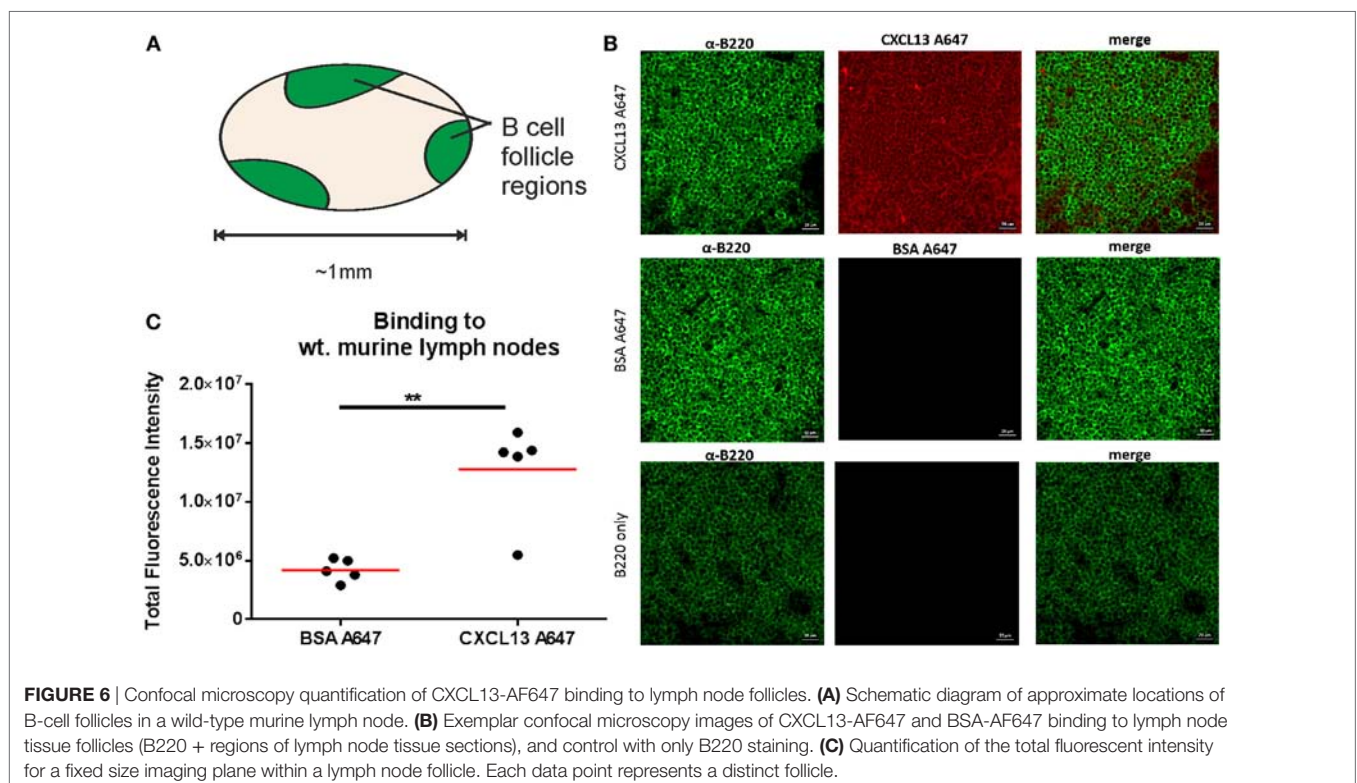
In the single-molecule microscopy experiments, we imaged and tracked CXCL13-AF647 in B-cell follicles of *ex vivo* murine lymph node tissue sections with super-resolution precision at ~2 ms timescales (Figure 7C; Video S6 in Supplementary Material), determining the precise location in the tissue using FITCB220 (B-cell-specific marker) (Figure S5 in Supplementary Material). Auto-fluorescent ECM components were localized by the tracking software and were segmented to allow discrimination of tracks from the immobile ECM and the diffusing chemokine (Figures 7C,D,F). When the same segmentation analysis was

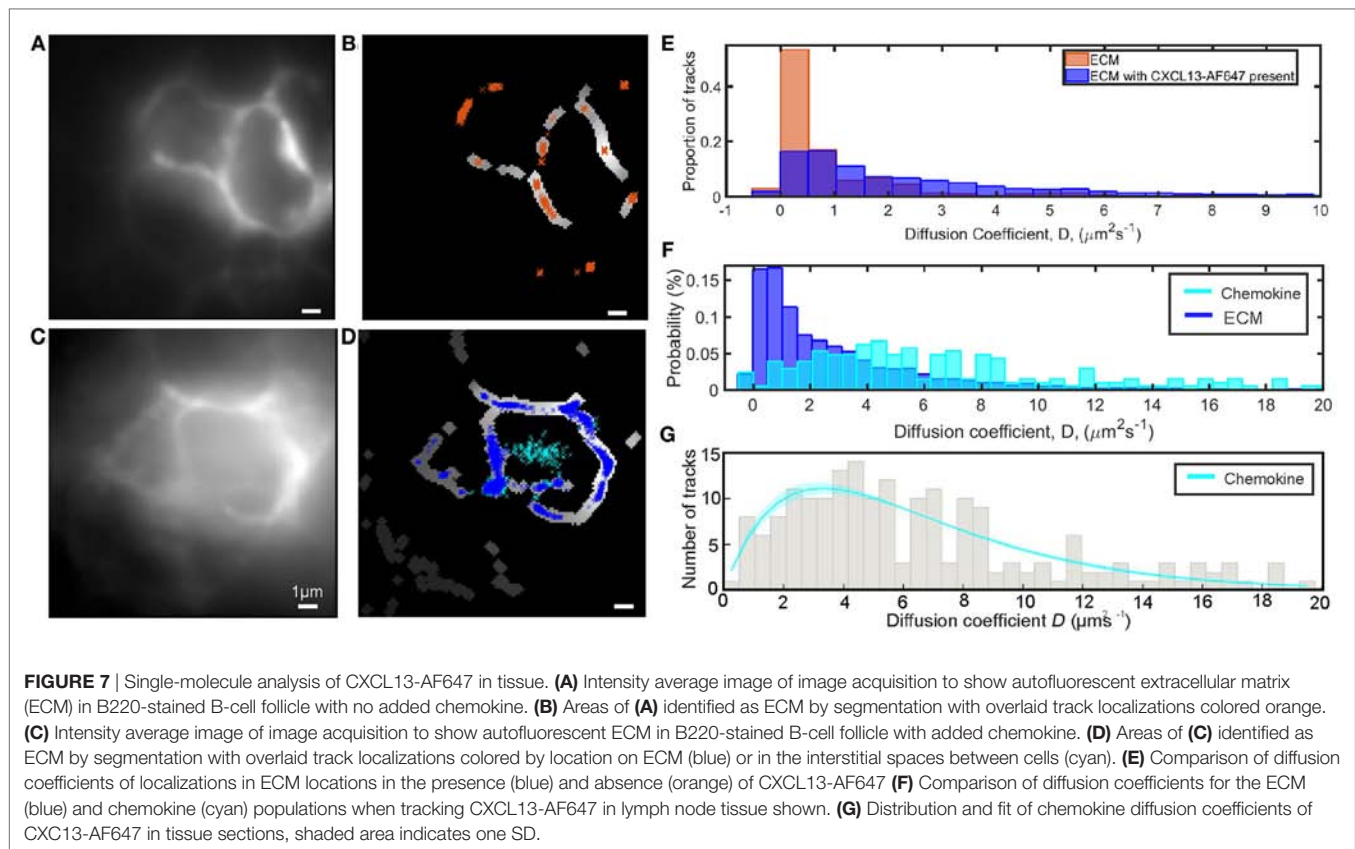
performed on control tissue sections prepared by the same protocol except without the addition of CXCL13-AF647, similar autofluorescent structures were seen and could be segmented (see Figures 7A,B). The observed diffusion coefficient distributions of tracked ECM regions within the B-cell follicle in the presence of chemokine were observed to be skewed toward higher mobility than those in the absence of chemokine (Figure 7E), further confirming the presence of specific binding of CXCL13-AF647 in the ECM regions.

Diffusion Coefficient of CXCL13 in Lymph Node Tissue Sections

For the same single-molecule imaging experiments performed at ~2 ms timescales described above, after discrimination of mobile and immobile tracks by segmentation, the diffusion coefficient of mobile tracked particles for the field of view shown was fitted with a single-gamma distribution indicating $D = 6.6 \pm 0.4 \mu\text{m}^2\text{s}^{-1}$ (Figure 7G). Due to the inclusion of a wash step in our sample preparation, we expect the majority of the mobile particles to be in the interstitial spaces between cells, although a small fraction may be within cut cells due to the preparation of tissue sections. The lack of fluorescent localizations in the central gap in the control sample is further evidence that the mobile population observed in the data is CXCL13-AF647.

To validate our result, we performed a simulation of the conditions in the tissue, with the mean background level and SD of the noise taken from the control data. A total of 1,000 frames of data of particles with diffusion coefficient $6.6 \mu\text{m}^2\text{s}^{-1}$ were generated,





with spot intensities and spot widths taken from the experimental data for tracking in tissue. Sample images from the simulation can be seen in Figure S6A in Supplementary Material. The result of fitting this simulation was $D = 7.0 \pm 0.4 \mu\text{m}^2\text{s}^{-1}$ (Figure S6B in Supplementary Material), in agreement with the simulated value. Whilst interstitial spaces are heterogeneous in their size, crowding, and local viscosity, taken together our results demonstrate the ability of our method to extract diffusion coefficients from challenging experimental data.

DISCUSSION

In this work, we have demonstrated the application of a high-speed single-molecule tracking microscopy system that is compatible with traditional widefield light microscopes. We compared our method with the traditional methods to measure molecular mobility of FCS and FRAP using one of the standard test molecules for molecular mobility (BSA). We applied this new approach to investigating hitherto unquantified molecular mobility of chemokines in complex environments, finding values of diffusion coefficients of $8.4 \pm 0.2 \mu\text{m}^2\text{s}^{-1}$ for CCL19-AF647 and $6.2 \pm 0.3 \mu\text{m}^2\text{s}^{-1}$ for CXCL13-AF647 in collagen, and of $6.6 \pm 0.4 \mu\text{m}^2\text{s}^{-1}$ for CXCL13-AF647 in example lymph node follicle sections, in addition to identifying a specifically bound CXCL13-AF647 population in the B-cell follicle. While we demonstrate the efficacy of the approach on chemokines, this is a proof-of-concept for a more general scheme that could be applied to signaling lipids and cytokines.

Our method enables single-molecule tracking of organic dye probes at submillisecond timescales, down to less than half a millisecond per imaging frame while still enabling 40-nm localization precision in realistic tissue mimetic microenvironments. In *ex vivo* lymph node tissue sections, we were able to perform rapid super-resolution sampling down to 2 ms per imaging frame and still achieve single-molecule detection sensitivity. To the best of our knowledge, this is the first application of submillisecond tracking of small fluorophores away from the coverslip interface.

We characterized the output of our single-molecule tracking tools with a range of simulations of mixed molecular mobility using realistic levels of signal and noise comparable to those exhibited at challenging single-molecule detection levels with very rapid submillisecond fluorescence sampling. We also tested our imaging and analysis methods using a fluorescently labeled version of the well-characterized molecule, BSA, and compared these with experiments using FRAP and FCS. The values of diffusion coefficient for mobile BSA determined using our rapid SMT method were in close agreement to expectations based on hydrodynamic modeling. Equivalent BSA mobility values estimated from using FRAP or FCS were less reliable. We compared the diffusion coefficients of BSA-AF647 measured by FCS, FRAP, and SMT, producing a comparison of these techniques away from the coverslip interface, and showing agreement in the relation of the measured values with most previous studies performed on lipid bilayers, or on live cell plasma membranes.

We measured the diffusion coefficients of CXCL13-AF647 and CCL19-AF647 in reconstituted collagen, finding values of 6.2 ± 0.3 and $8.4 \pm 0.2 \mu\text{m}^2 \text{s}^{-1}$, respectively. We further measured the diffusion coefficient of CXCL13-AF647 in *ex vivo* lymph node tissue section, finding a value in agreement with that measured in collagen of $6.6 \pm 0.4 \mu\text{m}^2 \text{s}^{-1}$. Fluorescent tags increase the mass of the labeled molecule and potentially affect the properties of diffusion. The fluorophore used in this work, AF647, was chosen for its small mass, which is especially important here given the small mass of chemokines. This choice resulted in a $\sim 10\%$ increase in mass of the labeled chemokine compared with the unlabeled chemokine, but this is lower than would have been achieved with other fluorophores. Following the assumptions of the Stokes–Einstein model of a spherical protein of uniform density, a 10% increase in mass would only decrease the observed diffusion coefficient by $\sim 3.2\%$. We observe a large discrepancy between our empirical measurements for chemokine diffusion rates and the higher estimated values derived on the basis of Stokes–Einstein relation. However, this discrepancy is of the same order of magnitude as that previously observed for the same theoretical calculation for GFP ($\sim 93 \mu\text{m}^2 \text{s}^{-1}$) and measured experimental values in *Escherichia coli* [$\sim 7.7\text{--}9 \mu\text{m}^2 \text{s}^{-1}$ (49, 50)]. This discrepancy may be indicative of additional factors that affect molecular mobility in tissues but are not captured in the simplistic Stokes–Einstein relation. These factors might include, for example, dynamic physical and chemical processes which result in more constrained mobility such as transient biochemical interactions within the localized microenvironment, as have been observed previously in studies which suggest that CXCL13 binds to ECM components (48).

Placed in an immunological context, our data show that chemokine mobility is multimodal in complex environments. Using our novel imaging approach, we were able to quantitatively identify a mobile and immobile fraction in collagen, and using a combination of confocal microscopy and single-molecule imaging we identified mobile and bound populations of CXCL13-AF647 in lymph node follicles. Thus, it is important to consider the contributions of both populations of CXCL13 upon cellular behaviors, rather than taking a view where it acts in either a soluble or an immobile way. The properties and likely highly constrained nature of CXCL13 diffusion within the follicle may provide an insight into how B cells can form such tightly compartmentalized microanatomical structures such as the follicle, or the germinal center light-zone.

Our novel high-speed microscopy and analysis outperform traditional molecular mobility tools of FRAP and FCS in being able to capture diffusional heterogeneity relevant to real, complex biological systems exemplified by underlying mobile, and immobile states. The high time resolution achieved with our system enables rapid diffusion to be quantified in heterogeneous aqueous environments typical of interstitial regions between cells in tissues, while still retaining super-resolution spatial precision and single-molecule detection sensitivity, enabling new insight into complex systems. A key advantage of our rapid single-particle tracking method is its ability to determine the underlying heterogeneity in the mobility of the molecular population exemplified here by chemokines that diffuse in different environments. While we demonstrate the efficacy of the approach on chemokines, this is a proof-of-concept for a more

general scheme that could be applied to lipids and cytokines. Our system is compatible with traditional widefield light microscopes as opposed to requiring expensive and dedicated super-resolution setups; this accessibility bodes well for establishing a significant future impact investigating multiple biological systems.

MATERIALS AND METHODS

Reagents

Human CXCL13 and CCL19 (Almac, CAF-12 and CAF-06, respectively) singly labeled with the far-red fluorescent tag AF647 (molecular mass 1,250 Da) were stored in water at $222 \mu\text{g mL}^{-1}$. This fluorophore was chosen because of its small molecular mass, which reduces the impact of increased mass on molecular mobility, and due to its excitation at the long, lower energy wavelength range of the spectrum, which reduces sample damage. Collagen samples contained type-I collagen extracted from rat tails (51) diluted in PBS to 3.3 mg mL^{-1} and chemokine at 111 ng mL^{-1} ; samples were set to pH7 with the addition of NaOH. BSA labeled with 3–6 AF647 was purchased from Thermo Fisher Scientific Inc. Ficoll 400 (Sigma-Aldrich) was diluted in PBS at 0.1 g mL^{-1} to create a 10% solution of viscosity 0.005 Pa s at room temperature (52).

Preparation of Collagen Matrix in Tunnel Slides

Samples for fluorescence microscopy were prepared in tunnel slides formed by placing two parallel lines of double-sided tape on a standard microscopy slide around 5 mm apart. A plasma-cleaned coverslip was placed on top and carefully tapped down (avoiding the imaging area) to create a water-tight tunnel.

For imaging in a collagen matrix tunnel slides were cooled to 4°C before addition of collagen and fluorescently labeled chemokines, and then incubated at 15°C for 30 min, followed by an additional 30-min incubation at 37°C . The collagen matrix was visualized using second harmonic imaging (47, 53).

Immobilized chemokine samples were prepared by incubating a plasma-cleaned coverslip in heparan sulfate (54) (50 mg mL^{-1}) (Sigma-Aldrich) in PBS for 30 min. Coverslips were washed with PBS and air dried for 30 min before tunnel slide assembly then 10-nM fluorescently labeled chemokine solution in PBS was introduced to the tunnel slide and incubated in a humidity chamber for 15 min at 20°C . Excess unbound chemokine was removed with a PBS wash.

SHIM Imaging

Second harmonic imaging microscopy was performed on a Zeiss LSM 780 MP with a Zeiss invert microscope. Excitation at 900-nm wavelength (Coherent Ultra Laser) through a plan-apochromat $63\times/1.4$ oil objective lens was incident on the sample. Up-converted light was collected *via* a 485-nm short pass filter onto a non-descanned detector.

Preparation of Lymph Node Tissue Sections

Six- to eight-week-old wild-type mice (C57BL/6) were housed in BSF at the University of York. All experiments conformed to

the ethical principles and guidelines approved by the University of York Institutional and Animal Care Use Committee. Popliteal Lymph Nodes were removed and excess fat or connective tissue removed with forceps. Samples were transferred to optimal cutting temperature medium (OCT, Tissue-Tek, Sakura Finetek) and snap frozen on dry ice samples and sectioned using a cryostat. 10- μ m thick sections were cut and collected onto poly-L-lysine coated microscope slides. Sections were dried overnight in the dark then stored at -20°C .

Before use, lymph node sections on poly-L-lysine slides were incubated at room temperature for 30 min. Sections were hydrated in PBS for 5 min then air dried. Wax ImmEdge pen (Vector Laboratories) was used to draw a hydrophobic circle around each section to retain liquid on the section during staining. Sections were incubated in a blocking buffer of PBS 5% goat serum (Sigma) at room temperature for 1 h. To determine where B-cell follicular structures were located in the tissue we used the marker B220, a protein expressed on the surface of all murine B lymphocytes. After blocking, sections were incubated with an FITC-conjugated antibody (RA3-6B2, purchased from eBioscience) that binds specifically to B220 diluted 1:200 in blocking buffer for 1 h at room temperature. After blocking, sections were incubated with an FITC-conjugated antibody (RA3-6B2, purchased from eBioscience) that binds specifically to B220 diluted 1:200 in blocking buffer for 1 h at room temperature. Samples were washed with PBS for 3 min $\times$ 5 min.

For confocal microscopy experiments where exogenous CXCL13-AF647/BSA-AF647 was used to measure binding to tissue, slides were stained with anti-B220 as described above followed by an incubation with 500 nM of each fluorescently labeled protein for 1 h at room temperature. Samples were then washed 1 min $\times$ 5 min in PBS. A drop of Prolong gold (Invitrogen) was added to each section, and then a No 1.5 glass coverslip (Fisher) mounted on top. The slides were incubated overnight at 4°C the next day slides were sealed using nail varnish and stored at 4°C . Immunofluorescent-stained sections were imaged using a Zeiss LSM 880 confocal microscope.

For single-molecule microscopy experiments sections were stained for B220 as described above, and then 1 μM of CXCL13-AF647 was added to the slides. Slides were incubated overnight at 4°C after which slides were washed for 30 s in PBS and a coverslip (thickness 0.1–0.17 mm, Menzel Gläser) mounted on top. Slides were then sealed and imaged.

Stokes Model of Diffusion

For a small sphere, the diffusion coefficient is given by

$$D = \frac{k_B T}{6\pi\eta r}$$

where k_B is the Boltzmann constant, T is room temperature, η is the dynamic viscosity of the media, and r is the radius of a sphere, calculated assuming that the molecule is a globular protein of density 1.35 g cm^{-3} (55). The theoretical value of the diffusion coefficient of BSA in 10% Ficoll 400 was calculated using the Stokes radius of BSA of 3.48 nm (56). The Stokes radius of a dimer was assumed to be double the Stokes radius of a monomer.

Faxen's Law

At distances probed by narrowfield fluorescence microscopy (approximately few hundred nanometers of the coverslip), the boundary effect of increased viscosity in the solution can be modeled by Faxen's law (57):

$$\eta(h) = \eta(\infty) \left(1 + P \left(\frac{r}{h} \right) \right)^{-1}$$

where $\eta(\infty)$ = dynamic laminar-flow viscosity in free solution, r is the radius of the particle, and h is the distance between the boundary and the center of the particle. To a fifth-order approximation:

$$P(\gamma) \approx -\frac{9\gamma}{16} + \left(\frac{\gamma}{8}\right)^3 - \left(\frac{45\gamma}{256}\right)^4 + \left(\frac{\gamma}{16}\right)^5$$

SEC-MALLS of BSA-AF647

The experimental system for SEC-MALLS experiments comprised a Wyatt HELEOS-II multi-angle light scattering detector and a Wyatt rEX refractive index detector attached to a Shimadzu HPLC system (SPD-20A UV detector, LC20-AD isocratic pump system, DGU-20A3 degasser and SIL-20A autosampler). 100 μL of 2.5 mg mL^{-1} BSA-AF647 sample was run at 0.5 mL min^{-1} flow rate at room temperature through superdex S200 columns (G.E. Healthcare) for 60 min in PBS running buffer. Peaks were integrated using Astra V software and the Zimm fit method with degree 1; a value of 0.183 was used for protein refractive index increment (dn/dc).

FCS and FRAP Microscopy

Fluorescence correlation spectroscopy and FRAP experiments were performed on a Zeiss LSM 880 microscope, using a GaAsP detector. Samples were prepared in MatTek glass bottom Petri dishes (1.5 coverglass, MatTek Corporation) and illuminated with a 633-nm wavelength laser.

For FCS the confocal volume was measured using a calibration sample of BSA-AF647 diffusion in water and constraining the diffusion coefficient to be $59 \mu\text{m}^2 \text{ s}^{-1}$ (58); this allowed the structural parameter, s , to be fixed at 6.6. Three repeats of 10 experiments were conducted; traces indicating the presence of multimeric clumps or proximity to the surface were excluded. Autocorrelation traces, $G(\tau)$, to account for transient dark states and translational diffusion were fitted with the following equation:

$$G(\tau) = 1 + \left(1 + \frac{T e^{-\left(\frac{\tau}{\tau_T}\right)}}{1 - T} \right) \left(\frac{1}{V_{\text{eff}} < C >} \left(\frac{1}{1 + \frac{\tau}{\tau_D}} \right) \sqrt{1 + \left(\frac{1}{s} \right)^2 \frac{\tau}{\tau_D}} \right)$$

where T is the triplet fraction, τ_T is the time constant of the dark state, τ_D is the time constant of translation across the confocal volume, V_{eff} , and $< C >$ is the average concentration. The diffusion coefficient, D , was calculated from τ_D via the following equation:

$$D_{FCS} = \frac{r_0^2}{4\tau_D}$$

where r_0 is the spot width (0.322 μm). For FRAP microscopy a square region defined in the Zeiss Zen software was bleached with the 633-nm wavelength focused laser in an axially thin sample that was treated as being 2D. When applied to immobilized fluorophore the shape of the bleached area (see **Figure 4B**) was found to be well approximated as a Gaussian spot of half-width $\omega = 4.9 \pm 0.1 \mu\text{m}$. To measure the diffusion coefficient of BSA-AF647, 30 recovery traces [intensity (I) vs. time (t)] were acquired and fitted in the Zeiss Zen software with the single exponential equation:

$$I = I_0 - I_1 e^{\left(-\frac{t}{\tau_1}\right)}$$

where I_0 is the initial intensity, I_1 is drop in intensity, and τ_1 is the decay constant. Thus, the diffusion coefficient, D , can be calculated as

$$D_{\text{FRAP}} = \frac{\omega^2}{8\tau_1}$$

Single-Molecule Fluorescence Microscopy

Bespoke fluorescence microscopy was performed on an inverted microscope body (Nikon Eclipse Ti-S) with a 100 $\times$ NA 1.49 Nikon oil immersion lens and illumination from a supercontinuum laser (Fianium SC-400-6, Fianium Ltd.), controlled with an acousto-optical tunable filter (AOTF) to produce excitation light centered on wavelength 619 nm (Figure S1 in Supplementary Material). A 633 nm dichroic mirror and 647 nm long-pass emission filter were used beneath the objective lens turret to exclude illumination light from the fluorescence images. The sample was illuminated with narrowfield excitation of 12 μm FWHM and an intensity of 2,300 W cm^{-2} . Images were recorded on an EMCCD camera (860 iXon+, Andor Technology Ltd.) cooled to -80°C . 128 $\times$ 128 pixel images were acquired with 1.98 ms exposure times and 128 $\times$ 29 pixel image strips were acquired with 0.59 ms exposure times, both for 1,000 frames at the full EM and pre-amplifier gains of 300 and 4.6, respectively.

Particle Tracking and Calculation of Diffusion Coefficients

All image data were recorded into .tiff files and analyzed in bespoke Matlab software. Single fluorescent molecules were identified and processed into super-resolution tracks using ADEMS code (41). The microscopic diffusion coefficient was calculated for each tracked particle from the gradient of a linear relation fitted to a plot of the mean square displacements against the four different step time intervals that can be calculated from the first four steps in a track. The microscopic diffusion coefficient distributions comprised an immobile fraction that had non-specifically adhered to the plasma-cleaned coverslip and a diffusive fraction. Microscopic diffusion coefficients were binned into histograms with bin width given by the localization precision of the immobilized (heparan sulfate) data. The probability distribution of diffusion coefficients was modeled by a gamma distribution (45, 59–61):

$$F(x, D, N) = \frac{\left(\frac{N}{D}\right)^N x^{N-1} e^{-\frac{Nx}{D}}}{(N-1)!}$$

where N is the number of independent steps in a track and D is the true diffusion coefficient. The histogram data were fitted iteratively with a two-gamma distribution to account for the mobile and immobile fractions. Initial fitting constraints conserved the number of tracks and assumed the number of independent steps in a track was 4 or less, giving a first estimation of the diffusion coefficients. Then fluorescence microscopy data with the found diffusion coefficients were simulated with and without noise, tracked, and the distribution of diffusion coefficients was fitted with the same constraints as the actual data. Fitting parameters were refined based on the results of fitting to the simulated data, and the experimental data were fitted with the refined constraints. The process of simulating the data, fitting the simulation to refine the constraints, and fitting the experimental data was repeated until the simulation represented the experimental data and the fit to the simulation data converged to the diffusion coefficient values simulated.

For immobilized spots, the N value was less than 1, implying that the steps are not independent. This is expected for immobile molecules as the localization precision uncertainty is larger than the diffusion distance. For mobile spots N was fixed at 2 as there are two steps that do not contain any common localizations when only the first four steps of a track are used.

Simulation of Fluorescence Microscopy Data

Image datasets were simulated in bespoke MATLAB software at given diffusion coefficients using foci intensity, foci spot width, background intensity, and foci density data from real images. Foci are created at random locations in the image frame with intensity randomly chosen from a localization in an experimental dataset. The new positions of a focus in the x - and y -directions after initial generation are each determined from a Gaussian distribution centered on the previous spot location with an SD of the mean square displacement of a particle in one direction, $2Dt$, where D is the simulated diffusion coefficient and t is the time interval between frames. To incorporate photobleaching and other effects causing truncation of trajectories foci were randomly reassigned to a new location 10% of the time; however, diffusion within a frame was not explicitly incorporated into the model beyond the use of the spot width of real localizations. The resulting image stack was used for no-noise simulations. Readout noise in the detector was incorporated in the simulations by the addition of zero mean Gaussian white noise, the intensity of which depended on the local intensity. For tissue data simulation, the mean background level and SD of the noise were taken from control data, away from autofluorescent ECM.

Bootstrapping

Errors on the found values of the diffusion coefficients from FRAP, FCS, and single-molecule tracking were found by bootstrapping (62, 63). In this method, a randomly chosen 80% of the data is fitted in the same way as the entire data set and the SD on each

parameter from ten repeats of this process is taken as the error on each fit parameter found from 100% of the data.

Single-Molecule Imaging in Tissue Sections

Tissue sections were stained with an FITC-conjugated antibody that binds B220 (RA3-6B2, purchased from eBioscience). Tissue sections were subsequently imaged at low (1.2 $\mu\text{m}/\text{pixel}$) magnification with green illumination (wavelength 470 nm (Figure S1 in Supplementary Material), 12- μm FWHM, intensity of 875 W cm^{-2}) to determine the location of the B-cell follicles, before switching to high (120 nm/pixel) magnification and red illumination to image chemokines in these areas.

Segmentation of Tissue Sections Images

Image acquisitions in tissue contain regions of autofluorescent ECM (see Supplementary Note and Figure S5 in Supplementary Material) which are identified by the tracking software. These images must be segmented to identify tracks due to fluorescently labeled chemokine or ECM. Intensity averages of the image acquisition were top hat filtered with a structuring element of radius 4 pixels. The resulting image was converted to binary form using a threshold defined by Otsu's method and the binary image used to enhance the ECM regions of the original image. Small holes in the thresholded region were filled by sequential erosion and dilation with a disk of radius 2 pixels as the structuring element.

Code Availability

All our bespoke software developed is freely and openly accessible via <https://sourceforge.net/projects/york-biophysics/>.

Statistics

Goodness-of-fit values for modeling of the distribution of microscopic diffusion coefficients were evaluated using χ^2 tests as detailed in the text. Experimentally measured stoichiometry distributions were compared against random overlap predictions in a pairwise fashion where appropriate using Pearson's χ^2 test.

ETHICS STATEMENT

All experiments conformed to the ethical principles and guidelines approved by the University of York Institutional and Animal Care Use Committee.

AUTHOR CONTRIBUTIONS

HM built the bespoke fluorescence microscope, overseen by ML. JC prepared biological samples overseen by MC. HM and JC performed the imaging except the confocal microscopy showing binding specificity of CXCL13-AF647 performed and analyzed by ET and ZZ. AW performed the overlap calculations. HM analyzed all other data with input from AW and ML. HM ran simulations of fluorescence data on code adapted from AW. PT oversaw FCS and FRAP microscopy. HM, AW, and ET prepared

the figures with input from all authors. HM, JC, and ML wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

This work was supported by the Biological Physical Sciences Institute (BPSI), MRC grants MR/K01580X/1 (to PT and ML), MC_PC_15073 (MC and ML), and BBSRC grant BB/N006453/1 (AW and ML). JC is supported by a studentship from the Wellcome Trust 4-year PhD programme (WT095024MA): Combating Infectious Disease: Computational Approaches in Translation Science. AW was supported by the Wellcome Trust [ref: 204829] through the Centre for Future Health (CFH) at the University of York, UK. The authors thank Jo Marrison and Andrew Leech (Bioscience Technology Facility, University of York) for technical assistance with FCS and FRAP microscopy, and for SEC-MALLs, respectively, Chris Power (Carl Zeiss Microscopy) for help with FCS, and Anne Theury for providing lymph node tissue sections.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01073/full#supplementary-material>.

VIDEO S1 | BSA-AF647 in PBS buffer. While some particles are tracked, many diffuse further than the tracking radius in each frame, and cannot be tracked. Video after 260 ms of imaging slowed 100x. Tracked molecules are overlaid with a white line, scale bar 1 μm .

VIDEO S2 | BSA-AF647 in 10% Ficoll 400, after 350 ms of imaging, slowed 100x. The increase in viscosity by a factor of 5.6 compared with buffer alone allows the particles to be tracked. Tracked molecules are not overlaid here to allow easier visualization of the actual level of signal and background noise, scale bar 1 μm .

VIDEO S3 | BSA-AF647 in 10% Ficoll 400, after 350 ms of imaging, slowed 100x. The same data as in video 3 is shown, with tracked molecules are overlaid with a white line, scale bar 1 μm .

VIDEO S4 | CCL19-AF647 diffusion in collagen, showing immobile population and stepwise photobleaching, slowed 100x. Tracked molecules are overlaid with a white line, scale bar 1 μm .

VIDEO S5 | CXCL13-AF647 diffusion in collagen, showing members of both the mobile and immobile populations, slowed 100x. Tracked molecules are overlaid with a white line, scale bar 1 μm .

VIDEO S6 | CXCL13-AF647 in lymph node tissue section. Tracks corresponding to both extracellular matrix and mobile chemokine are seen. 100 ms of imaging is shown, slowed 100x. Tracked molecules are overlaid with a white line, scale bar 1 μm .

VIDEO S7 | Heparan sulfate immobilized CCL19-AF647, showing a predominantly immobile population undergoing photoblinking behavior, slowed 100x. The first frames after laser illumination are included. Tracked molecules are overlaid with a white line, scale bar 1 μm .

VIDEO S8 | Heparan sulfate immobilized CXCL13-AF647, showing a predominantly immobile population, slowed 100x. Tracked molecules are overlaid with a white line, scale bar 1 μm .

REFERENCES

- Kienle K, Lämmermann T. Neutrophil swarming: an essential process of the neutrophil tissue response. *Immunol Rev* (2016) 273:76–93. doi:10.1111/imr.12458
- Buckley CD, Barone F, Nayar S, Bénézec C, Caamaño J. Stromal cells in chronic inflammation and tertiary lymphoid organ formation. *Annu Rev Immunol* (2015) 33:715–45. doi:10.1146/annurev-immunol-032713-120252
- Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol* (2006) 7:344–53. doi:10.1038/ni1330
- Pereira JP, Kelly LM, Cyster JG. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int Immunol* (2010) 22:413–9. doi:10.1093/intimm/dxq047
- Dustin ML, Baldari CT. The immune synapse: past, present, and future. In: Baldari C, Dustin M, editors. *The Immune Synapse. Methods in Molecular Biology*. (Vol. 1584), New York, NY: Humana Press (2017). p. 1–5.
- Andrecka J, Ortega Arroyo J, Takagi Y, de Wit G, Fineberg A, MacKinnon L, et al. Structural dynamics of myosin 5 during processive motion revealed by interferometric scattering microscopy. *Elife* (2015) 4:393–414. doi:10.7554/eLife.05413
- Fujiwara T, Ritchie K, Murakoshi H, Jacobson K, Kusumi A. Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J Cell Biol* (2002) 157:1071–81. doi:10.1083/jcb.200202050
- Leake MC. The physics of life: one molecule at a time. *Philos Trans R Soc Lond B Biol Sci* (2013) 368:20120248. doi:10.1098/rstb.2012.0248
- Miller H, Zhou Z, Shepherd J, Wollman AJM, Leake MC. Single-molecule techniques in biophysics: a review of the progress in methods and applications. *Rep Prog Phys* (2018) 81:24601. doi:10.1088/1361-6633/aa8a02
- Piliarik M, Sandoghdar V. Direct optical sensing of single unlabelled proteins and super-resolution imaging of their binding sites. *Nat Commun* (2014) 5:4495. doi:10.1038/ncomms5495
- Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt Lett* (1994) 19:780–2. doi:10.1364/OL.19.000780
- Schneider J, Zahn J, Maglione M, Sigrist SJ, Marquard J, Chojnacki J, et al. Ultrafast, temporally stochastic STED nanoscopy of millisecond dynamics. *Nat Methods* (2015) 12:827–30. doi:10.1038/nmeth.3481
- Balzarotti F, Eilers Y, Gwosch KC, Gynnä AH, Westphal V, Stefani FD, et al. Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Science* (2017) 355:606–12. doi:10.1126/science.aak9913
- Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science* (2006) 313:1642–5. doi:10.1126/science.1127344
- Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nature* (2006) 379:3–5. doi:10.1038/NMETH929
- Gustafsson MG. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J Microsc* (2000) 198:82–7. doi:10.1046/j.1365-2818.2000.00710.x
- Song L, Lu-Walther H-W, Förster R, Jost A, Kielhorn M, Zhou J, et al. Fast structured illumination microscopy using rolling shutter cameras. *Meas Sci Technol* (2016) 27:55401. doi:10.1088/0957-0233/27/5/055401
- Plank M, Wadhams GH, Leake MC. Millisecond timescale slimfield imaging and automated quantification of single fluorescent protein molecules for use in probing complex biological processes. *Integr Biol (Camb)* (2009) 1:602–12. doi:10.1039/b907837a
- Reyes-Lamothe R, Sherratt DJ, Leake MC. Stoichiometry and architecture of active DNA replication machinery in *Escherichia coli*. *Science* (2010) 328:498–501. doi:10.1126/science.1185757
- Juette MF, Bewersdorff J. Three-dimensional tracking of single fluorescent particles with submillisecond temporal resolution. *Nano Lett* (2010) 10:4657–63. doi:10.1021/nl1028792
- Ashley TT, Gan EL, Pan J, Andersson SB. Tracking single fluorescent particles in three dimensions via extremum seeking. *Biomed Opt Express* (2016) 7:3355–76. doi:10.1364/BOE.7.003355
- Hiramoto-Yamaki N, Tanaka KAK, Suzuki KGN, Hirokawa KM, Miyahara MSH, Kalay Z, et al. Ultrafast diffusion of a fluorescent cholesterol analog in compartmentalized plasma membranes. *Traffic* (2014) 15:583–612. doi:10.1111/tra.12163
- Wieser S, Moertelmaier M, Fuertbauer E, Stockinger H, Schütz GJ. (Un)confined diffusion of CD59 in the plasma membrane determined by high-resolution single molecule microscopy. *Biophys J* (2007) 92:3719–28. doi:10.1529/biophysj.106.095398
- van't Hoff M, de Sars V, Oheim M. A programmable light engine for quantitative single molecule TIRF and HILO imaging. *Opt Express* (2008) 16:18495. doi:10.1364/OE.16.018495
- Axelrod D, Koppel DE, Schlessinger J, Elson E, Webb WW. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys J* (1976) 16:1055–69. doi:10.1016/S0006-3495(76)85755-4
- Axelrod D, Ravdin P, Koppel DE, Schlessinger J, Webb WW, Elson EL, et al. Lateral motion of fluorescently labeled acetylcholine receptors in membranes of developing muscle fibers. *Proc Natl Acad Sci U S A* (1976) 73:4594–8. doi:10.1073/pnas.73.12.4594
- Edidin M, Zagayansky Y, Lardner T. Measurement of membrane protein lateral diffusion in single cells. *Science* (1976) 191:466–8. doi:10.1126/science.1246629
- Ehrenberg M, Rigler R. Rotational Brownian motion and fluorescence intensify fluctuations. *Chem Phys* (1974) 4:390–401. doi:10.1016/0301-0104(74)85005-6
- Magde D, Elson E, Webb WW. Thermodynamic fluctuations in a reacting system—measurement by fluorescence correlation spectroscopy. *Phys Rev Lett* (1972) 29:705–8. doi:10.1103/PhysRevLett.29.705
- Adkins EM, Samuvel DJ, Fog JU, Eriksen J, Jayanthi LD, Vaegter CB, et al. Membrane mobility and microdomain association of the dopamine transporter studied with fluorescence correlation spectroscopy and fluorescence recovery after photobleaching. *Biochemistry* (2007) 46:10484–97. doi:10.1021/bi700429z
- Calizo RC, Scarlata S. Discrepancy between fluorescence correlation spectroscopy and fluorescence recovery after photobleaching diffusion measurements of G-protein-coupled receptors. *Anal Biochem* (2013) 440:40–8. doi:10.1016/j.ab.2013.04.033
- Lagerholm BC, Andrade DM, Clausen MP, Eggeling C. Convergence of lateral dynamic measurements in the plasma membrane of live cells from single particle tracking and STED-FCS. *J Phys D Appl Phys* (2017) 50:63001. doi:10.1088/1361-6463/aa519e
- Machán R, Foo YH, Wohland T. On the equivalence of FCS and FRAP: simultaneous lipid membrane measurements. *Biophys J* (2016) 111:152–61. doi:10.1016/j.bpj.2016.06.001
- Guo L, Har JY, Sankaran J, Hong Y, Kannan B, Wohland T. Molecular diffusion measurement in lipid bilayers over wide concentration ranges: a Comparative Study. *Chemphyschem* (2008) 9:721–8. doi:10.1002/cphc.200700611
- Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokines grammar for immune cells. *Annu Rev Immunol* (2004) 22:891–928. doi:10.1146/annurev.immunol.22.012703.104543
- Colditz IG, Schneider MA, Pruenster M, Rot A. Chemokines at large: in-vivo mechanisms of their transport, presentation and clearance. *Thromb Haemost* (2007) 97:688–93. doi:10.1160/TH07-02-0105
- Schulz O, Hammerschmidt SI, Moschovakis GL, Förster R. Chemokines and chemokine receptors in lymphoid tissue dynamics. *Annu Rev Immunol* (2016) 34:203–42. doi:10.1146/annurev-immunol-041015-055649
- Bennett LD, Fox JM, Signoret N. Mechanisms regulating chemokine receptor activity. *Immunology* (2011) 134:246–56. doi:10.1111/j.1365-2567.2011.03485.x
- von Hundelshausen P, Agten SM, Eckardt V, Blanchet X, Schmitt MM, Ippel H, et al. Chemokine interactome mapping enables tailored intervention in acute and chronic inflammation. *Sci Transl Med* (2017) 9. doi:10.1126/scitranslmed.aah6650
- Einstein A. Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhenden Flüssigkeiten suspendierten Teilchen. *Ann Phys* (1905) 322:549–60. doi:10.1002/andp.19053220806
- Miller H, Zhou Z, Wollman AJM, Leake MC. Superresolution imaging of single DNA molecules using stochastic photoblinking of minor groove and intercalating dyes. *Methods* (2015) 88:81–8. doi:10.1016/j.ymeth.2015.01.010
- Robson A, Burrage K, Leake MC. Inferring diffusion in single live cells at the single-molecule level. *Philos Trans R Soc Lond B Biol Sci* (2013) 368:20120029. doi:10.1098/rstb.2012.0029
- Llorente-Garcia I, Lenn T, Erhardt H, Harriman OL, Liu L-N, Robson A, et al. Single-molecule in vivo imaging of bacterial respiratory complexes indicates delocalized oxidative phosphorylation. *Biochim Biophys Acta* (2014) 1837:811–24. doi:10.1016/j.bbabo.2014.01.020

44. Wollman AJ, Shashkova S, Hedlund EG, Friemann R, Hohmann S, Leake MC. Transcription factor clusters regulate genes in eukaryotic cells. *Elife* (2017) 6:e27451. doi:10.7554/eLife.27451
45. Qian H, Sheetz MP, Elson EL. Single particle tracking. Analysis of diffusion and flow in two-dimensional systems. *Biophys J* (1991) 60:910–21. doi:10.1016/S0006-3495(91)82125-7
46. Axelsson I. Characterization of proteins and other macromolecules by agarose gel chromatography. *J Chromatogr A* (1978) 152:21–32. doi:10.1016/S0021-9673(00)85330-3
47. Chen X, Nadiarynh O, Plotnikov S, Campagnola PJ. Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. *Nat Protoc* (2012) 7:654–69. doi:10.1038/nprot.2012.009
48. Monneau YR, Luo L, Sankaranarayanan NV, Nagarajan B, Vivès RR, Baleux F, et al. Solution structure of CXCL13 and heparan sulfate binding show that GAG binding site and cellular signalling rely on distinct domains. *Open Biol* (2017) 7:170133. doi:10.1098/rsob.170133
49. Elowitz MB, Surette MG, Wolf PE, Stock JB, Leibler S. Protein mobility in the cytoplasm of *Escherichia coli*. *J Bacteriol* (1999) 181:197–203.
50. Mullineaux CW, Nenninger A, Ray N, Robinson C. Diffusion of green fluorescent protein in three cell environments in *Escherichia coli*. *J Bacteriol* (2006) 188:3442–8. doi:10.1128/JB.188.10.3442-3448.2006
51. Barnes AL, Genever PG, Rimmer S, Coles MC. Collagen–Poly (N-isopropylacrylamide) hydrogels with tunable properties. *Biomacromolecules* (2016) 17:723–34. doi:10.1021/acs.biomac.5b01251
52. Rashid R, Chee SML, Raghunath M, Wohland T. Macromolecular crowding gives rise to microviscosity, anomalous diffusion and accelerated actin polymerization. *Phys Biol* (2015) 12:34001. doi:10.1088/1478-3975/12/3/034001
53. Cox G, Kable E, Jones A, Fraser I, Manconi F, Gorrell MD. 3-Dimensional imaging of collagen using second harmonic generation. *J Struct Biol* (2003) 141:53–62. doi:10.1016/S1047-8477(02)00576-2
54. Simon Davis DA, Parish CR. Heparan sulfate: a ubiquitous glycosaminoglycan with multiple roles in immunity. *Front Immunol* (2013) 4:470. doi:10.3389/fimmu.2013.00470
55. Fischer H, Polikarpov I, Craievich AF. Average protein density is a molecular-weight-dependent function. *Protein Sci* (2004) 13:2825–8. doi:10.1110/ps.04688204
56. Ikeda S, Nishinari K. Intermolecular forces in bovine serum albumin solutions exhibiting solidlike mechanical behaviors. *Biomacromolecules* (2000) 1:757–63. doi:10.1021/bm005587o
57. Happel J, Brenner H. *Low Reynolds Number Hydrodynamics: With Special Applications to Particulate Media*. Netherlands: Springer (1981).
58. Putnam F. *The Plasma Proteins: Structure, Function, and Genetic Control*. 2nd ed. New York, USA: Academic Press (1975).
59. Saxton MJ. Single-particle tracking: the distribution of diffusion coefficients. *Biophys J* (1997) 72:1744–53. doi:10.1016/S0006-3495(97)78820-9
60. Vrljic M, Nishimura SY, Brasselet S, Moerner WE, McConnell HM. Translational diffusion of individual class II MHC membrane proteins in cells. *Biophys J* (2002) 83:2681–92. doi:10.1016/S0006-3495(02)75277-6
61. Zawadzki P, Stracy M, Ginda K, Zawadzka K, Lesterlin C, Kapanidis AN, et al. The localization and action of topoisomerase IV in *Escherichia coli* chromosome segregation is coordinated by the SMC complex, MukBEF. *Cell Rep* (2015) 13:2587–96. doi:10.1016/j.celrep.2015.11.034
62. Asbury CL, Fehr AN, Block SM. Kinesin moves by an asymmetric hand-over-hand mechanism. *Science* (2003) 302:2130–4. doi:10.1126/science.1092985
63. Efron B, Tibshirani R. *An Introduction to the Bootstrap*. Chapman & Hall (1994).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sailing to and Docking at the Immune Synapse: Role of Tubulin Dynamics and Molecular Motors

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
Science and Technology Facilities
Council, United Kingdom

Reviewed by:

Michael Loran Dustin,
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Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 27 February 2018

Accepted: 11 May 2018

Published: 30 May 2018

Citation:

Martín-Cófreces NB and Sánchez-
Madrid F (2018) Sailing to and
Docking at the Immune Synapse:
Role of Tubulin Dynamics and
Molecular Motors.
Front. Immunol. 9:1174.
doi: 10.3389/fimmu.2018.01174

The different cytoskeleton systems and their connecting molecular motors move vesicles and intracellular organelles to shape cells. Polarized cells with specialized functions display an exquisite spatio-temporal regulation of both cytoskeletal and organelle arrangements that support their specific tasks. In particular, T cells rapidly change their shape and cellular function through the establishment of cell surface and intracellular polarity in response to a variety of cues. This review focuses on the contribution of the microtubule-based dynein/dynactin motor complex, the tubulin and actin cytoskeletons, and different organelles to the formation of the antigen-driven immune synapse.

Keywords: immune synapse, cytoskeleton, T cell receptor, centrosome, dynein, dynactin, microtubule, molecular motor

INTRODUCTION

The immune synapse (IS) is a highly organized structure at the interface between a T cell and an antigen-presenting cell (APC) that is initiated by antigen recognition through the T cell receptor (TCR) and supported by the complex network of cell skeletons (1–3). In particular, the role of tubulin- and actin-based skeletons has been studied on the polarization of intracellular organelles at the IS and the organization of specific adhesion molecules and signaling receptors at the plasma membrane (4–6). Candidates to regulate intracellular traffic and cell organization are the tubulin-based dynein/dynactin molecular motors (7, 8). Various strategies have been used to study this issue, e.g., omics-based techniques (proteomics and lipidomics) upon biochemical extraction and imaging of live or fixed cells through fluorescence and/or electron microscopy. Major issues include

Abbreviations: ADAP/SLAP130/FYB, adhesion and degranulation promoting adaptor protein/SLP-76-associated phospho-protein/fyn-binding protein; AKAP450, A kinase-anchoring protein of 450 kDa; APC, antigen-presenting cell; ATP, adenosine tri-phosphate; CD, cluster of differentiation; CK, casein kinase; CTL, cytotoxic T-lymphocyte; CTLA, cytotoxic T-lymphocyte-associated antigen; DAG, diacylglycerol; DC, dendritic cell; EB1, EB3, end-binding 1 or 3; eNOS, endothelial nitric oxide synthase; FH, formin homology; FIP, arfophilin; FMNL, formin-like protein; ICAM, intercellular adhesion molecule; IFT, intra-flagellar transport; INF2, inverted formin-2; IS, immune synapse; HDAC6, histone deacetylase 6; KIF, kinesin family member; KLC, kinesin light chain; KHC, kinesin heavy chain; LAT, linker for activation of T cells; Lck, leukocyte C-terminal Src kinase; LFA-1, lymphocyte function-associated antigen-1; LIS1, lysencephaly-1 protein; LLSM, lattice light-sheet microscopy; LSCM, laser scanning confocal microscopy; MAP, microtubule-associated protein; mDia, mammalian diaphanous-related-formin; MHC, major histocompatibility complex; MT, microtubule; NDE1, nuclear distribution protein nudE homolog 1; NDEL1, nuclear distribution protein nude-like 1; NO, nitric oxide; PALM, photo activated localization microscopy; PDK, 3'-phosphoinositide-dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; Rac, ras-related C3 botulinum toxin substrate-1; SKAP55, Src kinase-associated phosphoprotein; SNARE, N-ethylmaleimide-sensitive factor attachment protein receptors; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; TCR, T cell receptor; TIRFm, total internal reflection fluorescence microscopy; VAMP, vesicle-associated membrane protein.

determining the molecules that perform their function at the T cell surface, during T cell activation, the components delivered to the cell surface at/or near the IS that sustain or switch off T cell activation and the relevant mechanisms that control their transit to the IS upon activation. Motor protein complexes such as dynein/dynactin lie at the core of these issues. They regulate the movement and positioning of different cellular components, and generate internal forces.

Many studies support the principle of action and reaction processes during IS organization. The scanning of the APC by the T cell through initial adhesive contacts based mostly on lymphocyte function-associated antigen-1 (LFA-1), and the actin reorganization in the T cell impact on the ability of APCs, such as dendritic cells (DCs), to mobilize their intercellular adhesive molecules (ICAM-1 and -3) and subsequently the major histocompatibility complex class II molecules (MHC-II) (9). Recently, LFA-1 activity on T cells has been found to be important for ICAM-1 clustering at the DC, but not for MHC-II. The co-localization of MHC-II and ICAM-1 is mainly abrogated by drugs disrupting the actin cytoskeleton, which reduce MHC-II mobility while increasing ICAM-1 mobility (10). Most of the filamentous actin of a T cell engaged in an IS is found at a highly motile, contractile lamella used by the T cell to interact with the APC (11). The balance between actin filament polymerization and depolymerization establishes a retrograde flow that mediates continuous movement (12). Actin polymerization in filaments at the cell edge and depolymerization near the IS center directs the movement of different surface proteins, such as the TCR/CD3 complex (13). The organization of fluctuating molecules in clusters of different sizes allows the scaffolding of signaling networks that are highly efficient for the transmission of external cues to the intracellular milieu (14–16). All these processes can influence the APC. Forces usually come in pairs (action–reaction). Therefore, since protein-based complexes change their speed upon cell–cell contact, there must be an acceleration, which will depend on the net force applied and the mass of the object. This makes the actions exerted by T cell–APC contacts relevant for changes occurring in both cells. The mass of the objects (protein complexes) involved may change, as well as their ability to interact with large elements (cytoskeleton) that confers a kind of resistance and their capacity to undergo intramolecular changes that make the molecule itself different in terms of intramolecular stiffness or rigidity. All these events will affect the acceleration of the objects. In this regard, a conjugated T cell might not be considered as a “rigid body,” since it is highly plastic and its components change their position and shape, deforming the overall object (T cell). These circumstances confer relevance to every single event on receptors, cytoskeleton, and organelle dynamics during IS formation, therefore pointing to molecular motors.

The IS also behaves as an “active zone” acting as a platform for localized vesicular trafficking (17). This region corresponds to a low-actin area, which allows the microtubule (MT)-mediated transport of endosomes and vesicles toward the pericentrosomal region, near the IS and from there to the plasma membrane (18, 19). Although internalization of TCR/CD3 may occur randomly at any part of the cell surface, recycling is mainly focused to the T cell–APC contact area, leading to the polarized accumulation

of this receptor at the IS (20). The effective membrane traffic is a relevant, quantifiable process, both in resting and activated T cells, for the balance of the cellular localization of very different components, from the TCR to integrins and signaling components, such as kinases and adaptor proteins. It is also important to measure cell degranulation and secretion, as well as to evaluate the compartments dedicated to degradation, such as lysosomal-dependent autophagic or endosomal partitions. Hence, the contribution of lateral membrane motility to the recruitment of TCR/CD3 at the IS is facilitated by an intracellular pool of the complex associated with recycling endosomes to balance metabolic steady state (4). These endosomes are stores of signaling molecules and adaptor proteins and play a role in delivering them to the plasma membrane at the IS (21). The regulation of these internal movements depends on different molecular motors. In this review, we offer a perspective on the molecular players and mechanisms that may be contributing to the internal forces that control organelle positioning and function at the T cell–APC contact area; in particular, dynein/dynactin complexes.

COORDINATION OF ACTIN AND TUBULIN CYTOSKELETONS AT THE T-APC CONTACT AREA

The interplay of actin and MT skeletons with surface receptor complexes coordinates the forces applied on the T cell and those exerted by the T cell (**Figure 1**). A specific correlation between MT and actin areas has been largely analyzed in different cellular systems. It is clear that MTs and actin territories partially overlap. The activation of Rac1 regulates both actin polymerization and MT growth at the leading edge during migration (22). At the IS, MTs growing from the translocated centrosome (18) may benefit from TCR and integrin-mediated activation of Rac1, paralleling the retrograde flux of actin (**Figure 2**). Activated Rac1 collaborates with Rab11 and FIP3 at endosomes to control actin dynamics and tracking forces at the IS (23) and endosomal clathrin coordinates actin polymerization at the same location, thereby controlling T cell activation (24). Since endosomes re-localize near the IS thanks to the centrosomal positioning, it is conceivable that MT skeleton collaborates with actin for sustained tracking forces. This may be done through changes in the MT network by polymerization and depolymerization at plus ends of MTs during centrosomal repositioning and once it is located at the IS (18, 25, 26). However, disruption of Aurora A activity or expression, which reduces MT growing from the polarized centrosome (but not its polarization) and vesicular traffic at the IS, does not prevent actin dynamics in CD4 T cells. Aurora A activates leukocyte C-terminal Src kinase (Lck), a tyrosine-kinase involved in early TCR/CD3 phosphorylation at the plasma membrane, but the blockade of Aurora A does not affect Lck activity enough to prevent the docking of the centrosome in CD4 T cells (27). Lck activity on CD3 ITAMs is required for correct centrosome polarization (28). This cascade of kinases might be rapidly activated by Ca^{2+} influx, since Ca^{2+} promotes immediate Aurora A activation (29). It has been postulated that CD8 T cells require Fyn tyrosine-kinase for centrosome movement and Lck for docking (30). Previous work

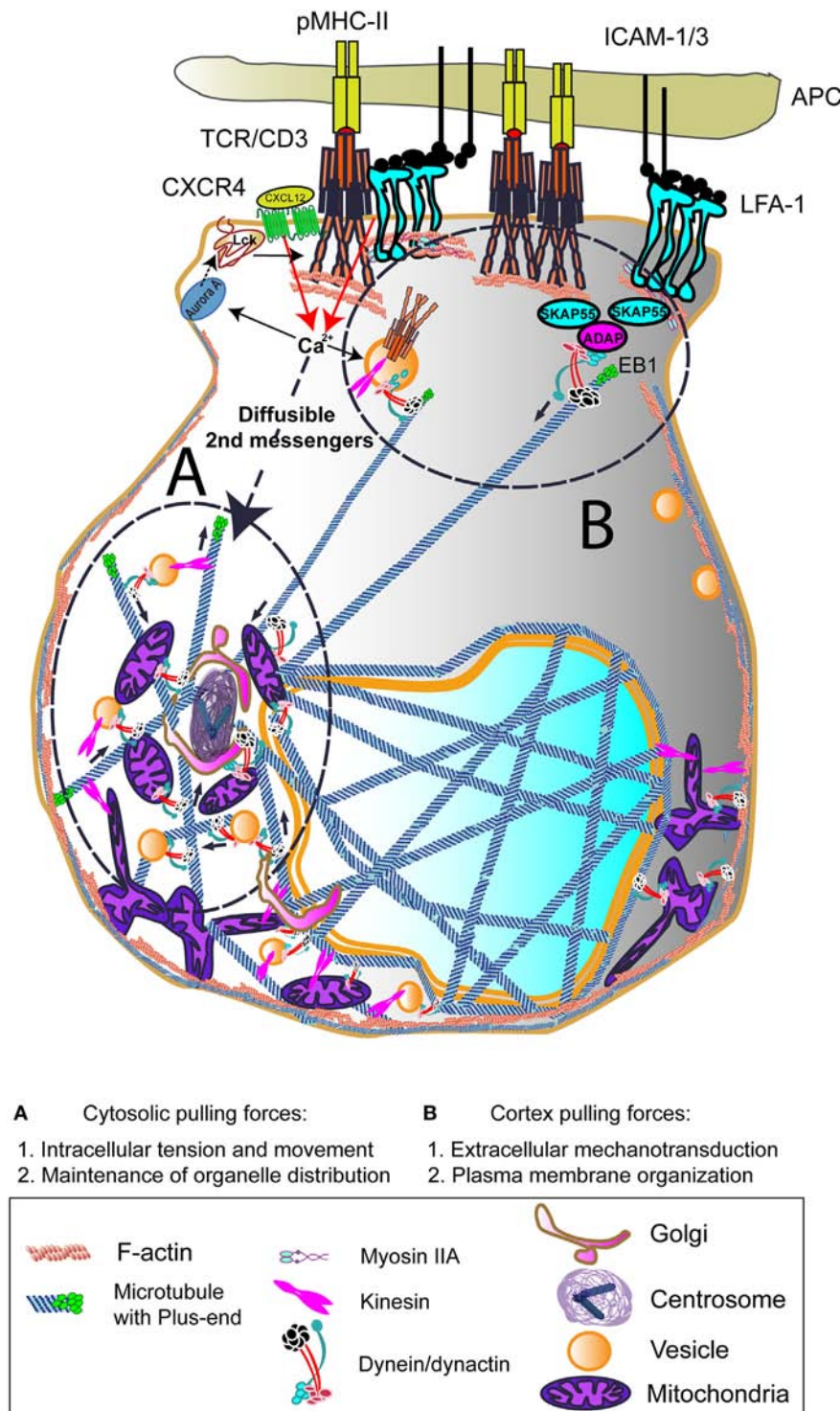


FIGURE 1 | Dynein-driven forces acting on centrosome polarization during early T cell activation. Centrosome polarization upon T cell receptor (TCR) activation can be mediated by cortex and/or cytosolic pulling forces. TCR, lymphocyte function-associated antigen-1 (LFA-1), and CXCR4 binding to its ligands involve rapid increases in Ca^{2+} intracellular influx. This can activate Aurora A, which will favor leukocyte C-terminal Src kinase (Lck) activation and subsequent CD3 ITAMs phosphorylation at the CD3/TCR complex. The activation of the centrosome and associated molecules is probably due to diffusible secondary messengers such as the Ca^{2+} . The interaction of dynein/dynactin complexes and other motors with intracellular organelles and the cytoskeleton may induce the force needed to move the centrosome toward the immune synapse (IS). At the IS, the interaction with TCR/CD3/SKAP55/ADAP or LFA-1/SKAP55/ADAP may serve to dock growing microtubules (MTs) and to pull the centrosome to the IS. End-binding 1 (EB1) may interact directly to CD3 ζ subunit of the CD3/TCR complex. The growth and shrinkage of MTs at this zone would also create pulling forces. The images in the figure are not scaled.

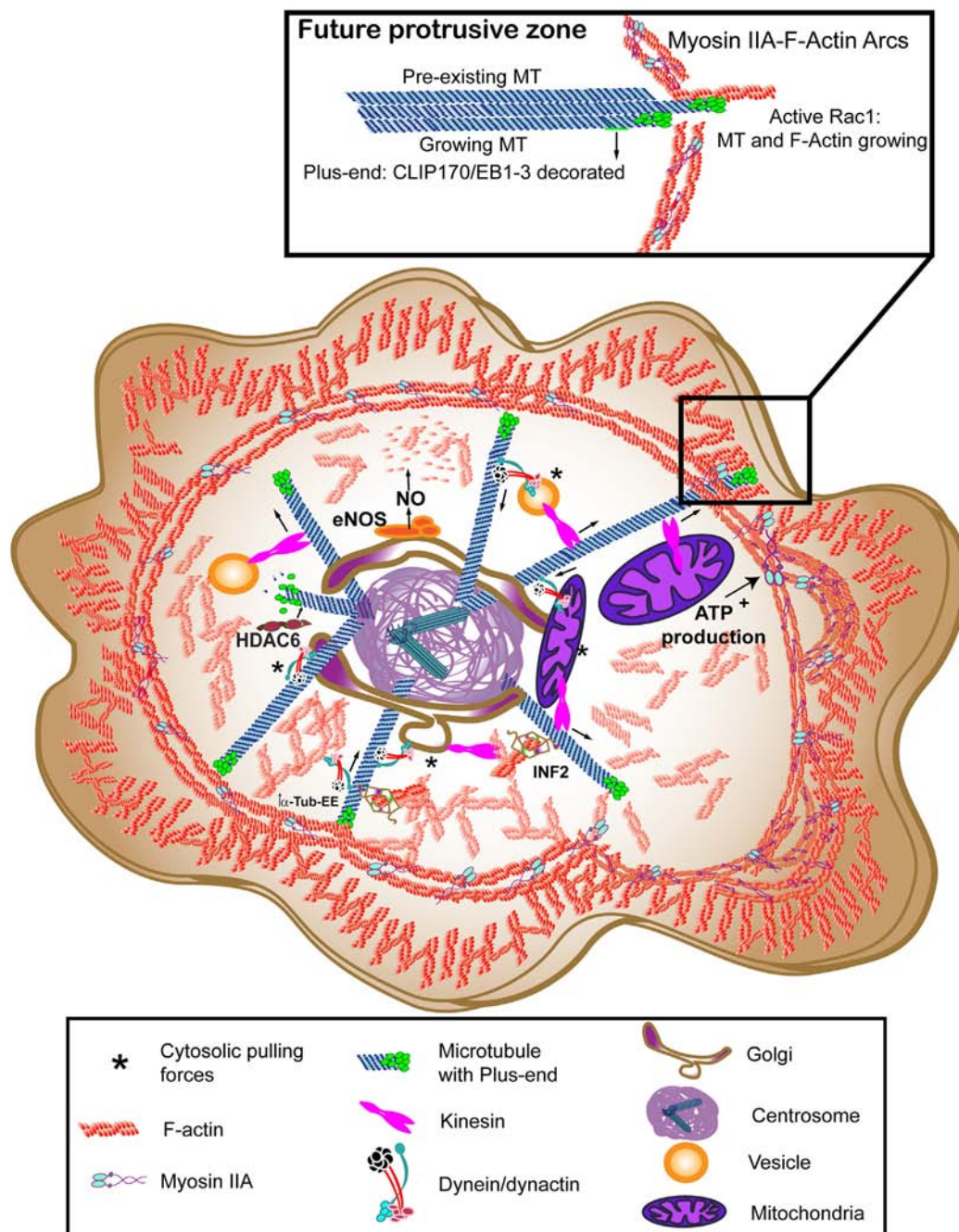


FIGURE 2 | Molecular motors at motion to rearrange the cytoskeleton at the immune synapse (IS). Myosin IIA provides the lymphocyte function-associated antigen-1 (LFA-1)-dependent actin ring with contractile activity, thereby helping the centripetal movement of surface proteins. Dynein/dynactin may interact directly with receptors or move vesicles to allow recycling, walking toward the minus-end of microtubules (MTs) (centrosome). Kinesin-1 helps the traffic from the centrosome to the periphery. Vesicular traffic allows secretion and mitochondria can provide the adenosine tri-phosphate (ATP) needed for Myosin IIA activation. The forces exerted by these motors between the organelles and the cytoskeleton constitute the cytosolic pulling forces, that may provide a docking mechanism for the centrosome. The translocated centrosome provides the IS with multiple signaling, scaffold and modifying proteins that can regulate relevant post-translational modifications (PTMs) for actin or tubulin cytoskeletons, such as endothelial nitric oxide synthase (eNOS) for β -actin nitrosylation, histone deacetylase 6 (HDAC6) for deacetylation, or inverted formin-2 (INF2) to allow detyrosination of MTs. The images in the figure are not scaled.

showed that Fyn is relevant for centrosomal polarization mainly in the absence of Lck. Full localization of centrosome at the IS is prevented in CD8 Fyn-deficient T cells when anti-CD3 ϵ -coated

beads are used. Moreover, Fyn-deficient OTI CD8 cells show maximal inhibition of centrosomal polarization under low stimulation conditions, such as partial agonist and antagonist peptides,

but only mild effect when activated with agonist peptide (31). Thereby, other elements involved in the activation of the T cell, such as LFA-1 integrin and MHC-TCR interaction may direct the complete stimulation of centrosomal polarization.

A dual action of the centrosome on actin dynamics may exist. On the one hand, it can provide positive regulators from the associated Golgi apparatus and secretory machinery to increase cell–cell adhesion. On the other hand, negative regulators of actin polymerization such as nitric oxide produced by endothelial nitric oxide synthase (eNOS) (32) can help the fine-tuning of actin dynamics to prepare the clearance of actin structures at the center of the contact for secretion. The final recovery of actin at the IS and the switch off of the activated T cell can be achieved through the delivery of negative regulators, such as cytotoxic T-lymphocyte-associated antigen 4, to the IS from the endosomal associated systems (33). The organization of the polar retrograde flux of actin in the geometrical shape generated at the interface between the T cell and the APC generates a low-viscosity “sink” for inward flow of signaling microcluster in the T cell (34). There, actin regulators probably cluster, such as cofilin, profilin, and coronin. Golgi-resident eNOS coordinates centrosomal positioning at the IS with actin dynamics by decreasing the actin retrograde flux through modification of the actin binding to profilin by β -actin nitrosylation (32). Profilin is a major actin-binding protein in different cells (35), which makes it a predominant substrate for actin polymerization. Recently, two independent studies have demonstrated its collaboration with Ena/VASP complex and with formins to organize actin polymerization rather than Arp2/3 complex. Through cooperation with profilin, actin increases its ratio of incorporation to formin-bound filaments and helps Ena/VASP complex to elongate the distal lamellipodia (36, 37). The ability of different formins to increase actin polymerization can also help the initiation of finger-like protrusions at the plasma membrane in coordination with lamellipodia extension. FMLN3 formin, in cooperation with mammal diaphanous-related-formin (mDia)2, favors this process; however, this is not the case for FMLN2 (38). FMLN1 and mDia1 targeting showed no effect on filopodia formation and actin accumulation during T cell interaction with APC. The knockdown of either Arp2 or Arp3 converted the lamellipodia-based scanning of the APC into a filopodia-based interaction (39). It has been described that the formation of Arp2/3-dependent F-actin foci at TCR microclusters at the IS may facilitate the formation of protrusions toward the APC (40). Filopodia—also referred as microvilli or microspikes—seem relevant to the initial scan of the APC by the T cell through a tyrosine kinase- and actin-independent TCR–pMHC interaction (41). Therefore, the actin organization upon activation relies largely in Arp2/3 and formin activities. Recently, FRAP analysis of the cell cortex of T cells has determined the presence of two F-actin subsets: formin-nucleated, long filaments of about 500 nm showing long turnover times and Arp2/3-nucleated, short filaments of 50 nm with fast turnover times and actin free barbed ends. Also, Arp2/3 activity was more prominent than formin upon TCR activation, but the formin activity endorsed longer filaments on the external lamellipodia. The use of both super-resolution stimulated emission depletion (STED) microscopy and lattice light-sheet microscopy (LLSM), allowed the

identification of a more internal network of actin at the IS, different from the lamellipodia and complementary to it, that may help the intracellular traffic (42). In this regard, profilin ability to bind formins can help the interconnection between F-actin and MTs, as formins can bind simultaneously both elements through their formin homology (FH)1 and FH2 domains (43), which may be also a relevant mechanism to generate protrusions at the plasma membrane (see **Figure 2**).

Microtubules are polymers of α - and β -tubulin heterodimers bound in a head-to-tail manner. This organization gives rise to MT polarity, with plus- and minus-ends, depending on their rate of polymerization (44). The conventional formin mDia has been shown to bind end-binding 1 (EB1), CLIP170, and APC, a group of proteins involved in the growth of MTs at their plus-ends, or tips (43). mDia-deficient mouse presents different alterations in T cell development and activation (45). Indeed, Arp2/3 accounts for TCR recycling and cell–cell adhesion in conjugates, without affecting centrosomal positioning, whereas mDia and formin-like protein 1, two canonical formins, affect centrosomal localization. Their effect on actin is antagonistic (39). Inverted formin-2 (INF2), a non-canonical formin, regulates the centrosome translocation, but does not seem to affect actin during IS in T cells (46). At any rate, Rac1 activity seems relevant for centrosomal polarization to the IS (39, 46). Both actin exclusion and centrosome recruitment at the center of the IS, together with the vesicle and secretory machinery of the cell, allow the correct signaling and recycling of receptor microclusters and also focus secretion (6, 47, 48). In cytotoxic T-lymphocytes (CTLs), the secretion of lytic granules recruited to the polarized centrosome at the target cell area (49) is facilitated by the clearance of central cortical actin in coordination with calcium influx (50, 51). On the other hand, local clearance of actin at the site of docking and delivery of the granules has been defined in the killing immunological synapse organized by NK cells. This effect is dependent on the action of Coronin 1A, a protein able to interact with F-actin and MTs that uses Arp2/3 to destabilize F-actin (52, 53). Coronin 1A seems to be dispensable in T cells for antigen-recognition events, but not for migration (54). To fulfill their ability to engage different target cells serially, CTLs seem to recover cortical actin upon secretion, thereby stopping this process (55). The role of the centrosome in driving the localization of the lytic granules at the target cell area, and the possible role of centrosome-associated, Golgi-resident eNOS in actin clearance point to a role of tubulin skeleton in fine-tuning actin-based cytoskeleton dynamics.

DYNEIN MOTORS SHAPE THE IS

Experimental evidence on this issue arises from the observation of the polarization of the cytoskeleton at the IS and the associated changes of intracellular organization. In the T cell, the centrosome, together with the Golgi apparatus, secretory and recycling machinery and mitochondria, localize at the IS. An active growth of MTs from the centrosomal area organizes an MT network that helps traffic at the IS (56). An array of molecular motors is able to walk between the two ends of MTs, transporting different organelles along these trails. Also, actin-based molecular motors can act on actin structures to increase tracking forces at

the IS, probably helping movement. These molecular motors are important for cell polarity and are mostly represented by dyneins, kinesins, and myosins (**Figures 1 and 2**).

Cytoplasmic dynein complex belongs to a large family of MT motor proteins involved in intracellular transport; it is an MT minus-end directed, motor protein complex of about 1 MDa, that comprises two heavy chains containing the AAA motor and MT binding site, two different intermediate chains, among them the p74 subunit (DIC) and several light and intermediate light chains. These chains provide interaction with a plethora of proteins in cells, conferring to the motor the ability to interact with multiple cargoes. p74 interacts with dynactin p150^{Glued} subunit, and p150^{Glued} is able to interact with MT through its first 200 amino acid residues at the N-terminus CAP-Gly (cytoskeleton-associated protein glycine rich) domain and a basic region. Dynactin complex enhances dynein processivity while regulating its localization (57, 58). Cytoplasmic dynein accumulates at the periphery of the T-APC contact and associates with ADAP (adhesion and degranulation promoting adaptor protein; SLAP130/Fyb) (59), as does dynactin (60). This interaction may generate the pulling force needed to polarize the centrosome to the IS; e.g., cortex pulling forces (**Figure 1**). These cortex pulling forces would be exerted from the ring of activated LFA-1 integrin, favored by ADAP upon TCR activation. Accordingly, the knockout mouse for ADAP shows deficient T cell LFA-1-mediated adhesion (61, 62). Although actin polymerization is normal in the knockout cells, the absence of ADAP is essential for T cell proliferation and adhesion. Indeed, ADAP is a scaffold protein that connects to SKAP55 and regulates its stability and half-life by preventing its degradation at the proteasome (63). SKAP55 connects with the actin cytoskeleton and its deficiency causes an effect similar to that of ADAP (64). Therefore, it is conceivable that, through connection with ADAP, cytoplasmic dynein might exert a regulatory role on interconnecting MTs and cortical actin at the IS, producing the pulling forces at the cortex. The disruption of the dynein/dynactin complex de-localized LFA-1 from the external zone of the IS, showing a scattered pattern (60). In these conditions, the centrosome was not polarized at the IS, without affecting the number of conjugates formed with APCs. Indeed, dynein has also been proposed to move TCR/CD3 complexes along MT toward the center of the IS, enhancing their motility and signal termination in mouse cells (65). Coordinated dynein/dynactin activity was also found essential for sustained T cell activation, based on centrosome polarization (60).

Cytoplasmic pulling forces are now matter of study and exemplify a mechanism *via* dynein/dynactin complexes to generate traction during intracellular transport and cell shape maintenance (**Figure 1**). Cytoplasmic pulling forces are based on the net forces applied by molecular motors to get together the components of organelles such as the Golgi, as well as the traction exerted on skeletons for movement. Dynein/dynactin-mediated cytosolic pulling forces may be relevant for the localization of the centrosome, given the high number of organelles and vesicles which are interconnected by MTs around it, and their proximity to the IS (24, 66). The study of large protein complexes in cells is difficult due to the high number of subunits and the ability of cells to compensate some effects when protein complexes are disturbed or the protein expression of their subunits diminished. In the case of dynein/

dynactin, either the silencing of cytoplasmic dynein heavy chain 1 or a high overexpression of the p50-dynamin-GFP subunit of dynactin in human T cells prevented the correct polarization of the centrosome. A sustained, long-term overexpression of p50-dynamin-GFP [obtaining a ratio of more than 4:1 for p50-dynamin:p150^{Glued} proportions in the dynactin complex (67, 68)] in Jurkat cells prevented the interaction between p74-dynein intermediate chain and p150^{Glued}. This effect correlated with a dispersed localization of the TCR, as well as with a de-localized centrosomal positioning (60). A recent study shows that dynein motor, which can form different complexes in cells by changing its partners, may play a dual role in T cell activation, depending on whether the interaction is with nuclear distribution protein nudE homolog 1 (NDE1) or p150^{Glued} (69). NDE1 protein is involved in the intracellular organization of the Golgi through interaction with nuclear distribution protein nude-like 1 (NDEL1), lysen-cephaly-1 protein, and dynein; silencing of NDE1 and NDEL1 disorganizes the Golgi, makes the endocytic compartment collapse toward the plasma membrane and abrogates cortical dynein localization (70). The palmitoylation of either NDE1 or NDEL1 prevents interaction with dynein and intracellular traffic (71), thereby pointing to a relevant spatial mechanism to regulate dynein complexes composition and action. In this regard, the silencing of p150^{Glued} does not seem to exert an effect on centrosome localization at the IS in this study (69). Other authors have observed that the direct knockdown of dynein heavy chain does not affect the translocation of the centrosome in mouse cells (65). However, a number of studies support dynein/dynactin role in centrosome polarization in lymphocytes (25, 60, 69, 72, 73).

The full deletion of p150 or *Glued* is lethal early in embryo development in *D. melanogaster*. Genetic experiments to analyze the survival of deficient cells in wild-type adult tissues were unable to recapitulate the cell functionality (74). This indicates that p150^{Glued} is essential for cells to survive, divide, or participate in tissue-level organization, although dynactin complex is not required for dynein sustained motility (75). Therefore, a partial silencing of p150^{Glued} would allow the initial activity of some dynein/dynactin complexes, without major requirements for later movement and generation of pulling forces, but with a high replacement/interchange rate of dynactin between complexes. Dynein/dynactin supercomplex has a definitive different behavior in the use of different MTs as tracks, which can be due to the different post-translational modifications (PTMs) of tubulin (**Figure 3**); partly through the action of different carboxipeptidases at α -tubulin ends (α -Tub-EEY) (6, 76). Dynein can move on detyrosinated MTs once the movement is initiated without the participation of dynactin. The movement of centrosomes in *C. elegans* embryos depends on the interaction of dynactin with tyrosinated MTs, the cytoplasmic pulling forces exerted through its binding to dynein complex and the initiation of intracellular traffic (77). Also, dynactin interacts preferentially with tyrosinated MTs through p150^{Glued} or with the EEY-ends of end-binding (EB) proteins bound to MTs (75). The formin INF2 regulates the tyrosinated state of MTs in T cells during activation; MTs near the translocated centrosome are detyrosinated (α -Tub-EE) and TCR activation promotes the increase of detyrosinated MTs (46). A possibility is that dynactin would help dynein to initiate

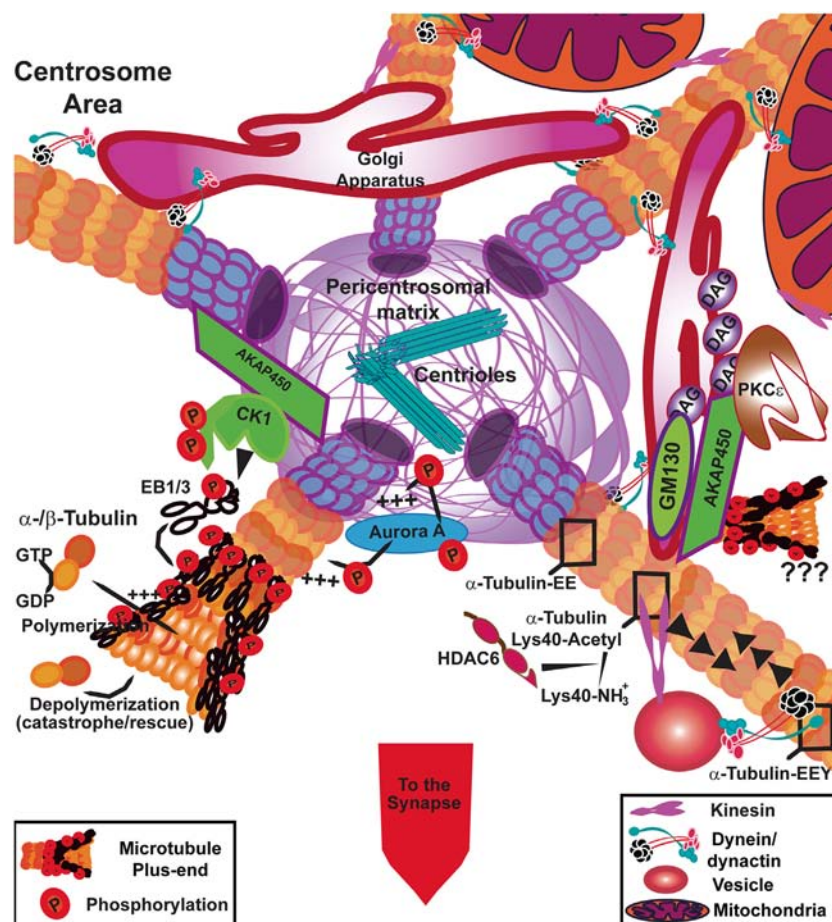


FIGURE 3 | Signaling at the centrosome area to fuel tubulin polymerization. In T cells, the polymerization of microtubules (MTs) from the centrosome is controlled by casein kinase Iδ (CK1δ) through phosphorylation of end-binding 1 (EB1). AKAP450 anchors CK1 to the pericentrosomal matrix. Aurora A also promotes the incorporation of α/β -tubulin heterodimers into MTs at the centrosome through its kinase activity. AKAP450 can also dock at the Golgi apparatus where it may collaborate with GM130 to facilitate tubulin polymerization. The Golgi apparatus is formed by diacylglycerol (DAG)-enriched membranes, where protein kinase C (PKC) ϵ anchors. AKAP450 binds to hypophosphorylated PKC ϵ , which can constitute a reservoir for the non-activated kinase. The post-translational modifications of the MTs can affect the binding of molecular motors; kinesin likely interacts preferentially with acetylated MTs and dynein/dynactin interact with tyrosinated MT (α -tubulin-EEY) through dynactin binding, although dynein can move along deetyrosinated (α -tubulin-EE) and tyrosinated MTs. Both motors are in charge of movement around the centrosomal area, of organelles such as vesicles and mitochondria, whereas dynein is the main responsible for Golgi apparatus shape and stability. The images in the figure are not scaled.

its processive movement to transport cargoes on tyrosinated MTs until the area of deetyrosinated MTs near the centrosome is reached. Alternatively, dynactin can use EB1 or EB3 at the plus-ends of MTs. Conceivably, high inhibition of dynein/dynein interaction by sustained overexpression of p50-dynamitin or complete knockdown of p150^{Glued} would affect dynein initial interaction with MTs, preventing intracellular traffic and localization of the centrosome at the IS and the organization of organelles due to lack of cytosolic pulling forces.

THE IS AND ITS AXONEMAL CONNECTIONS

The connection between IS and axonemal components is being established. Axonemal dynein is very important to allow the

movement of flagella, based on its interaction with the axonemal MTs and its AAA motor activity; a “coup de force” (57, 58), which may be also a possible mechanism at the IS. During the biogenesis of the cilium, including centrosomal and acentrosomal processes, the basal body connects to cenexin-centriolin-Rab11a-Rabin8-Rab8 complex and organizes the retrograde and anterograde transport of vesicles along MTs through dynein and kinesin, respectively. These molecules also act at the IS; Rab8 and vesicle-associated membrane protein (VAMP)3 complex regulates recycling of TCRs (78). Rab8a and Rab11a dissociate from the pericentriolar region by casein kinase 1 (CK1δ) action (79). The centrosomal docking of CK1δ is mediated by AKAP450 (80) (Figure 3) and is needed to form the basal body of the primary cilium. In CD4 T cells, AKAP450 inhibition delocalizes the centrosome from the IS and decreases TCR and integrin activation and clustering (81). In other cell types, AKAP450 has

an important role in MT polymerization from the centrosome and through the Golgi mediated by interaction with GM130 (82). Therefore, it can be also of relevance for cytosolic pulling forces. CK1 δ silencing causes a 50% reduction in the centrosomal positioning at the IS in Jurkat cells. CK1 δ forms a complex with EB1, phosphorylates it, and activates its function. It might interact with dynein/dynactin directly or through EB1, but these can represent different complexes recovered by co-immunoprecipitation and EB1-GST pulldown, respectively. CK1 δ phosphorylation of EB1 can activate the protein and promote MT growing from the centrosome (**Figure 3**). This effect is dependent on the dynamics of CK1 δ localization at the centrosome since its persistent localization at this organelle prevents the correct centrosomal polarization at the IS (83). The deletion of EB1 does not prevent centrosomal positioning at the IS, but abrogates TCR signaling at linker for activation of T cells (LAT)/phospholipase C (PLC) γ 1 signalosome and regulates the traffic of CD3 ζ vesicles at the IS (18). EB3, which is also expressed in T cells, could replace EB1 in allowing centrosomal positioning, since overexpression of EB1-CT as a dominant negative mutant for EB1 protein–protein interactions has an effect on centrosomal positioning (39, 84). Polymerization of MTs has been described to be important for centrosomal polarization, assayed through the use of low doses of nocodazol (25). In this study, the centrosomal relocation at the IS was defined through two different phases with different mean speeds: a first one to position the centrosome near the synapse and a second one to center and dock it. In this work, the silencing or chemical inhibition of dynein with ciliobrevin D and overexpression of a dominant negative mutant of p150^{Glued} had a negative effect on centrosomal positioning and docking (25), corroborating previous results (60). Major effects on both phases were observed upon taxol and ciliobrevin D treatment to prevent depolymerization of MTs and dynein-driven force. Taxol alone prevented partly the repositioning, as did low doses of nocodazol (loss of polymerization); therefore, a kind of internal “scanning” of the cell cortex from MT plus-ends in collaboration with dynein/dynactin was proposed as a model for docking the centrosome at the IS (25).

Acetylation, a PTM of α -tubulin, is a hallmark of stable MTs which is also detected in the cilium. HDAC6, a histone deacetylase with activity on α -tubulin or cortactin (85), is able to interact with dynein to transport unfolded proteins (86) and is also important for lymphocyte migration as a scaffold protein (87). Its role in migration in other cell types is linked to EB1 protein (88). Notably, HDAC6, which also has a role in the cilium disassembly under the control of Aurora A (89), influences CD4⁺ T cell activation at the IS, since its overexpression precludes centrosome positioning and the interaction of important signaling molecules from the TCR pathways with MTs. HDAC6-silenced CD4 T cells showed a similar hyper-acetylation of tubulin than taxol-treated cells and even higher centrosome polarization than control cells (90). Likewise, in HDAC6-deficient CD8 T cells, the polarization of the centrosome is also higher than in control cells (it is closer to the IS) and tubulin acetylation is increased (91). Taxane (paclitaxel and docetaxel) binding to MTs was mapped to the β -tubulin subunit on the MT inner surface (92); the initially accepted model proposed that taxanes and other MT-stabilizing

agents reach the binding pocket by diffusing through the MT wall. However, the kinetics of binding determined that diffusion could not account for the process (93). More recently, the application of different computational techniques to the MT structure showed that a possible external binding pocket would allow an initial binding and later the entry of the drugs (94). Therefore, depending on the amount of drug present, taxol may be affecting the binding of different microtubule-associated proteins (MAPs) at both surfaces of the MT. This may account for the different consequences of taxol treatment depending on the amount of drug used (from 1 to 20 nM; minutes to hours), as known for stimulation of MT growth *in vitro* (95), formation of MT bundles due to high polymerization and stabilization (96) and cell death and mitotic inhibition (97). In the work of Yi et al., the concentration of taxane used did not seem to provoke great changes in the overall shape of MT skeleton (pre-treatment with 0.5 μ M for 10 min and then, stimulation), but prevented catastrophes, and therefore stabilized MT growth. They observed a defect in both phases: repositioning and docking. The combined treatment with ciliobrevin D and taxane produced the major effect, blocking centrosome movement. Indeed, inhibition of dynein with ciliobrevin D promoted the disorganization of intracellular organelles and vesicles (25). In sum, treatment with taxanes initially promotes an ever-growing MT skeleton, depending on the dose and time of treatment, with a resulting paralyzed skeleton and high acetylation of MTs. In addition, the inhibition of molecular motors such as dynein/dynactin prevents correct organellar disposition. This may have differential consequences on the activity of molecular motors both on cytosolic and cortical pulling forces, depending on the status of the MT cytoskeleton, the organelle positioning, and the interaction with cortical surfaces.

MOTORING THE SYNAPTIC ORGANELLES TO FUEL CYTOSKELETAL DYNAMICS

Mitochondria localize at the IS (98) and this localization is also dependent on the centrosome polarization to the IS, since mitochondria are accumulated around the de-localized centrosome and perinuclear region in T cells overexpressing p50-dynamitin (72). The perinuclear localization of mitochondria upon p50-dynamitin-GFP overexpression was primarily observed in Hela cells. The recruitment of dynamin-related protein 1 (drp1), a protein involved in fission of mitochondria, to the dynactin/dynein complex was shown to sustain the retrograde transport. The size and shape of mitochondria was irregular in these cells, with some of them presenting T- and V-shapes (99). Drp1 helps the correct localization of mitochondria at the uropod, the trailing edge of migrating polarized lymphocytes, prior to stimulation to form an IS. Through mitochondria localization at the uropod, the lymphocyte regulates its ability to migrate (100). These mitochondria surround the centrosomal area, and can provide adenosine tri-phosphate (ATP) to the intracellular traffic for LFA-1 recycling and Myosin II contraction needed to sustain the motility and polarity of the lymphocyte (101). Upon TCR activation, drp1-disrupted T cells allowed the centrosomal

localization during IS formation, but mitochondria were not correctly polarized to the IS. There was no effective movement of the mitochondria toward the minus-end of MTs. The required ATP production for energy fueling at the IS was also diminished (72). The effect of drp1 delocalization from mitochondria upon p50-overexpression may also be due to a defective fission of mitochondria, which are then poorly transported in an anterograde mode by kinesin-1.

Kinesin-1, also called conventional kinesin, is a MT plus-end-oriented motor complex with different subunits. Kinesin-1 is a heterotetramer of ~380 kDa and comprises two kinesin heavy chains (KHCs) with motor activity and two kinesin light chains (102). It is important to note that kinesins are an extended family of proteins, with about 45 genes coding for them. Most of them [kinesin family members (KIFs)] have their motor domain at the N-terminus, but also as a central domain or at the C-terminus, determining whether they walk toward the plus-ends (N-KIF) or the minus-end (C-KIF) of the MT (103). Kinesin-1, an N-KIF, uses adaptor proteins to fix the cargo; for mitochondria effective movement forward to the cell cortex (MT plus-ends), Miro-1 forms a triade with Milton and kinesin-1 (104). The role of Miro-1 protein has been reported in the localization of mitochondria during T cell–endothelial contact for transmigration from blood vessels to tissues during inflammation. Miro-1 is needed to relocate the mitochondria around the centrosome, which is recruited from the uropod (trailing edge) to the T cell–endothelial cell contact area and congregates the mitochondria there (73). Miro-1 interacts with the dynactin subunit p150^{Glued} and dynein heavy chain in these lymphocytes, but the possible complex formed with kinesin-1 was not explored. Therefore, motors might coordinately interchange at the surface of the cargoes, to regulate the retrograde and anterograde transport through MT, using the centrosome as a crossroads.

Kinesin-1 is indeed involved in the final transport and delivery of lytic granules at the killing IS in CTLs, forming a complex with Slp3 and Rab27a (105). In fact, the knockout mouse for *Kif5b*, the KHC involved, is embryonic lethal, showing perinuclear clustering of lysosomes and mitochondria. Kinesin-1 is helped in the transport of lytic granules to the IS by the action of HDAC6 (91). In HDAC6 knockout T cells, the acetylation of MTs is highly increased, but the centrosomal polarization to the IS of either silenced CD4 or knockout CD8 T cells was even enhanced (90, 91). Kinesin-1 ability to bind and move over MT is increased by acetylation at Lys40 of α -tubulin in the lumen of MTs (106). This is in concert with the long-term increase in acetylated MTs at the IS (90) and would facilitate the kinesin-driven movement of vesicles from centrosomal region to the plasma membrane at the IS. Indeed, the use of cell-extracts of intact MT networks and single fluorescently labeled motor proteins to study motility through total internal reflection fluorescence microscopy (TIRFm) unveiled that acetylated MTs are predominantly bundled, which enhances the number of kinesin binding sites and run lengths of the motor (107). However, in the case of HDAC6 knockout CD8 T cells, the kinesin-1 interaction with p150^{Glued} was defective, correlating with a defect in the final delivery of lytic granules at the IS and their degranulation, even though acetylation of MTs was highly increased (91).

Histone deacetylase 6 may also play a role in the biogenesis and degradation of organelles through interaction with dynein/dynactin. These proteins are well-known partners for the transport of ubiquitinated, misfolded proteins to the aggresome formed near the centrosome for degradation through autophagy. Its interaction with dynein/dynactin takes part through a region different from its two catalytic domains for acetylation and its C-terminus (86). Parkin coordinates the E2 enzyme UbcH13/Uev1a to mediate K63-linked polyubiquitination of misfolded proteins (108). Under conditions of proteasomal impairment, the machinery and membranes for autophagosome are recruited to the aggresome and the fusion with lysosomes allows protein clearance. Indeed, the recruitment of Parkin to the centrosome in these conditions is dependent on HDAC6. This accumulation was reversible and HDAC6 used either dynein or kinesin-1 for bidirectional movements (109). HDAC6 binds preferentially to K63-ubiquitin modified proteins (instead of K48) through its ubiquitin-binding domain, at the C-terminal (110). It may bind both mono or polyubiquitin chains (111, 112), although it seems that it prefers polyubiquitinated chains (108). This precise relationship between HDAC6 and the dynein motor is likely to be of relevance for mitochondrial shape and health, since HDAC6 and Parkin are both involved in the process of mitophagy (56). Therefore, the possible connections in the cytosolic and cortex pulling forces generated by dynein and their relationship with kinesin complexes and their ability to interconnect organelles and to move components inside the cell is a field for intense research. In this context, the MTs, their PTMs, and enzymatic modifiers will be extremely relevant.

Myosins are a superfamily of motor proteins that bind to actin and use the energy of ATP hydrolysis to generate force and movement along actin filaments. There are about 18 classes of myosins. They play significant roles in cell movement, muscle contraction, cytokinesis, membrane trafficking, and signal transduction (113). They consist of a motor domain, a neck region, and a tail region; most myosins form a dimer of two heavy chains with the supplementary binding of two light chains (MLC) per heavy chain to their neck region. Regulatory MLCs can be phosphorylated for regulation of the motor activity (103). Non-muscle myosin IIA (encoded by gene *Myh9*) has been involved in the accumulation of the TCR to the center of the IS to be recycled (114). The lack of mitochondria polarization at the IS by inhibition of drp1 prevented MLC phosphorylation at Ser19 at the actin-rich lamella, thereby unleashing TCRs from the retrograde flow, which showed a less concentrated appearance. However, the centrosome was correctly positioned at the IS (72). The collaboration between myosin IIA and dynein has been recently shown in mouse cells through the use of photoactivatable peptide-MHC complexes. This evidence supports the action of Myosin IIA in pushing the centrosome toward the IS while dynein would pull it from the IS. The inhibition of each one separately did not exert apparent high effects on centrosomal positioning in this study. Indeed, inhibition of Myosin IIA did not alter the signaling from the TCR (115). Dynein localization at the plasma membrane has been suggested to precede the centrosome polarization at the IS rapidly upon TCR activation. The gradient of diacylglycerol (DAG) organized at the IS by active PLC γ 1 would be the polarizing signal to direct

centrosomal localization (116). In this sense, the MAP4 knock-down reduces the stability of MTs and makes the centrosome to move slower until it reaches the IS, although PLC γ 1 is more active and DAG accumulation increases at the IS. It is feasible that centrosomal polarization acts as a negative regulator for DAG production. DAG accumulation at the IS is also observed when Ct-AKAP450-GFP is overexpressed in T cells (117), a construct that displaces AKAP450 from the centrosome and prevents its translocation to the IS (81). Indeed, if DAG production is disturbed, the centrosome does not position correctly (116). To control centrosomal positioning, there is a specific and temporal recruitment of three different protein kinases C (PKCs) to the IS; essentially PKC ϵ and PKC η come first followed by PKC θ (118). All of them bind to DAG and phorbol esters and need phosphorylation by 3'-phosphoinositide-dependent protein kinase 1 at their activation loop to be fully active (119). PKC ϵ controls its localization through second messengers and is dependent on G-protein-coupled receptors activation. It can bind to Myosin IIA and actin in fibroblasts, thereby connecting to actin cytoskeleton (120). In this regard, the exposure to the CXCL12 chemokine strengthens the IS shape, as an additive signal to CD3 and CD28 (121), and CXCR4, the G-protein-coupled receptor for CXCL12, is localized at the IS through connection with actin cytoskeleton (122). CXCL12 binding to its receptor allows Ca²⁺ influx and rapid activation of Rac1 (123) and its internalization seems dependent on MIIA interaction (124). Immature hypophosphorylated PKC ϵ associates to AKAP450 (125) and this can be related to its high basal localization to DAG-enriched membranes, such as the Golgi (126) (**Figure 3**). PKC θ is a well-known modulated kinase during T cell activation, which recruitment to the IS depends on CD28 costimulation (127). PKC θ clustering at the center of the IS correlates with its activity (127) and is highly dependent on actin dynamics; Golgi-resident eNOS translocated to the IS together with the centrosome lowers the actin retrograde flux and enhances PKC θ activity (32). The regulation of PKC θ downstream activity by the control of Carma1 localization at the IS by the plus-end directed, kinesin molecular motor GAKIN has also been described. GAKIN can walk on the MTs toward the periphery of the IS, displacing Carma1 from Bcl10 and the central part of the IS (128). Recently, the identification of a protein complex comprising CD28, Lck, and PKC θ has explained the dependence of PKC θ on CD28 activation. The unique domain V3 from PKC θ interacts with the SH3 domain of Lck, which in turn docks at the phosphorylated tail of CD28 (129). The mutation of the PI3K interaction site in CD28 prevented the recruitment of PKC θ at the IS and transcription of IL-2 mRNA (130), determining the relevance of the targeting to the IS in T cells. The localization through Lck at the IS may explain also why PKC θ docks at the IS in CD28-deficient Jurkat T cells (32). A different mechanism seems to operate in regulatory T cells (Tregs), with PKC θ preferentially located at the distal pole of the T cell, far from the T-APC contact (131). The use of knockout mice or chemical inhibitors for PKC θ has rendered distinct results in the Treg subset. Hence, Tregs from *Pkc θ ^{-/-}* showed similar activity than wild type, although their numbers were diminished due to developmental problems (132), while chemical inhibition clearly enhanced Treg function (131). IL-2 production by effector T cells is essential for CD4

Treg differentiation and function (133), which could affect the numbers in the *Pkc θ ^{-/-}* mice. Naïve T cells from *Pkc θ ^{-/-}* mice have been analyzed for stability of the IS, and the absence of the kinase allows the IS to be formed for longer periods of time without loss of symmetry, therefore preventing the formation of different IS by the same T cell (134). Therefore, the interconnection between skeletons and signaling is clear again, but there is still much information lacking to understand precisely which is the signaling cascades controlling the motor activities and the dynamics of the actin-based and tubulin-based skeletons at the IS.

PROTEIN MULTIPLEXING HIGHLIGHTS THE COMPLEXITY OF TRANSPORT SYSTEMS

The use of different imaging techniques together with biochemical identification of proteins and different drugs against cytoskeletal components has allowed the understanding of different routes for transport of vesicles at the IS. The intracellular traffic at the IS has been analyzed mainly through wide-field fluorescence microscopy, laser scanning confocal microscopy (LSCM), and, to a lesser extent, TIRFm (48, 135). Endocytosed TCRs enter a pathway to recycling endosomes marked by Rab4 and Rab11. Rab4-positive endosomes are early endosomes involved in rapid shuttling of internalized receptors to the plasma membrane in an MT-independent manner. The endosomes marked by Rab11 cluster deeper inside the cell (next to the centrosome) and follow a slower route to return to the plasma membrane along MT. Rab35 and other Rab GTPases regulate the endosomal trafficking together with Wiskott-Aldrich syndrome protein and SCAR homolog (WASH), which controls actin polymerization. WASH activates Arp2/3 complex and also interacts with tubulin cytoskeleton in both early and late endosomes, promoting the local actin polymerization that may provide the force for their movement along MT (78). Later, actin clearance at specific sites of the IS allows the fusion of the vesicles with the plasma membrane. The corresponding N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) at the vesicles (v-SNARE) and on target membranes (t-SNAREs) mediate this process. A complex between two t-SNAREs (syntaxin-3 or -4 and SNAP-23 in non-neuronal cells) and one v-SNARE such as VAMP3 (136) allows the docking and priming of the vesicle, which fuses with the plasma membrane in the presence of high Ca²⁺. In contrast to TCR/CD3 vesicles that are controlled by VAMP3, the endosomal recruitment and docking of LAT to the cortical region of the IS are dependent on the VAMP7 v-SNARE (although CD3 vesicles may also interact with VAMP7) (137). The presence of two traveling LAT pools at the IS was described through fluorescence microscopy (138). Endosomal pools of LAT are localized in different subpopulations of recycling endosomes marked by Rab27 and Rab37. This suggests that early phosphorylation of LAT upon TCR activation depends on the clustering of the LAT pool at the plasma membrane rather than on the LAT subset at endosomes. The latter seems to be more involved, however, in stabilizing signaling mediators close to the TCR (19, 139). The growing of MTs at the IS, analyzed by TIRFm through imaging of EB1 and

EB3 (18), allows the movement of TCR/CD3-enriched cortical vesicles underneath the plasma membrane and their encounter with LAT-enriched vesicles, thereby helping sustained activation of LAT and PLC γ 1 upon TCR triggering (18). Indeed, an important pool of Lck in Rab11b⁺MAL⁺ endosomes is detected during T cell activation. MAL is involved in the targeting of Lck to the plasma membrane and the correct sorting of Lck and LAT to membrane subdomains at the IS (84, 140). Rab11b interacts with myosin 5B, a motor protein able to interact with actin cables and vesicles, through the adaptor protein uncoordinated 119 (Unc 119). This complex allows the final delivery of the vesicles, from the MTs of the pericentrosomal region to the cortical actin at the IS (4). This kind of collaboration between motors and skeletons for intracellular traffic is essential for correct T cell activation, and probably favors cytosolic pulling forces.

An unexpected player was recently described as a regulator of TCR recycling at the IS: the intraflagellar transport (IFT) system. IFT are multimeric protein complexes relevant for the biogenesis and maintenance of the primary cilium. T cells express different IFT constituents such as IFT20, 52, 57, and 88, which participate in the recycling of the TCR to the endosomal system upon centrosome positioning at the IS. The polarization of the Golgi apparatus and the centrosome drives the building of both structures, the IS and the cilium. They direct the growing of MT and the traffic of vesicles toward the plasma membrane. These membranes are both highly enriched in cholesterol and sphingolipids. They act as signaling platforms for extracellular cues (21). IFT20, together with IFT88, IFT52 and IFT57, are recruited to the IS in association with the Golgi apparatus and centrioles (141). IFT20 sustains TCR clustering and signaling but is dispensable for polarization of the Golgi and the centrosome. IFT20 couples internalized TCR/CD3 complexes with Rab5⁺ early endosomes and promotes their transit to recycling endosomes. Since IFT20 co-localizes with the TCR in Rab11⁺Rab4⁺ endosomes, it is possible that it accompanies this receptor during recycling. In this regard, IFT20 also interacts with the transferrin receptor (TfR), which also undergoes polarized recycling at the IS (141, 142). Indeed, tubulin heterodimers can be transported by the IFT system to the end of the cilium, thereby facilitating its elongation (143). An increase of available heterodimers helps reaching the critical concentration needed for polymerization of MTs. A similar process may take place at the IS, providing the IFT proteins expressed by the T cell can also perform this task. Therefore, the transport of molecules and vesicles and its relationship with the centrosome and the MTs arising from it seem to be of special relevance in different cell systems used for sensing changes in the extracellular medium, such as the cilium and the IS. Intracellular traffic and the cytoskeleton are tightly related in the regulation of the IS and the cilium. Tubulin tracks, as identified by cryo-tomography or transmission electron microscopy near mitochondria and the endoplasmic reticulum (24, 66, 72) connect different organelles, and establish tensional forces between them, as well as with the plasma membrane. Pulling and pushing forces would help the scission and fusion of vesicles from and to Golgi or endosomes, respectively. For instance, EB1, which is involved in MT growth from the polarized centrosome and regulation of vesicular traffic in T cells, is also related to the vesicular transport for cilium formation (144). Indeed, during cilium formation, the

tubule scission from the Golgi by spastin, an MT-severing protein, is achieved through the interaction with the ESCRT complex (145). Therefore, the linkage between intracellular organelles and cytoskeleton is needed to organize a productive IS, and this is in part mediated by different molecular complexes that coordinate their action.

CONCLUDING REMARKS

Signaling constituents transported to the IS by polarized vesicles trafficking are a crucial piece of the information transmitted from the plasma membrane to the nucleus and other organelles (18, 19, 146). This is needed to sustain T cell activation and is activated from the TCR, costimulatory molecules such as CD28 and adhesive receptors such as LFA-1. The different cytoskeletal systems are absolutely required to coordinate and activate the plethora of molecules involved in these processes. Molecular motors facilitate these events by exerting forces of different orientations along the cytoskeletal track. These pulling and pushing forces are critical for cell shaping and movement. Future experimentation will profit from new technical advances to analyze complete cells in three dimensions with higher resolution and low toxicity for live cells such as LLSM or 3D-SIM. The super-resolution techniques such as STED and photo activated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM) that have been developed will allow to analyze in more detail the already known structures [for revision of imaging techniques, see in this topic (147) and for protocols and methods (148)]. To unveil the role of motors in T cell organization, it is essential to study the specific composition of the complexes they form, the organelles they bind to and their relationship with the dynamics of the cytoskeleton systems, in particular with the PTMs that can fine-tune their functional activity and direct the activation of T cells. Dynein/dynactin is a crucial motor complex in this context. It directs the rearrangement of different MT-associated organelles, such as the Golgi and mitochondria. The sum of the forces exerted from the cell cortex and the cytosolic elements will determine the shape of the IS.

AUTHOR CONTRIBUTIONS

NM-C wrote the manuscript and composed the figures. FS-M wrote the manuscript.

ACKNOWLEDGMENTS

We want to apologize to those authors whose works have been not cited herein. We want to thank Drs. M. Gómez and J. M. Serrador for critical reading of the manuscript.

FUNDING

This manuscript has been funded by grants SAF2014-55579-R and SAF 2017/82886-R from the Spanish Ministry of Economy and Competitiveness, CAM S2017/BMD-3671 from the Comunidad de Madrid, grant PIE13/00041 from Instituto de Salud Carlos III, the European Regional Development Fund (ERDF) and ERC-2011-AdG 294340-GENTRIS.

REFERENCES

- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) 395:82–6. doi:10.1038/25764
- Wulfig C, Davis MM. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* (1998) 282:2266–9. doi:10.1126/science.282.5397.2266
- Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* (1999) 285:221–7. doi:10.1126/science.285.5425.221
- Soares H, Lasserre R, Alcover A. Orchestrating cytoskeleton and intracellular vesicle traffic to build functional immunological synapses. *Immunol Rev* (2013) 256:118–32. doi:10.1111/immr.12110
- Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, et al. Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol* (2010) 28:79–105. doi:10.1146/annurev-immunol-030409-101308
- Martin-Cofreces NB, Baixauli F, Sanchez-Madrid F. Immune synapse: conductor of orchestrated organelle movement. *Trends Cell Biol* (2014) 24:61–72. doi:10.1016/j.tcb.2013.09.005
- Barlan K, Gelfand VI. Microtubule-based transport and the distribution, tethering, and organization of organelles. *Cold Spring Harb Perspect Biol* (2017) 9:a025817. doi:10.1101/cshperspect.a025817
- Carter AP, Diamant AG, Urnavicius L. How dynein and dynactin transport cargos: a structural perspective. *Curr Opin Struct Biol* (2016) 37:62–70. doi:10.1016/j.sbi.2015.12.003
- de la Fuente H, Mittelbrunn M, Sanchez-Martin L, Vicente-Manzanares M, Lamana A, Pardi R, et al. Synaptic clusters of MHC class II molecules induced on DCs by adhesion molecule-mediated initial T-cell scanning. *Mol Biol Cell* (2005) 16:3314–22. doi:10.1091/mbc.e05-01-0005
- Comrie WA, Li S, Boyle S, Burkhardt JK. The dendritic cell cytoskeleton promotes T cell adhesion and activation by constraining ICAM-1 mobility. *J Cell Biol* (2015) 208:457–73. doi:10.1083/jcb.201406120
- Bunnell SC, Kapoor V, Tribble RP, Zhang W, Samelson LE. Dynamic actin polymerization drives T cell receptor-induced spreading: a role for the signal transduction adaptor LAT. *Immunity* (2001) 14:315–29. doi:10.1016/S1074-7613(01)00112-1
- Melki R, Fievez S, Carlier MF. Continuous monitoring of Pi release following nucleotide hydrolysis in actin or tubulin assembly using 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine-nucleoside phosphorylase as an enzyme-linked assay. *Biochemistry* (1996) 35:12038–45. doi:10.1021/bi961325o
- Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol* (2002) 158:1263–75. doi:10.1083/jcb.200203043
- Mossman KD, Campi G, Groves JT, Dustin ML. Altered TCR signaling from geometrically repatterned immunological synapses. *Science* (2005) 310:1191–3. doi:10.1126/science.1119238
- Cemerski S, Das J, Giuriso E, Markiewicz MA, Allen PM, Chakraborty AK, et al. The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. *Immunity* (2008) 29:414–22. doi:10.1016/j.immuni.2008.06.014
- Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc Natl Acad Sci U S A* (2006) 103:18992–7. doi:10.1073/pnas.0609009103
- Martin-Cofreces NB, Alarcon B, Sanchez-Madrid F. Tubulin and actin interplay at the T cell and antigen-presenting cell interface. *Front Immunol* (2011) 2:24. doi:10.3389/fimmu.2011.00024
- Martin-Cofreces NB, Baixauli F, Lopez MJ, Gil D, Monjas A, Alarcon B, et al. End-binding protein 1 controls signal propagation from the T cell receptor. *EMBO J* (2012) 31:4140–52. doi:10.1038/emboj.2012.242
- Soares H, Henriques R, Sachse M, Ventimiglia L, Alonso MA, Zimmer C, et al. Regulated vesicle fusion generates signaling nanoterritories that control T cell activation at the immunological synapse. *J Exp Med* (2013) 210:2415–33. doi:10.1084/jem.20130150
- Das V, Nal B, Dujancourt A, Thoulouze MI, Galli T, Roux P, et al. Activation-induced polarized recycling targets T cell antigen receptors to the immunological synapse; involvement of SNARE complexes. *Immunity* (2004) 20:577–88. doi:10.1016/S1074-7613(04)00106-2
- Finetti F, Baldari CT. Compartmentalization of signaling by vesicular trafficking: a shared building design for the immune synapse and the primary cilium. *Immunol Rev* (2013) 251:97–112. doi:10.1111/immr.12018
- Wittmann T, Bokoch GM, Waterman-Storer CM. Regulation of leading edge microtubule and actin dynamics downstream of Rac1. *J Cell Biol* (2003) 161:845–51. doi:10.1083/jcb.200303082
- Bouchet J, Del Rio-Iniguez I, Lasserre R, Agüera-Gonzalez S, Cuhe C, Danckaert A, et al. Rac1-Rab11-FIP3 regulatory hub coordinates vesicle traffic with actin remodeling and T-cell activation. *EMBO J* (2016) 35:1160–74. doi:10.15252/emboj.201593274
- Calabria-Linares C, Robles-Valero J, de la Fuente H, Perez-Martinez M, Martin-Cofreces N, Alfonso-Perez M, et al. Endosomal clathrin drives actin accumulation at the immunological synapse. *J Cell Sci* (2011) 124:820–30. doi:10.1242/jcs.078832
- Yi J, Wu X, Chung AH, Chen JK, Kapoor TM, Hammer JA. Centrosome repositioning in T cells is biphasic and driven by microtubule end-on capture-shrinkage. *J Cell Biol* (2013) 202:779–92. doi:10.1083/jcb.201301004
- Lasserre R, Charrin S, Cuhe C, Danckaert A, Thoulouze MI, de Chaumont F, et al. Ezrin tunes T-cell activation by controlling Dlg1 and microtubule positioning at the immunological synapse. *EMBO J* (2010) 29:2301–14. doi:10.1038/emboj.2010.127
- Blas-Rus N, Bustos-Moran E, Perez de Castro I, de Carcer G, Borroto A, Camafeite E, et al. Aurora A drives early signalling and vesicle dynamics during T-cell activation. *Nat Commun* (2016) 7:11389. doi:10.1038/ncomms11389
- Lowin-Kropf B, Shapiro VS, Weiss A. Cytoskeletal polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-dependent mechanism. *J Cell Biol* (1998) 140:861–71. doi:10.1083/jcb.140.4.861
- Plotnikova OV, Pugacheva EN, Dunbrack RL, Golemis EA. Rapid calcium-dependent activation of Aurora-A kinase. *Nat Commun* (2010) 1:64. doi:10.1038/ncomms1061
- Tsun A, Qureshi I, Stinchcombe JC, Jenkins MR, de la Roche M, Kleczkowska J, et al. Centrosome docking at the immunological synapse is controlled by Lck signaling. *J Cell Biol* (2011) 192:663–74. doi:10.1083/jcb.201008140
- Martin-Cofreces NB, Sancho D, Fernandez E, Vicente-Manzanares M, Gordon-Alonso M, Montoya MC, et al. Role of Fyn in the rearrangement of tubulin cytoskeleton induced through TCR. *J Immunol* (2006) 176:4201–7. doi:10.4049/jimmunol.176.7.4201
- Garcia-Ortiz A, Martin-Cofreces NB, Ibiza S, Ortega A, Izquierdo-Alvarez A, Trullo A, et al. eNOS S-nitrosylates beta-actin on Cys374 and regulates PKC-theta at the immune synapse by impairing actin binding to profilin-1. *PLoS Biol* (2017) 15:e2000653. doi:10.1371/journal.pbio.2000653
- Chikuma S, CTLA-4, an essential immune-checkpoint for T-cell activation. *Curr Top Microbiol Immunol* (2017) 410:99–126. doi:10.1007/82_2017_61
- Beemiller P, Jacobelli J, Krummel MF. Integration of the movement of signaling microclusters with cellular motility in immunological synapses. *Nat Immunol* (2012) 13:787–95. doi:10.1038/ni.2364
- Kaiser DA, Vinson VK, Murphy DB, Pollard TD. Profilin is predominantly associated with monomeric actin in *Acanthamoeba*. *J Cell Sci* (1999) 112 (Pt 21):3779–90.
- Suarez C, Carroll RT, Burke TA, Christensen JR, Bestul AJ, Sees JA, et al. Profilin regulates F-actin network homeostasis by favoring formin over Arp2/3 complex. *Dev Cell* (2015) 32:43–53. doi:10.1016/j.devcel.2014.10.027
- Rotty JD, Wu C, Haynes EM, Suarez C, Winkelman JD, Johnson HE, et al. Profilin-1 serves as a gatekeeper for actin assembly by Arp2/3-dependent and -independent pathways. *Dev Cell* (2015) 32:54–67. doi:10.1016/j.devcel.2014.10.026
- Young LE, Heimsath EG, Higgs HN. Cell type-dependent mechanisms for formin-mediated assembly of filopodia. *Mol Biol Cell* (2015) 26:4646–59. doi:10.1091/mbc.E15-09-0626
- Gomez TS, Kumar K, Medeiros RB, Shimizu Y, Leibson PJ, Billadeau DD. Formins regulate the actin-related protein 2/3 complex-independent polarization of the centrosome to the immunological synapse. *Immunity* (2007) 26:177–90. doi:10.1016/j.immuni.2007.01.008
- Kumari S, Depoil D, Martinelli R, Judokusumo E, Carmona G, Gertler FB, et al. Actin foci facilitate activation of the phospholipase C-gamma in primary T lymphocytes via the WASP pathway. *Elife* (2015) 4:e04953. doi:10.7554/eLife.04953

41. Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) 356:eaa13118. doi:10.1126/science.aal3118
42. Fritzsche M, Fernandes RA, Chang VT, Colin-York H, Clausen MP, Felce JH, et al. Cytoskeletal actin dynamics shape a ramifying actin network underpinning immunological synapse formation. *Sci Adv* (2017) 3:e1603032. doi:10.1126/sciadv.1603032
43. Chesarone MA, DuPage AG, Goode BL. Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nat Rev Mol Cell Biol* (2010) 11:62–74. doi:10.1038/nrm2816
44. Howard J, Hyman AA. Growth, fluctuation and switching at microtubule plus ends. *Nat Rev Mol Cell Biol* (2009) 10:569–74. doi:10.1038/nrm2713
45. Eisenmann KM, West RA, Hildebrand D, Kitchen SM, Peng J, Sigler R, et al. T cell responses in mammalian diaphanous-related formin mDia1 knock-out mice. *J Biol Chem* (2007) 282:25152–8. doi:10.1074/jbc.M703243200
46. Andres-Delgado L, Anton OM, Bartolini F, Ruiz-Saenz A, Correia I, Gundersen GG, et al. INF2 promotes the formation of deetyrosinated microtubules necessary for centrosome reorientation in T cells. *J Cell Biol* (2012) 198:1025–37. doi:10.1083/jcb.201202137
47. Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* (2006) 7:247–55. doi:10.1038/ni1304
48. Onnis A, Finetti F, Baldari CT. Vesicular trafficking to the immune synapse: how to assemble receptor-tailored pathways from a basic building set. *Front Immunol* (2016) 7:50. doi:10.3389/fimmu.2016.00050
49. Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* (2006) 443:462–5. doi:10.1038/nature05071
50. Lyubchenko TA, Wurth GA, Zweifach A. Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. *Immunity* (2001) 15:847–59. doi:10.1016/S1074-7613(01)00233-3
51. Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen BC, Gawden-Bone C, et al. Actin depletion initiates events leading to granule secretion at the immunological synapse. *Immunity* (2015) 42:864–76. doi:10.1016/j.immuni.2015.04.013
52. Mace EM, Orange JS. Lytic immune synapse function requires filamentous actin deconstruction by Coronin 1A. *Proc Natl Acad Sci U S A* (2014) 111:6708–13. doi:10.1073/pnas.1314975111
53. Brown AC, Oddos S, Dobbie IM, Alakoskela JM, Parton RM, Eissmann P, et al. Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol* (2011) 9:e1001152. doi:10.1371/journal.pbio.1001152
54. Fager N, Rangell L, Danilenko DM, Chan AC. Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. *Science* (2006) 313:839–42. doi:10.1126/science.1130563
55. Ritter AT, Kapnick SM, Murugesan S, Schwartzberg PL, Griffiths GM, Lippincott-Schwartz J. Cortical actin recovery at the immunological synapse leads to termination of lytic granule secretion in cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A* (2017) 114:E6585–94. doi:10.1073/pnas.1710751114
56. Bustos-Moran E, Blas-Rus N, Martín-Cófreces NB, Sánchez-Madrid F. Orchestrating lymphocyte polarity in cognate immune cell–cell interactions. *Int Rev Cell Mol Biol* (2016) 327:195–261. doi:10.1016/bs.ircmb.2016.06.004
57. Fu MM, Holzbaur EL. Integrated regulation of motor-driven organelle transport by scaffolding proteins. *Trends Cell Biol* (2014) 24:564–74. doi:10.1016/j.tcb.2014.05.002
58. Kikkawa M. Big steps toward understanding dynein. *J Cell Biol* (2013) 202:15–23. doi:10.1083/jcb.201304099
59. Combs J, Kim SJ, Tan S, Ligon LA, Holzbaur EL, Kuhn J, et al. Recruitment of dynein to the Jurkat immunological synapse. *Proc Natl Acad Sci U S A* (2006) 103:14883–8. doi:10.1073/pnas.0600914103
60. Martín-Cófreces NB, Robles-Valero J, Cabrero JR, Mittelbrunn M, Gordon-Alonso M, Sung CH, et al. MTOC translocation modulates IS formation and controls sustained T cell signaling. *J Cell Biol* (2008) 182:951–62. doi:10.1083/jcb.200801014
61. Peterson EJ, Woods ML, Dmowski SA, Derimanov G, Jordan MS, Wu JN, et al. Coupling of the TCR to integrin activation by Slap-130/Fyb. *Science* (2001) 293:2263–5. doi:10.1126/science.1063486
62. Griffiths EK, Krawczyk C, Kong YY, Raab M, Hyduk SJ, Bouchard D, et al. Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science* (2001) 293:2260–3. doi:10.1126/science.1063397
63. Huang Y, Norton DD, Precht P, Martindale JL, Burkhardt JK, Wange RL. Deficiency of ADAP/Fyb/SLAP-130 destabilizes SKAP55 in Jurkat T cells. *J Biol Chem* (2005) 280:23576–83. doi:10.1074/jbc.M413201200
64. Wang H, Moon EY, Azouz A, Wu X, Smith A, Schneider H, et al. SKAP-55 regulates integrin adhesion and formation of T cell-APC conjugates. *Nat Immunol* (2003) 4:366–74. doi:10.1038/ni913
65. Hashimoto-Tane A, Yokosuka T, Sakata-Sogawa K, Sakuma M, Ishihara C, Tokunaga M, et al. Dynein-driven transport of T cell receptor microclusters regulates immune synapse formation and T cell activation. *Immunity* (2011) 34:919–31. doi:10.1016/j.immuni.2011.05.012
66. Ueda H, Morpheus MK, McIntosh JR, Davis MM. CD4+ T-cell synapses involve multiple distinct stages. *Proc Natl Acad Sci USA* (2011) 108:17099–104. doi:10.1073/pnas.1113703108
67. Burkhardt JK, Echeverri CJ, Nilsson T, Vallee RB. Overexpression of the dynamin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J Cell Biol* (1997) 139:469–84. doi:10.1083/jcb.139.2.469
68. Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol* (1996) 132:617–33. doi:10.1083/jcb.132.4.617
69. Nath S, Christian L, Tan SY, Ki S, Ehrlich LI, Poenie M. Dynein separately partners with NDE1 and dynactin To orchestrate T cell focused secretion. *J Immunol* (2016) 197:2090–101. doi:10.4049/jimmunol.1600180
70. Lam C, Vergnolle MA, Thorpe L, Woodman PG, Allan VJ. Functional interplay between LIS1, NDE1 and NDEL1 in dynein-dependent organelle positioning. *J Cell Sci* (2010) 123:202–12. doi:10.1242/jcs.059337
71. Shmueli A, Segal M, Sapir T, Tsutsumi R, Noritake J, Bar A, et al. Ndel1 palmitoylation: a new mean to regulate cytoplasmic dynein activity. *EMBO J* (2010) 29:107–19. doi:10.1038/emboj.2009.325
72. Baixauli F, Martín-Cófreces NB, Morlino G, Carrasco YR, Calabia-Linares C, Veiga E, et al. The mitochondrial fission factor dynamin-related protein 1 modulates T-cell receptor signalling at the immune synapse. *EMBO J* (2011) 30:1238–50. doi:10.1038/emboj.2011.25
73. Morlino G, Barreiro O, Baixauli F, Robles-Valero J, Gonzalez-Granado JM, Villa-Bellosta R, et al. Miro-1 links mitochondria and microtubule Dynein motors to control lymphocyte migration and polarity. *Mol Cell Biol* (2014) 34:1412–26. doi:10.1128/MCB.01177-13
74. Harte PJ, Kankel DR. Genetic analysis of mutations at the Glued locus and interacting loci in *Drosophila melanogaster*. *Genetics* (1982) 101:477–501.
75. McKenney RJ, Huynh W, Vale RD, Sirajuddin M. Tyrosination of alpha-tubulin controls the initiation of processive dynein-dynactin motility. *EMBO J* (2016) 35:1175–85. doi:10.15252/emboj.201593071
76. Barisic M, Maiato H. The tubulin code: a navigation system for chromosomes during mitosis. *Trends Cell Biol* (2016) 26:766–75. doi:10.1016/j.tcb.2016.06.001
77. Barbosa DJ, Duro J, Prevo B, Cheerambathur DK, Carvalho AX, Gassmann R. Dynactin binding to tyrosinated microtubules promotes centrosome centration in *C. elegans* by enhancing dynein-mediated organelle transport. *PLoS Genet* (2017) 13:e1006941. doi:10.1371/journal.pgen.1006941
78. Finetti F, Patrussi L, Galgano D, Cassioli C, Perinetti G, Pazour GJ, et al. The small GTPase Rab8 interacts with VAMP-3 to regulate the delivery of recycling T-cell receptors to the immune synapse. *J Cell Sci* (2015) 128:2541–52. doi:10.1242/jcs.171652
79. Greer YE, Westlake CJ, Gao B, Bharti K, Shiba Y, Xavier CP, et al. Casein kinase 1delta functions at the centrosome and Golgi to promote ciliogenesis. *Mol Biol Cell* (2014) 25:1629–40. doi:10.1091/mbc.e13-10-0598
80. Sillibourne JE, Milne DM, Takahashi M, Ono Y, Meek DW. Centrosomal anchoring of the protein kinase CK1delta mediated by attachment to the large, coiled-coil scaffolding protein CG-NAP/AKAP450. *J Mol Biol* (2002) 322:785–97. doi:10.1016/S0022-2836(02)00857-4
81. Robles-Valero J, Martín-Cófreces NB, Lamana A, Macdonald S, Volkov Y, Sánchez-Madrid F. Integrin and CD3/TCR activation are regulated by the scaffold protein AKAP450. *Blood* (2010) 115:4174–84. doi:10.1182/blood-2009-12-256222

82. Rivero S, Cardenas J, Bornens M, Rios RM. Microtubule nucleation at the cis-side of the Golgi apparatus requires AKAP450 and GM130. *EMBO J* (2009) 28:1016–28. doi:10.1038/emboj.2009.47
83. Zyss D, Ebrahimi H, Gergely F. Casein kinase I delta controls centrosome positioning during T cell activation. *J Cell Biol* (2011) 195:781–97. doi:10.1083/jcb.201106025
84. Anton OM, Andres-Delgado L, Reglero-Real N, Batista A, Alonso MA. MAL protein controls protein sorting at the supramolecular activation cluster of human T lymphocytes. *J Immunol* (2011) 186:6345–56. doi:10.4049/jimmunol.1003771
85. Valenzuela-Fernandez A, Cabrero JR, Serrador JM, Sanchez-Madrid F. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. *Trends Cell Biol* (2008) 18:291–7. doi:10.1016/j.tcb.2008.04.003
86. Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* (2003) 115:727–38. doi:10.1016/S0092-8674(03)00939-5
87. Cabrero JR, Serrador JM, Barreiro O, Mittelbrunn M, Naranjo-Suarez S, Martín-Cófreces N, et al. Lymphocyte chemotaxis is regulated by histone deacetylase 6, independently of its deacetylase activity. *Mol Biol Cell* (2006) 17:3435–45. doi:10.1091/mbc.e06-01-0008
88. Li D, Xie S, Ren Y, Huo L, Gao J, Cui D, et al. Microtubule-associated deacetylase HDAC6 promotes angiogenesis by regulating cell migration in an EB1-dependent manner. *Protein Cell* (2011) 2:150–60. doi:10.1007/s13238-011-1015-4
89. Mergen M, Engel C, Muller B, Follo M, Schafer T, Jung M, et al. The nephronophthisis gene product NPHP2/Inversin interacts with Aurora A and interferes with HDAC6-mediated cilia disassembly. *Nephrol Dial Transplant* (2013) 28:2744–53. doi:10.1093/ndt/gft316
90. Serrador JM, Cabrero JR, Sancho D, Mittelbrunn M, Urzainqui A, Sanchez-Madrid F. HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. *Immunity* (2004) 20:417–28. doi:10.1016/S1074-7613(04)00078-0
91. Nunez-Andrade N, Iborra S, Trullo A, Moreno-Gonzalo O, Calvo E, Catalan E, et al. HDAC6 regulates the dynamics of lytic granules in cytotoxic T lymphocytes. *J Cell Sci* (2016) 129:1305–11. doi:10.1242/jcs.180885
92. Andreu JM, Bordas J, Diaz JF, Garcia de Ancos J, Gil R, Medrano FJ, et al. Low resolution structure of microtubules in solution. Synchrotron X-ray scattering and electron microscopy of taxol-induced microtubules assembled from purified tubulin in comparison with glycerol and MAP-induced microtubules. *J Mol Biol* (1992) 226:169–84. doi:10.1016/0022-2836(92)90132-4
93. Diaz JF, Barasoain I, Andreu JM. Fast kinetics of Taxol binding to microtubules. Effects of solution variables and microtubule-associated proteins. *J Biol Chem* (2003) 278:8407–19. doi:10.1074/jbc.M211163200
94. Magnani M, Maccari G, Andreu JM, Diaz JF, Botta M. Possible binding site for paclitaxel at microtubule pores. *FEBS J* (2009) 276:2701–12. doi:10.1111/j.1742-4658.2009.06994.x
95. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly in vitro by taxol. *Nature* (1979) 277:665–7. doi:10.1038/277665a0
96. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci U S A* (1980) 77:1561–5. doi:10.1073/pnas.77.3.1561
97. Torres K, Horwitz SB. Mechanisms of taxol-induced cell death are concentration dependent. *Cancer Res* (1998) 58:3620–6.
98. Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, et al. T cell activation requires mitochondrial translocation to the immunological synapse. *Proc Natl Acad Sci U S A* (2007) 104:14418–23. doi:10.1073/pnas.0703126104
99. Varadi A, Johnson-Cadwell LI, Cirulli V, Yoon Y, Allan VJ, Rutter GA. Cytoplasmic dynein regulates the subcellular distribution of mitochondria by controlling the recruitment of the fission factor dynamin-related protein-1. *J Cell Sci* (2004) 117:4389–400. doi:10.1242/jcs.01299
100. Campello S, Lacalle RA, Bettella M, Manes S, Scorrano L, Viola A. Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. *J Exp Med* (2006) 203:2879–86. doi:10.1084/jem.20061877
101. Sanchez-Madrid F, Serrador JM. Bringing up the rear: defining the roles of the uropod. *Nat Rev Mol Cell Biol* (2009) 10:353–9. doi:10.1038/nrm2680
102. Hirokawa N, Tanaka Y. Kinesin superfamily proteins (KIFs): various functions and their relevance for important phenomena in life and diseases. *Exp Cell Res* (2015) 334:16–25. doi:10.1016/j.yexcr.2015.02.016
103. Hirokawa N, Niwa S, Tanaka Y. Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* (2010) 68:610–38. doi:10.1016/j.neuron.2010.09.039
104. Glater EE, Megeath LJ, Stowers RS, Schwarz TL. Axonal transport of mitochondria requires Milton to recruit kinesin heavy chain and is light chain independent. *J Cell Biol* (2006) 173:545–57. doi:10.1083/jcb.200601067
105. Kurowska M, Goudin N, Nehme NT, Court M, Garin J, Fischer A, et al. Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1/Slp3/Rab27a complex. *Blood* (2012) 119:3879–89. doi:10.1182/blood-2011-09-382556
106. Reed NA, Cai D, Blasius TL, Jih GT, Meyhofer E, Gaertig J, et al. Microtubule acetylation promotes kinesin-1 binding and transport. *Curr Biol* (2006) 16:2166–72. doi:10.1016/j.cub.2006.09.014
107. Balabanian L, Berger CL, Hendricks AG. Acetylated microtubules are preferentially bundled leading to enhanced kinesin-1 motility. *Biophys J* (2017) 113:1551–60. doi:10.1016/j.bpj.2017.08.009
108. Olzmann JA, Chin LS. Parkin-mediated K63-linked polyubiquitination: a signal for targeting misfolded proteins to the aggresome-autophagy pathway. *Autophagy* (2008) 4:85–7. doi:10.4161/auto.5172
109. Jiang Q, Ren Y, Feng J. Direct binding with histone deacetylase 6 mediates the reversible recruitment of parkin to the centrosome. *J Neurosci* (2008) 28:12993–3002. doi:10.1523/JNEUROSCI.2860-08.2008
110. Olzmann JA, Li L, Chudav MV, Chen J, Perez FA, Palmiter RD, et al. Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. *J Cell Biol* (2007) 178:1025–38. doi:10.1083/jcb.200611128
111. Seigneurin-Berny D, Verdel A, Curtet S, Lemerrier C, Garin J, Rousseaux S, et al. Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways. *Mol Cell Biol* (2001) 21:8035–44. doi:10.1128/MCB.21.23.8035-8044.2001
112. Hook SS, Orian A, Cowley SM, Eisenman RN. Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes. *Proc Natl Acad Sci U S A* (2002) 99:13425–30. doi:10.1073/pnas.172511699
113. Foth BJ, Goedecke MC, Soldati D. New insights into myosin evolution and classification. *Proc Natl Acad Sci U S A* (2006) 103:3681–6. doi:10.1073/pnas.0506307103
114. Ilani T, Vasiliver-Shamis G, Vardhana S, Bretscher A, Dustin ML. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. *Nat Immunol* (2009) 10:531–9. doi:10.1038/ni.1723
115. Liu X, Kapoor TM, Chen JK, Huse M. Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin II. *Proc Natl Acad Sci U S A* (2013) 110:11976–81. doi:10.1073/pnas.1306180110
116. Quann EJ, Merino E, Furuta T, Huse M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol* (2009) 10:627–35. doi:10.1038/ni.1734
117. Bustos-Moran E, Blas-Rus N, Martín-Cófreces NB, Sánchez-Madrid F. Microtubule-associated protein-4 controls nanovesicle dynamics and T cell activation. *J Cell Sci* (2017) 130:1217–23. doi:10.1242/jcs.199042
118. Quann EJ, Liu X, Altan-Bonnet G, Huse M. A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat Immunol* (2011) 12:647–54. doi:10.1038/ni.2033
119. Toker A. The biology and biochemistry of diacylglycerol signalling. Meeting on molecular advances in diacylglycerol signalling. *EMBO Rep* (2005) 6:310–4. doi:10.1038/sj.embor.7400378
120. England K, Ashford D, Kidd D, Rumsby M. PKC epsilon is associated with myosin IIA and actin in fibroblasts. *Cell Signal* (2002) 14:529–36. doi:10.1016/S0898-6568(01)00277-7
121. Cascio G, Martín-Cófreces NB, Rodríguez-Frade JM, López-Cotarelo P, Criado G, Pablos JL, et al. CXCL12 regulates through JAK1 and JAK2 formation of productive immunological synapses. *J Immunol* (2015) 194:5509–19. doi:10.4049/jimmunol.1402419
122. Perez-Martinez M, Gordon-Alonso M, Cabrero JR, Barrero-Villar M, Rey M, Mittelbrunn M, et al. F-actin-binding protein drebrin regulates CXCR4 recruitment to the immune synapse. *J Cell Sci* (2010) 123:1160–70. doi:10.1242/jcs.064238
123. Vicente-Manzanares M, Cruz-Adalia A, Martín-Cófreces NB, Cabrero JR, Dosil M, Alvarado-Sánchez B, et al. Control of lymphocyte shape and

- the chemotactic response by the GTP exchange factor Vav. *Blood* (2005) 105:3026–34. doi:10.1182/blood-2004-07-2925
124. Rey M, Valenzuela-Fernandez A, Urzainqui A, Yanez-Mo M, Perez-Martinez M, Penela P, et al. Myosin IIA is involved in the endocytosis of CXCR4 induced by SDF-1 α . *J Cell Sci* (2007) 120:1126–33. doi:10.1242/jcs.03415
 125. Takahashi M, Mukai H, Oishi K, Isagawa T, Ono Y. Association of immature hypophosphorylated protein kinase cepsilon with an anchoring protein CG-NAP. *J Biol Chem* (2000) 275:34592–6. doi:10.1074/jbc.M005285200
 126. Newton AC. Protein kinase C: poised to signal. *Am J Physiol Endocrinol Metab* (2010) 298:E395–402. doi:10.1152/ajpendo.00477.2009
 127. Monks CR, Kupfer H, Tamir I, Barlow A, Kupfer A. Selective modulation of protein kinase C- θ during T-cell activation. *Nature* (1997) 385:83–6. doi:10.1038/385083a0
 128. Lamason RL, Kupfer A, Pomerantz JL. The dynamic distribution of CARD11 at the immunological synapse is regulated by the inhibitory kinesin GAKIN. *Mol Cell* (2010) 40:798–809. doi:10.1016/j.molcel.2010.11.007
 129. Kong KF, Yokosuka T, Canonigo-Balancio AJ, Isakov N, Saito T, Altman A. A motif in the V3 domain of the kinase PKC- θ determines its localization in the immunological synapse and functions in T cells via association with CD28. *Nat Immunol* (2011) 12:1105–12. doi:10.1038/ni.2120
 130. Sanchez-Lockhart M, Marin E, Graf B, Abe R, Harada Y, Sedwick CE, et al. Cutting edge: CD28-mediated transcriptional and posttranscriptional regulation of IL-2 expression are controlled through different signaling pathways. *J Immunol* (2004) 173:7120–4. doi:10.4049/jimmunol.173.12.7120
 131. Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M, et al. Protein kinase C- θ mediates negative feedback on regulatory T cell function. *Science* (2010) 328:372–6. doi:10.1126/science.1186068
 132. Gupta S, Manicassamy S, Vasu C, Kumar A, Shang W, Sun Z. Differential requirement of PKC- θ in the development and function of natural regulatory T cells. *Mol Immunol* (2008) 46:213–24. doi:10.1016/j.molimm.2008.08.275
 133. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* (2002) 196:851–7. doi:10.1084/jem.20020190
 134. Sims TN, Soos TJ, Xenias HS, Dubin-Thaler B, Hofman JM, Waite JC, et al. Opposing effects of PKC θ and WASp on symmetry breaking and relocation of the immunological synapse. *Cell* (2007) 129:773–85. doi:10.1016/j.cell.2007.03.037
 135. Bouchet J, Del Rio-Iniguez I, Alcover A. Imaging vesicular traffic at the immune synapse. *Methods Mol Biol* (2017) 1584:129–42. doi:10.1007/978-1-4939-6881-7_9
 136. Hay JC. SNARE complex structure and function. *Exp Cell Res* (2001) 271:10–21. doi:10.1006/excr.2001.5368
 137. Alcover A, Alarcon B, Di Bartolo V. Cell biology of T cell receptor expression and regulation. *Annu Rev Immunol* (2017) 36:103–25. doi:10.1146/annurev-immunol-042617-053429
 138. Bonello G, Blanchard N, Montoya MC, Aguado E, Langlet C, He HT, et al. Dynamic recruitment of the adaptor protein LAT: LAT exists in two distinct intracellular pools and controls its own recruitment. *J Cell Sci* (2004) 117:1009–16. doi:10.1242/jcs.00968
 139. Larghi P, Williamson DJ, Carpiér JM, Dogniaux S, Chemin K, Bohineust A, et al. VAMP7 controls T cell activation by regulating the recruitment and phosphorylation of vesicular Lat at TCR-activation sites. *Nat Immunol* (2013) 14:723–31. doi:10.1038/ni.2609
 140. Anton O, Batista A, Millan J, Andres-Delgado L, Puertollano R, Correias I, et al. An essential role for the MAL protein in targeting Lck to the plasma membrane of human T lymphocytes. *J Exp Med* (2008) 205:3201–13. doi:10.1084/jem.20080552
 141. Finetti F, Patrussi L, Masi G, Onnis A, Galgano D, Lucherini OM, et al. Specific recycling receptors are targeted to the immune synapse by the intraflagellar transport system. *J Cell Sci* (2014) 127:1924–37. doi:10.1242/jcs.139337
 142. Finetti F, Paccani SR, Riparbelli MG, Giacomello E, Perinetti G, Pazour GJ, et al. Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse. *Nat Cell Biol* (2009) 11:1332–9. doi:10.1038/ncb1977
 143. Bhogaraju S, Cajanek L, Fort C, Blisnick T, Weber K, Taschner M, et al. Molecular basis of tubulin transport within the cilium by IFT74 and IFT81. *Science* (2013) 341:1009–12. doi:10.1126/science.1240985
 144. Schroder JM, Larsen J, Komarova Y, Akhmanova A, Thorsteinsson RI, Grigoriev I, et al. EB1 and EB3 promote cilia biogenesis by several centrosome-related mechanisms. *J Cell Sci* (2011) 124:2539–51. doi:10.1242/jcs.085852
 145. Allison R, Lumb JH, Fassier C, Connell JW, Ten Martin D, Seaman MN, et al. An ESCRT-spastin interaction promotes fission of recycling tubules from the endosome. *J Cell Biol* (2013) 202:527–43. doi:10.1083/jcb.201211045
 146. Purbhoo MA, Liu H, Oddos S, Owen DM, Neil MA, Paeon SV, et al. Dynamics of subsynaptic vesicles and surface microclusters at the immunological synapse. *Sci Signal* (2010) 3:ra36. doi:10.1126/scisignal.2000645
 147. Calvo V, Izquierdo I. Imaging polarized secretory traffic at the immune synapse in living T lymphocytes. *Front Immunol* (2018) 9:684. doi:10.3389/fimmu.2018.00684
 148. Baldari CT, Dustin ML. *The Immune Synapse*. New York: Humana Press (2017).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Super-Resolution Correlative Light and Electron Microscopy (SR-CLEM) Reveals Novel Ultrastructural Insights Into Dendritic Cell Podosomes

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 30 May 2018

Accepted: 02 August 2018

Published: 22 August 2018

Citation:

Joosten B, Willemse M, Fransen J,
Cambi A and van den Dries K (2018)
Super-Resolution Correlative Light and
Electron Microscopy (SR-CLEM)
Reveals Novel Ultrastructural Insights
Into Dendritic Cell Podosomes.
Front. Immunol. 9:1908.
doi: 10.3389/fimmu.2018.01908

Podosomes are multimolecular cytoskeletal structures that coordinate the migration of tissue-resident dendritic cells (DCs). They consist of a protrusive actin-rich core and an adhesive integrin-rich ring that contains adaptor proteins such as vinculin and zyxin. Individual podosomes are typically interconnected by a dense network of actin filaments giving rise to large podosome clusters. The actin density in podosome clusters complicates the analysis of podosomes by light microscopy alone. Here, we present an optimized procedure for performing super-resolution correlative light and electron microscopy (SR-CLEM) to study the organization of multiple proteins with respect to actin in podosome clusters at the ventral plasma membrane of DCs. We demonstrate that our procedure is suited to correlate at least three colors in super-resolution Airyscan microscopy with scanning electron microscopy (SEM). Using this procedure, we first reveal an intriguing complexity in the organization of ventral and radiating actin filaments in clusters formed by DCs which was not properly detected before by light microscopy alone. Next, we demonstrate a differential organization of vinculin and zyxin with respect to the actin filaments at podosomes. While vinculin mostly resides at sites where the actin filaments connect to the cell membrane, zyxin is primarily associated with filaments close to and on top of the core. Finally, we reveal a novel actin-based structure with SEM that connects closely associated podosome cores and which may be important for podosome topography sensing. Interestingly, these interpodosomal connections, in contrast to the radiating and ventral actin filaments appear to be insensitive to inhibition of actin polymerization suggesting that these pools of actin are not dynamically coupled. Together, our work demonstrates the power of correlating different imaging modalities for studying multimolecular cellular structures and could potentially be further exploited to study processes at the ventral plasma membrane of immune cells such as clathrin-mediated endocytosis or immune synapse formation.

Keywords: super-resolution microscopy, scanning electron microscopy, dendritic cells, podosomes, actin, vinculin, zyxin

INTRODUCTION

Correlative light and electron microscopy (CLEM) bridges the gap between light microscopy (LM) and electron microscopy (EM). With CLEM, the EM adds structural and cellular context to the LM images while the LM provides a specific molecular context to the EM images, thereby offering unique and complementary information from the same sample (1). CLEM data obtained with EM (lateral resolution <2 nm) and diffraction limited LM (lateral resolution ~ 250 nm) are usually hard to interpret since the detailed structures from the EM are not easily identified in the LM images, something which is often referred to as the resolution gap. The developments of various super-resolution (SR) LM techniques over the past decades have greatly improved the lateral resolution of LM to ~ 140 nm– ~ 10 nm depending on the technique of choice (2), thus creating new opportunities for correlating SR-LM with EM (SR-CLEM) (3, 4).

Podosomes are cytoskeletal structures used by osteoclasts to create a tight sealing zone to facilitate bone resorption and by innate immune cells such as macrophages and dendritic cells (DCs) to cross basement membranes and slowly migrate through peripheral tissues (5, 6). Moreover, podosomes have been shown to be involved in the uptake of foreign antigens by DCs (7). Podosomes consist of an actin-rich protrusive core that is surrounded by an integrin-rich adhesive ring. Typically, hundreds of podosomes are grouped into large clusters, where individual podosomes are interconnected by radiating actin filaments (8). Importantly, the detailed actin organization of podosomes is difficult to study by LM alone, since podosome cores are small (~ 700 nm), actin dense structures and the interconnecting actin filaments are only resolved by super-resolution microscopy (8). Therefore, scanning EM (SEM) of ventral plasma membranes, where podosomes are assembled, has been applied before to study the ultrastructural organization of podosomes in osteoclasts (9–11). Although these studies provided invaluable insight into the ultrastructural organization of podosomes, the lack of information on the nanoscale organization of specific proteins makes interpretation of the SEM images alone challenging. The individual limitations of LM and SEM highlight the need for correlative imaging approaches to study the podosome ultrastructure.

Podosomes contain dozens of proteins that are either primarily associated with the actin core or the integrin ring where also the radiating actin filaments are located. Vinculin and zyxin are two cytoskeletal adaptor proteins involved in cell adhesion and classically associated with the ring (12). The function, however, of vinculin and zyxin is very different with vinculin being directly involved in connecting actin to integrins (13), while zyxin may be more involved in the stress fiber repair (14), suggesting that their localization may also be different. Interestingly, by averaging the fluorescent signal from hundreds of podosomes in diffraction limited microscopy, we have shown before that zyxin resides more close to the core than vinculin (15). Moreover, it has been shown by SR-LM for focal adhesions that vinculin and zyxin reside in different vertically separated regulatory layers (16). Studying the exact mutual localization of multiple proteins with respect to actin structures in podosomes

is instrumental for our understanding of how the dozens of functionally different proteins together regulate podosome function and requires a procedure that combines multicolor SR-LM microscopy with the ultrastructural actin organization as imaged by SEM.

Airyscan imaging is a relatively new laser scanning imaging technique that is based on a concentrically arranged hexagonal detector array, and achieves nearly twice the resolution (lateral resolution ~ 140 nm) in all three dimensions compared to conventional confocal laser scanning microscopy. Compatible with virtually all conventional fluorophores, Airyscan imaging allows simultaneous labeling of multiple proteins and Airyscan SR-CLEM therefore offers new opportunities to correlate the SR-LM information from multiple fluorescently labeled cellular constituents with the ultrastructural organization imaged by SEM. This prompted us to develop a novel imaging pipeline to correlate these imaging modalities for the detailed investigation of the mutual organization of actin, vinculin, and zyxin in podosomes.

Here, we present a novel SR-CLEM imaging pipeline that enables the sequential imaging of multicolor samples using Airyscan SR-LM imaging followed by SEM image acquisition. We present experimental results confirming the feasibility of our SR-CLEM procedure to obtain an optimal overlay of the fluorescence information of multiple podosome proteins with the ultrastructural information obtained by SEM across entire cells. Combining the two imaging modalities allowed us to precisely determine the mutual localization of vinculin and zyxin in DC podosomes. Also, we identify a novel actin-rich structure that connects closely associated podosome cores on flat surfaces as well as on topographical cues and we have investigated the effects of inhibition of actin polymerization on these different actin structures. We foresee that the relatively straightforward procedure that we developed can also be applied to study various other multi-molecular structures at the ventral plasma membrane such as clathrin-coated pits and the immune synapse.

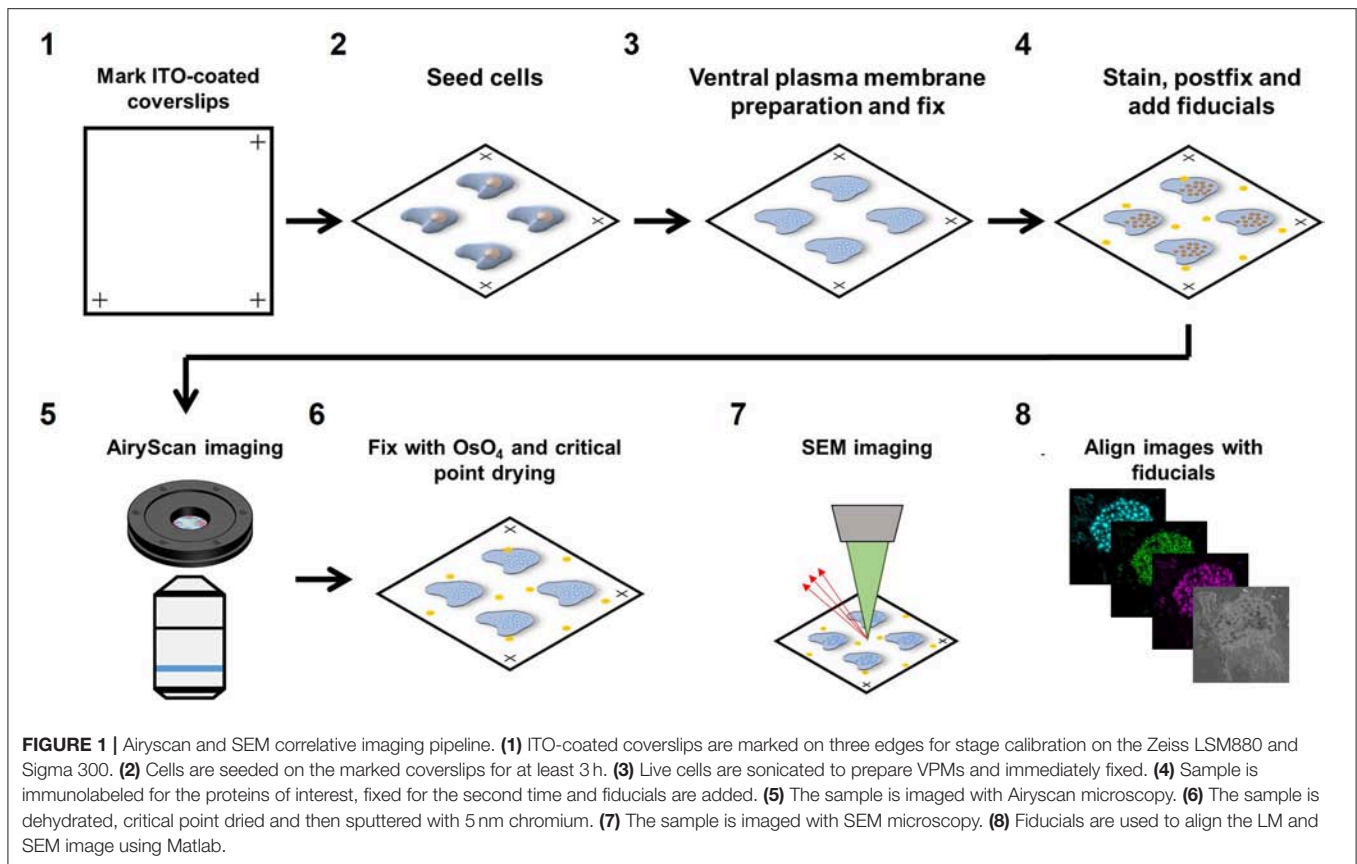
MATERIALS AND METHODS

Airyscan and SEM Correlative Imaging Pipeline

We developed a novel workflow to correlate SEM with multicolor super-resolution Airyscan. Briefly, samples were first prepared for Airyscan imaging under wet conditions (PBS) after which the sample was dehydrated with ethanol followed by critical point drying to perform SEM imaging. Below, we provide a step-by-step detailed protocol for sample preparation (Figure 1).

Step 1. Mark Indium-Tin-Oxide (ITO) Coated Coverslips

22×22 mm ITO coated coverslips were marked in three corners with an engraving pen with diamond tip. Marking the corners of the coverslips allowed calibration of the motorized stages on both the Zeiss LSM880 confocal microscope with Airyscan detector (Carl Zeiss AG, Jena, Germany) as well as the Zeiss Sigma 300 Scanning Electron Microscope (Carl Zeiss AG, Jena, Germany),



saving time and effort in finding back the same cells on both instruments. After marking, coverslips were washed in 100% ethanol for sterilization and washed three times in PBS before seeding the cells.

Step 2. Seed Cells

Marked coverslips were placed in a 6 well plate and 0.2×10^6 cells/well were added to the well and left to adhere to the coverslips for 3 h in RPMI medium supplemented with 10% FCS before preparing the ventral plasma membranes (VPM). For DCs, 3 h is sufficient for strong cell adhesion to allow VPM preparation but other cell types may need a longer adherence time. To study the role of actin polymerization, cells were stimulated with $2.5 \mu\text{g/ml}$ cytochalasin D (CytoD) for 10 min prior to VPM preparation.

Step 3. Ventral Plasma Membrane Preparation

To prepare VPMs, cells were briefly sonicated. Sonication was performed using a Sartorius Labsonic P sonicator with cycle set at 1 and amplitude at 20% output. First, the sonicator tip was placed in a glass beaker containing 100 ml prewarmed hypotonic PHEM buffer (20% PHEM: 6 mM PIPES, 5 mM HEPES, 0.4 mM Mg_2SO_4 , 2 mM EGTA). Next, coverslips were taken from the 6 well plate and held 1–2 cm below the sonicator tip at a 45 degrees angle in the hypotonic PHEM solution. Cells were sonicated for ~ 1 s and directly after sonication, coverslips were transferred to a 6 well plate containing a prewarmed (37°C) PBS

solution with 4% paraformaldehyde and 0.05% glutaraldehyde and incubated for 30 min at room temperature. VPM efficiency was about 50% within the area that was closest to the tip (**Supplementary Figure 1A**).

Step 4. Stain, Fix and Add Fiducials

For staining, samples were first blocked for 60 min with PBS containing 20 mM glycine and subsequently incubated with the appropriate primary antibodies for 60 min. Samples were subsequently incubated for 60 min with the appropriate fluorescently labeled secondary antibodies together with fluorescently labeled phalloidin to visualize the actin cytoskeleton. Since Airyscan imaging is performed under wet conditions, conventional fluorophores could be used for protein visualization (Alexa488, Alexa568, and Alexa647). After staining, samples were post-fixed with 2% paraformaldehyde and fiducials were added (1:2,000 diluted $0.2 \mu\text{m}$ Tetraspeck beads). Tetraspeck beads are visible by both SEM and LM and can therefore be used as fiducials for precise image alignment after image acquisition.

Step 5. Airyscan Imaging

Prior to Airyscan imaging, coverslips were mounted into a self-designed low drift magnetic imaging chamber. First, the three marks at the edges of the coverslip were located and the coordinates of the marks were stored in the shuttle and find option in the Zeiss ZEN software. Images were obtained

at room temperature with a 63 × Plan Apochromat (1.4 NA) oil objective on the Airyscan array detector and processed using the Airyscan processing toolbox in the ZEN software. Comparing confocal with Airyscan laser scanning microscopy demonstrated the 1.7 increased resolution and the ability of Airyscan imaging to properly resolve diffraction limited actin filaments (**Supplementary Figure 2**) Only cells with a sufficient number of Tetraspeck beads (≥ 3) in the field of view and good VPM preservation were selected for correlative imaging.

Step 6. Fix With OsO₄ and Critical Point Drying (CPD)

After Airyscan imaging, samples were postfixed with 1% OsO₄ in 0.1 M phosphate buffer for 10 min, and washed in MQ. Samples were subsequently dehydrated in a graded ethanol series before critical point drying (Polaron E3000, Quorum Technologies Ltd., East Sussex, UK). Samples were finally sputtered with 5 nm chromium (Quorum Q150TS, Quorum Technologies Ltd., East Sussex, UK) before SEM imaging.

Step 7. SEM Imaging

SEM imaging was performed in a Zeiss Sigma 300 microscope (Carl Zeiss AG, Jena, Germany) equipped with the ATLAS 5 external scanner and software (Fibics, Canada) and the shuttle and find option in the Zeiss ZEN Blue software. The coordinates of the marks were recorded allowing the retrieval of the same field of view as acquired in the LSM880 Airyscan. With the SEM, unroofed cells were easily identified (**Supplementary Figure 1B**). SEM images were acquired at 3–5 kV, with a 30 nm aperture and a working distance of 7 mm using the InLens detector. Brightness and contrast were set at ~50 and ~30%, respectively. Large field of view images were recorded using the Zeiss Atlas software with a pixel resolution of 2 nm.

Step 8. Align Images Using Fiducials

After image acquisition, the immunofluorescence and SEM images were aligned using the Tetraspeck beads in the field of view. The center of mass of the fiducials was collected in the green fluorescence channel as well as the SEM image with ImageJ. Since the sample is distorted minimally from the LM to the SEM, we initially reasoned that a combination of scaling, translation and rotation would be sufficient to align the SEM and LM image. This, however, resulted in a poor image alignment and additional shearing was essential for proper image alignment (**Supplementary Figure 1C**). For image alignment, the *cpt2tform* and *imtransform* functions in Matlab were used to apply a 2D affine spatial transformation to the SEM image. Confirmation of alignment accuracy was done by post-alignment center of mass determination of fiducials and the mean alignment error was <10 nm. Alignment between the three fluorescence channels was not necessary as the beads already showed a near to perfect overlap between these channels.

Merging LM-SEM Images

After image alignment, images were prepared for LM-SEM merge using Fiji. First, LM images were thresholded with a local threshold with radius 5 (mean for actin images and phalloidin for vinculin and zyxin images). The resulting mask values of

255 were set to 90 and a Gaussian blur filter with radius 10 was applied. Appropriate lookup tables were applied (cyan, green and magenta) and the resulting images were merged with the SEM image.

Preparation of Human DCs

DCs were generated from monocytes, which were isolated from peripheral blood mononuclear cells as described previously (17, 18). Monocytes were derived either from buffy coats or leukapheresis products, purchased at Sanquin blood bank, Nijmegen, the Netherlands. Plastic-adherent monocytes were cultured for 6 days in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Greiner Bio-One, Kremsmünster, Austria), 1 mM Ultraglutamine (BioWhittaker, Inc., Walkersville, MD, USA), antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B; Gibco, Grand Island, NY, USA), IL-4 (300 U/mL), and GM-CSF (450 U/mL) in a humidified, 5% CO₂ containing atmosphere.

Antibodies and Materials

The following antibodies were used: mouse anti-vinculin (Sigma-Aldrich) and goat anti-zyxin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Alexa488-conjugated phalloidin (Invitrogen Corporation, Carlsbad, CA, USA) was used to stain F-actin. Cytochalasin D was purchased from Sigma-Aldrich. ITO coated coverslips were purchased from SPI supplies (West Chester, PA, USA) and were rubbed (3 strokes) over P400 grit size sandpaper to generate topographical cues. 0.2 µm Tetraspeck beads were purchased from Invitrogen (Carlsbad, CA, USA).

Fluorescence Profile Analysis

To quantify the localization vinculin, zyxin and actin in podosomes as a function of x, y, and z, we used a semi-automatic self-developed ImageJ macro that (1) recognizes the podosome core centers based on the actin image, (2) draws a vertical line of ~3 µm through the center of the core that rotates around its center (36 steps of 10 degrees) and collects an orthogonal view for every line, and (3) produces an average radial orthogonal view for each podosome core. We used these radial orthogonal views to retrieve the fluorescence profiles as a function of x, y, and z. For the x, y profiles, horizontal lines were drawn through the orthogonal views and intensity profiles were taken at the four corresponding z-sections for each channel for each podosome. For the z profiles, a diagonal line was drawn through the orthogonal views at a distance of 200 nm (zyxin), 360 nm (vinculin), 0 nm (core actin) and 1,000 nm (network actin) from the podosome core center. All profiles were normalized to the minimum and maximum (of all z-sections) for visualization and comparison. For the CytoD fluorescence intensity profiles as a function of the distance from the podosome center, the values were normalized to the control values on VPMs for comparison. All ImageJ macro codes used for the analyses in this manuscript are available upon request.

RESULTS

Ventral Plasma Membrane (VPM) Preparation and Critical Point Drying (CPD) Preserves Podosome Structure

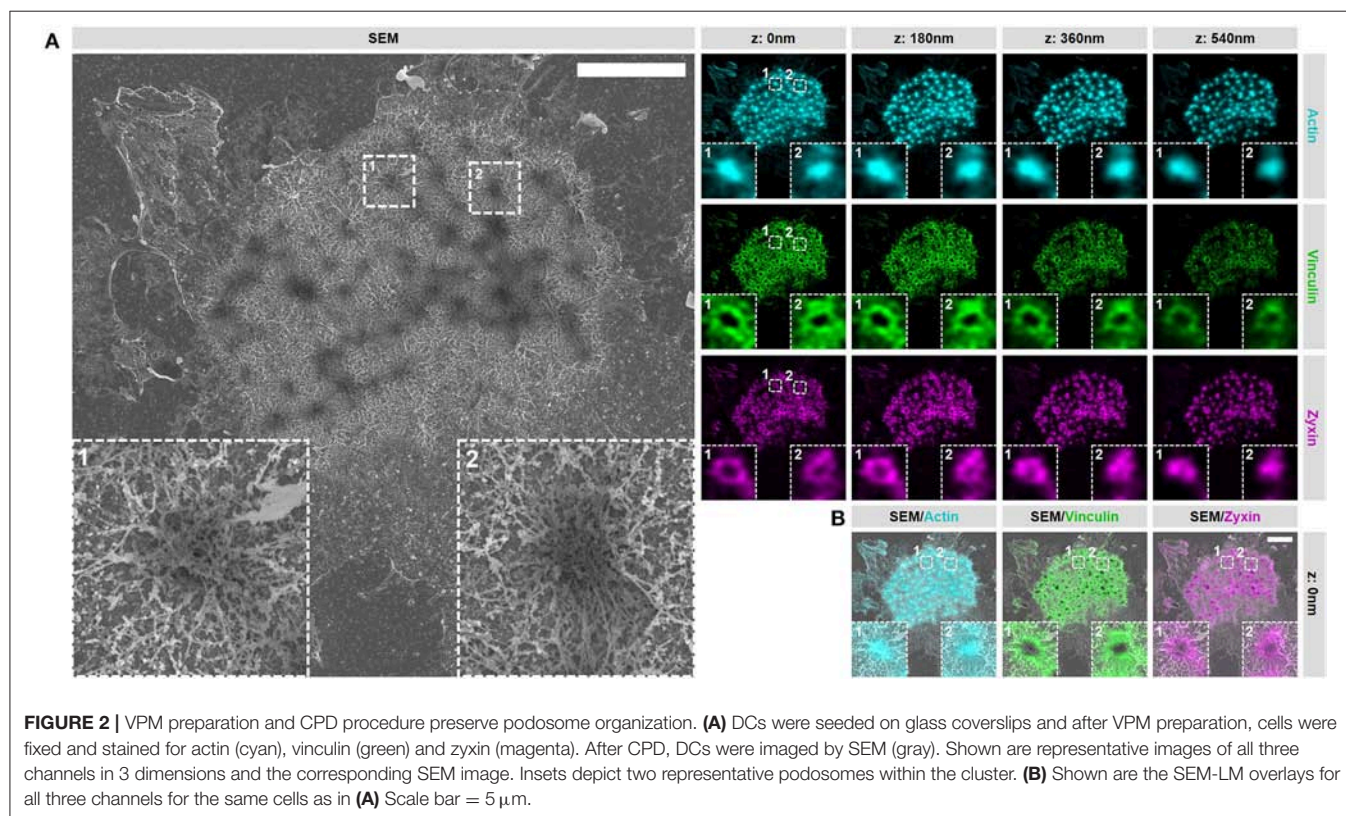
In order to gain access to the ultrastructure of podosomes by SEM, the cell has to be unroofed (VPM preparation) and dried by CPD, both harsh procedures that could alter the structure of cellular structures. To investigate whether cellular actin features are sufficiently preserved, we examined the organization and structure of podosomes after SR-CLEM sample preparation. For this, we first prepared VPMs and labeled them for actin to visualize the podosome core and radiating filaments, and for vinculin and zyxin to label the podosome ring. By acquiring 3D image stacks with Airyscan super-resolution microscopy, we observed that the actin cores as well as the radiating actin filaments were preserved after VPM preparation (Figure 2A). To note, Airyscan microscopy, in contrast to conventional confocal microscopy, resolves the network of diffraction limited actin filaments that have been shown to radiate from podosome cores before by SEM (9) and STORM super-resolution microscopy (8). We also found that vinculin and zyxin were still present in the podosome cluster after VPM preparation (Figure 2A). Moreover, we observed a specific localization of vinculin to the radiating actin filaments as we had observed previously with STORM super-resolution microscopy (8). We next performed CPD and imaged the VPMs by SEM. We observed that podosome clusters, individual podosomes and the associated network of radiating

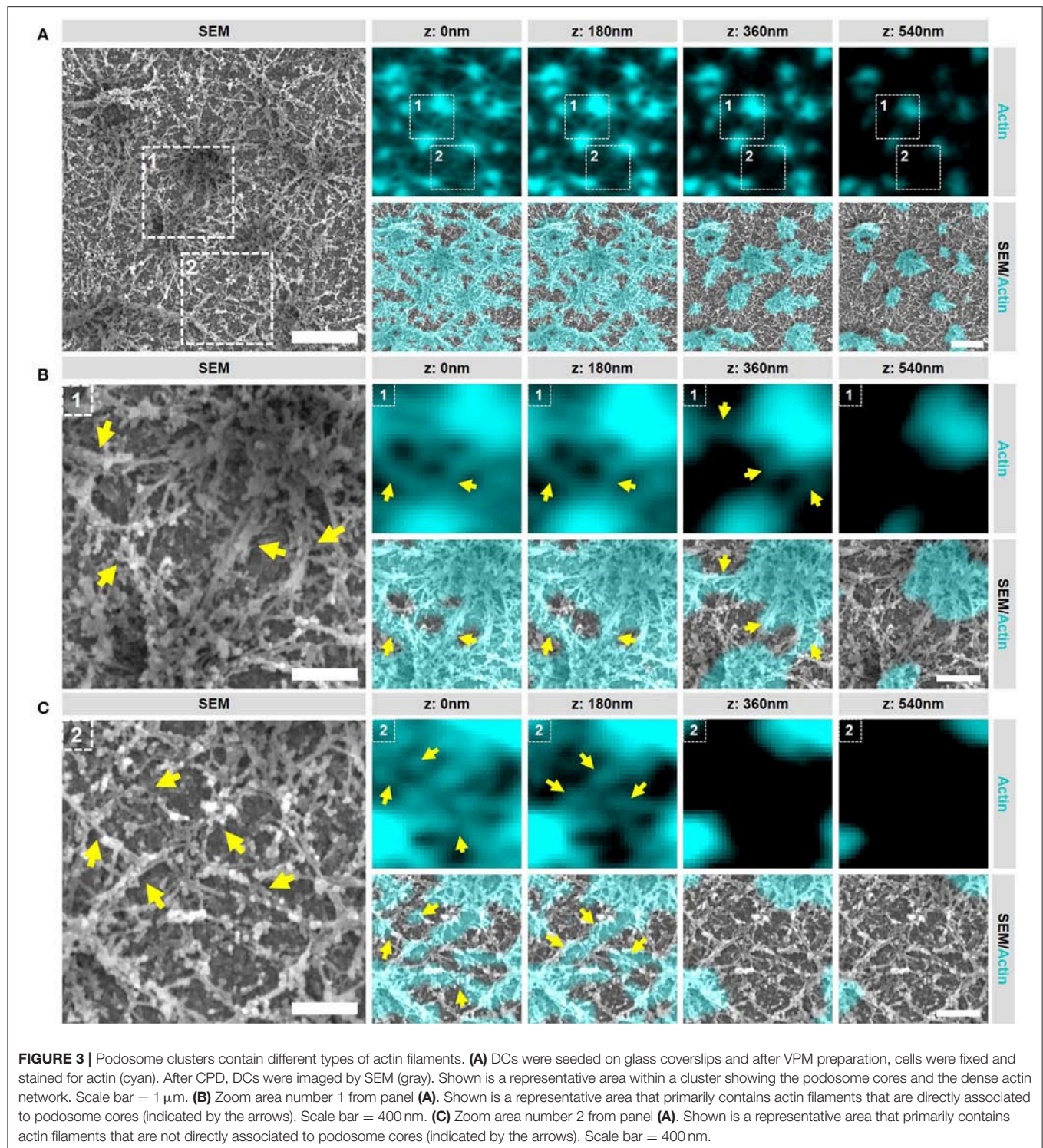
filaments were easily identified in the SEM image (Figure 2A). Moreover, after correlating the Airyscan and SEM images, we noted a near perfect overlay of these podosomal features, which can be observed by both imaging modalities, across the entire cell (Figure 2B).

Together, these results indicate that proteins associated with the core and ring are still present within podosome clusters on VPMs and demonstrate that the podosome structure is sufficiently preserved after VPM preparation for further investigation. Moreover, they also demonstrate that our SR-CLEM imaging pipeline allows the simultaneous visualization of at least three proteins in LM, all of which can be correlated with the ultrastructural information obtained by SEM across entire cells.

Podosome Clusters Contain Different Types of Actin Filaments

Having established our SR-CLEM imaging pipeline, we next aimed to study several podosome characteristics that have proved to be challenging by LM alone. As such, we first investigated the ultrastructural organization of actin in podosome clusters of human DCs. Interestingly, when observed by SEM, the cluster clearly stands out from the rest of the VPM with respect to the density of filamentous structures (Supplementary Figure 3). In fact, the entire cluster appears to be characterized by a very thick layer of filaments and correlating the SEM with the LM indicated that these filaments, as expected, are composed of actin (Figure 3A). Interestingly, at least two types of actin





filaments can be identified in the podosome cluster based on SR-CLEM. Firstly, and as anticipated, the cluster contains actin filaments that radiate from the podosome core (**Figure 3B**). These filaments are associated with the top of the core and run down to the plasma membrane where, in many cases, they appear to associate with filaments that originate from neighboring cores.

Secondly, and much less anticipated, there are many filaments that do not appear to be associated with podosome cores (**Figure 3C**). These filaments are restricted to the ventral side of the plasma membrane and appear to assemble a thick layer of cortical actin that is present at the bottom of the entire podosome cluster.

These results indicate that multiple types and layers of actin filaments are present in the podosome cluster and reveal that the actin network around podosomes in DCs is much more complex than previously anticipated. They further indicate that the SEM ultrastructural information provides a unique insight into the actin organization of podosome clusters in DCs which can be used to further study the localization of podosome associated proteins.

Vinculin and Zyxin Associate With Different Structures in Podosomes

We have shown before that vinculin and zyxin have a different organization in podosome rings, with the vinculin ring being slightly larger compared to the zyxin ring (15). Furthermore, we have shown that vinculin recruitment is dependent on the integrity of the radiating actin filaments while the recruitment of zyxin is not (12). Considering that both these proteins are

classified as ring proteins, these differences in the organization and regulation of vinculin and zyxin prompted us to study the detailed organization of these adaptor proteins by our SR-CLEM procedure. **Supplementary Figure 4** and **Figure 4** show a representative individual podosome with long radiating actin filaments that is located within a large cluster. By analyzing the localization of vinculin, we found that it is very much enriched around the core and clearly present in the ring region. By correlating the vinculin fluorescence with the SEM actin ultrastructural information, we observed that vinculin is especially enriched at sites where the radiating filaments seem to connect the ventral plasma membrane, resulting in a non-continuous ring around the core (**Figure 4B**, arrows). Unexpectedly, vinculin is also localized to the smaller ventral actin filaments that do not appear to associate with a podosome core (**Figure 4B**, lower 2 planes). Overall, the 3D Airyscan images clearly indicate that the majority of vinculin is localized very

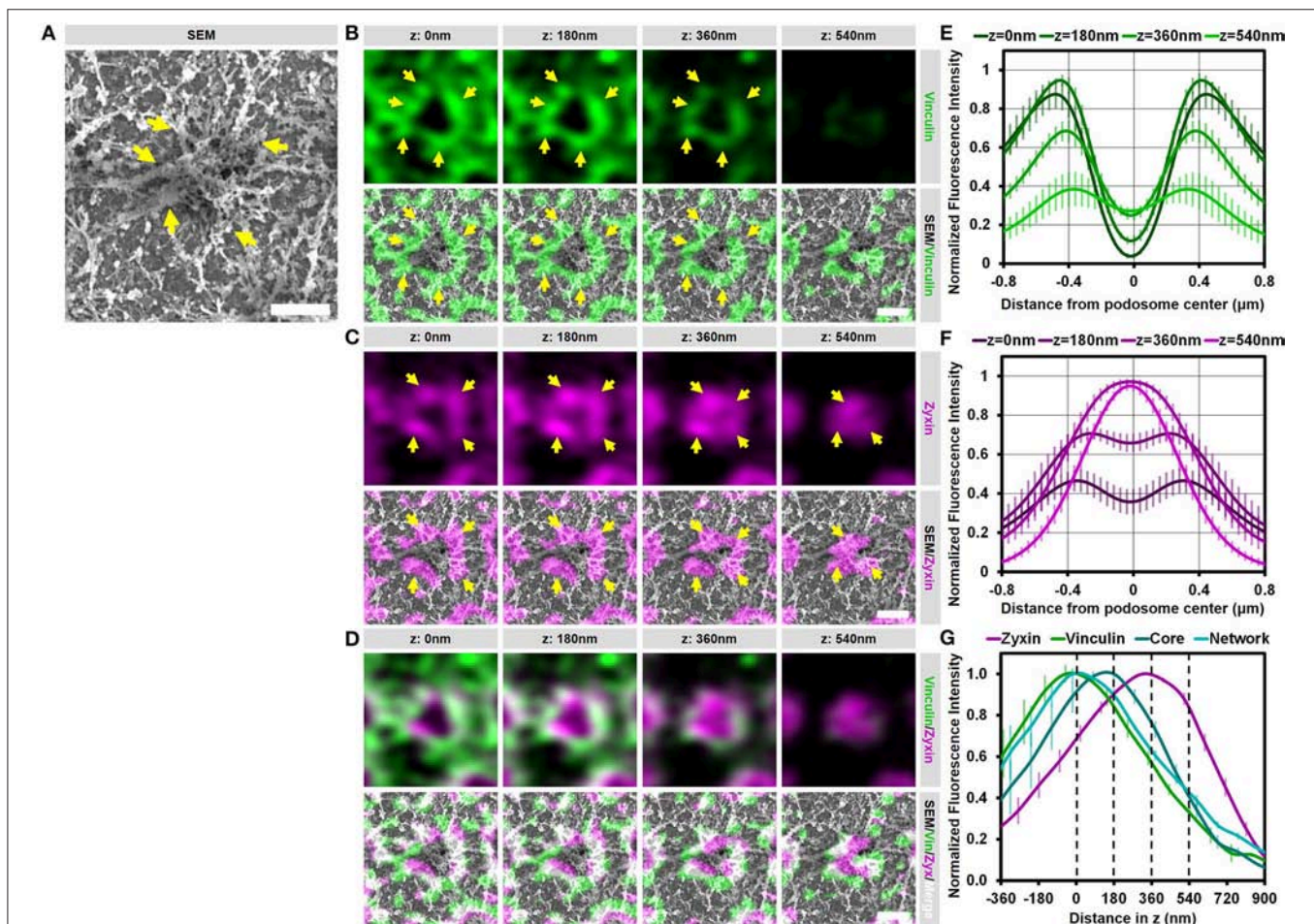


FIGURE 4 | Vinculin and zyxin associate with different structures in podosomes. (A–D) DCs were seeded on glass coverslips and after VPM preparation, cells were fixed and stained for vinculin (green, **B**), zyxin (magenta, **C**) and actin (cyan, **Supplementary Figure 4**). Overlay between vinculin and zyxin is shown in white (**D**). After CPD, DCs were imaged by SEM (gray, **A**). Shown is a representative podosome with radiating filaments that are clearly observed in the SEM image. Arrows indicate areas enriched for vinculin or zyxin, which overlap with the radiating actin filaments (also indicated by arrows). Scale bar = 500 nm. (E–F) Radial fluorescence profile analysis of vinculin (**E**) and zyxin (**F**) at each of the four z-sections depicted in (B–D). Shown is the average ± SEM ($n = 312$ podosomes, 5 cells). (G) Quantification of the localization in z of zyxin, vinculin, core actin and network actin in podosomes. The z-sections shown in (B–D) are represented by the dashed lines in the graph. Shown is the average ± SEM ($n = 312$ podosomes, 5 cells).

proximal to the plasma membrane. For zyxin, we observed a surprisingly different organization. Firstly, zyxin is almost completely absent from the small actin filaments that do not associate with podosome cores (**Figure 4C**, lower 2 planes). Secondly, although zyxin, like vinculin, is enriched at sites where the radiating filaments are located, zyxin localizes more closely to the podosome cores and is especially enriched at the top of podosomes (**Figure 4C**, arrows). Thirdly, zyxin localizes more distal from the plasma membrane compared to vinculin (**Figures 4C,D**).

To further substantiate our visual inspection, we quantified the localization of vinculin, zyxin, and actin for hundreds of podosomes in multiple cells in both the lateral and the axial direction. For this, fluorescence intensity profiles of vinculin, zyxin and actin were generated both as a function of the distance from the podosome center (**Figures 4E,F** and **Supplementary Figure 4**) as well as the distance in *z* (**Figure 4G**). First, the intensity profiles as a function of the distance from the podosome center clearly indicated that vinculin is localized more distant from the podosome center compared to zyxin (**Figures 4E,F**). Importantly, while vinculin localization remains ring-shaped at all *z*-sections analyzed, zyxin is clearly not at higher planes, strongly suggesting that zyxin is not only associated to the radiating actin filaments but also to the top of the podosome core. Second, the intensity profiles as a function of distance in *z* clearly indicated that vinculin is localized more close to the membrane compared to zyxin (**Figure 4G**). While vinculin intensity completely overlaps with the actin network intensity, zyxin intensity peaks at much higher *z*-sections, most likely corresponding to the top of podosome cores. Importantly, we performed the same quantitative analysis in intact cells and observed a very similar organization for vinculin (**Supplementary Figure 5**), zyxin and actin suggesting that our results on the VPMs are not a result from an artifact from the complex sample preparation.

Together, these correlative data and quantitative analyses reveal that vinculin and zyxin have a very different organization and strongly suggest that vinculin is specifically recruited to sites where actin filaments connect to the membrane while zyxin appears to decorate the podosome core from the middle to the top.

Closely Associated Podosomes Are Connected by Actin Filaments Positive for Zyxin

Podosomes undergo continuous fission and fusion events resulting in closely associated neighboring podosomes (19, 20). These events are mechanistically very poorly understood and we therefore decided to study them in greater detail with our SR-CLEM procedure. Importantly, for this study, podosomes were defined as closely associated when two separate actin cores were surrounded by one continuous vinculin ring. With these criteria, we selected two representative events of closely associated podosomes (**Figure 5** and **Supplementary Figures 6, 7**). Firstly, we observed that, similar to individual podosomes described above, vinculin and zyxin are very differently organized

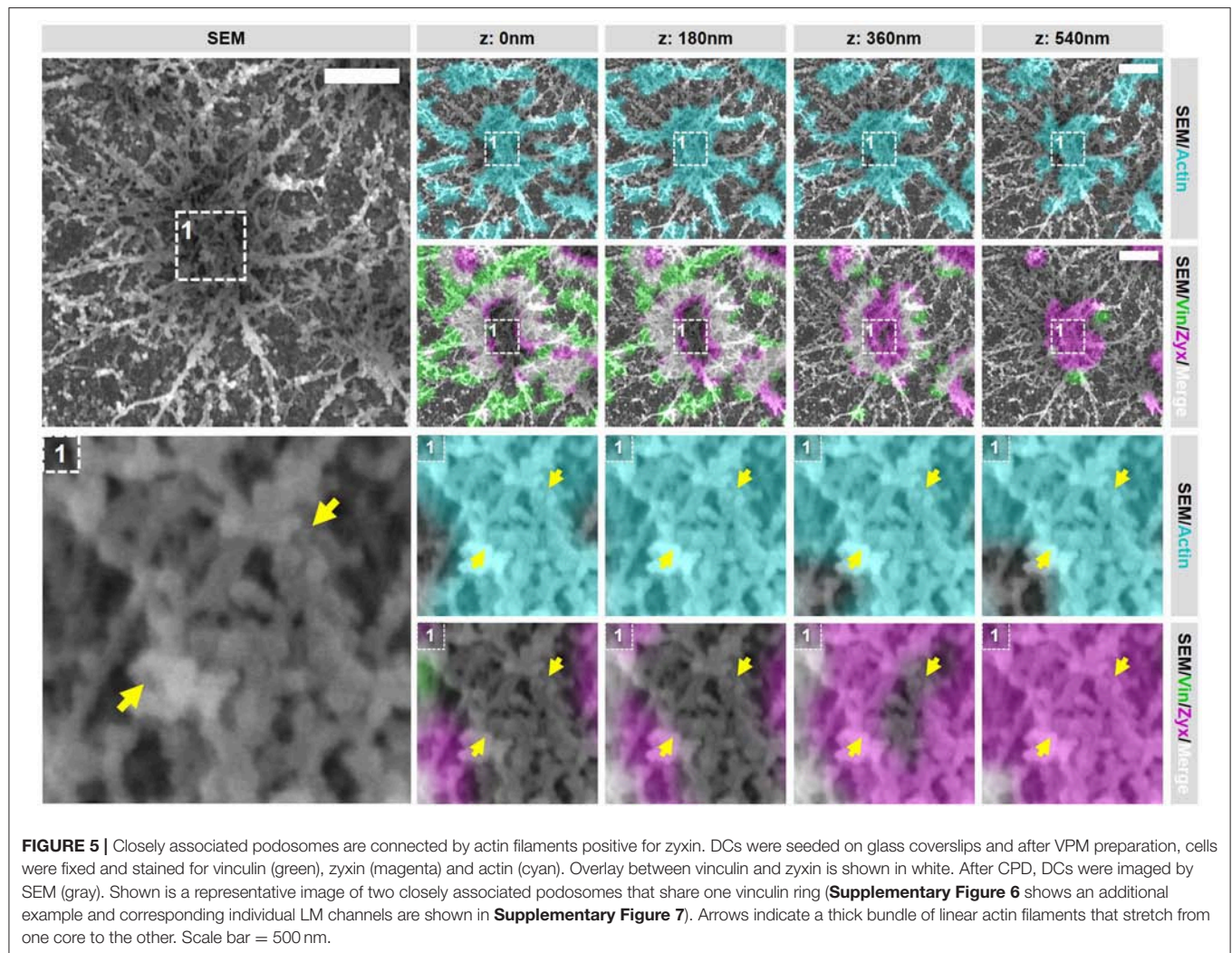
in these closely associated podosomes. Vinculin is present proximal to the plasma membrane and associates with the actin filaments that radiate from the two podosome cores while zyxin is localized more closely to the two cores (**Figure 5**, **Supplementary Figures 6, 7**). Moreover, while at this stage, vinculin is present around the two cores and not in between, resulting in one continuous vinculin ring around the two actin cores, zyxin is clearly associated with and entirely covers the two separate cores (**Figure 5**, **Supplementary Figure 6**). Together, these data strengthen the notion that vinculin and zyxin have very different functions at podosomes. While vinculin may specifically stabilize the filaments around podosome cores, zyxin is likely involved in stabilizing filaments that cover the podosome core.

Importantly, when closely analyzing the SEM ultrastructural information of these closely associated podosomes, we noted a structure that has not been described before. In both representative examples, the closely associated podosomes appear to be connected by a thick bundle of linear actin filaments that stretch from one core to the other (**Figure 5** and **Supplementary Figure 6**, arrows). This bundle of actin filaments was almost always observed [41 out of 44 (93%) events analyzed], strongly suggesting that this structure is a general and important feature of podosomes that are closely associated. Interestingly, these filaments are associated with zyxin at the top but not with vinculin suggesting that they do not associate with the plasma membrane and may originate from the core actin.

Substrate Topology Induces Interpodosomal Connections

We previously demonstrated that substrate topographical cues induce the close association of podosomes (21). To study if this induced association of podosomes by substrate topology is similar to the closely associated podosomes on flat surfaces, we seeded DCs on coverslips with manually made scratches and investigated the organization of podosomes on top of these scratches with SR-CLEM. As shown before, we first noticed that the surface topography induced the alignment of podosomes and their close association (**Supplementary Figure 8**). To analyze the localization of vinculin and zyxin as well as the SEM ultrastructural information, we selected two representative arrays of multiple closely associated podosomes, where the different actin cores could still be recognized (**Figure 6A** and **Supplementary Figures 9, 10**). Interestingly, similar to the closely associated podosomes on a flat surface, vinculin was specifically present at the actin filaments around the cores but not in between, resulting in one large vinculin ring encircling sometimes as many as 5 actin cores (**Supplementary Figure 8**). Also for zyxin, we noted a similar localization when compared to the podosomes on a flat surface, with zyxin being closely associated with the side and top of each individual podosome core.

To substantiate these findings for podosomes on topographical cues, we generated fluorescence intensity profiles as a function of the distance from the podosome center (**Figure 6B,C** and **Supplementary Figure 11**) and the distance in *z* (**Figure 6D**). The intensity profiles as a function of the distance



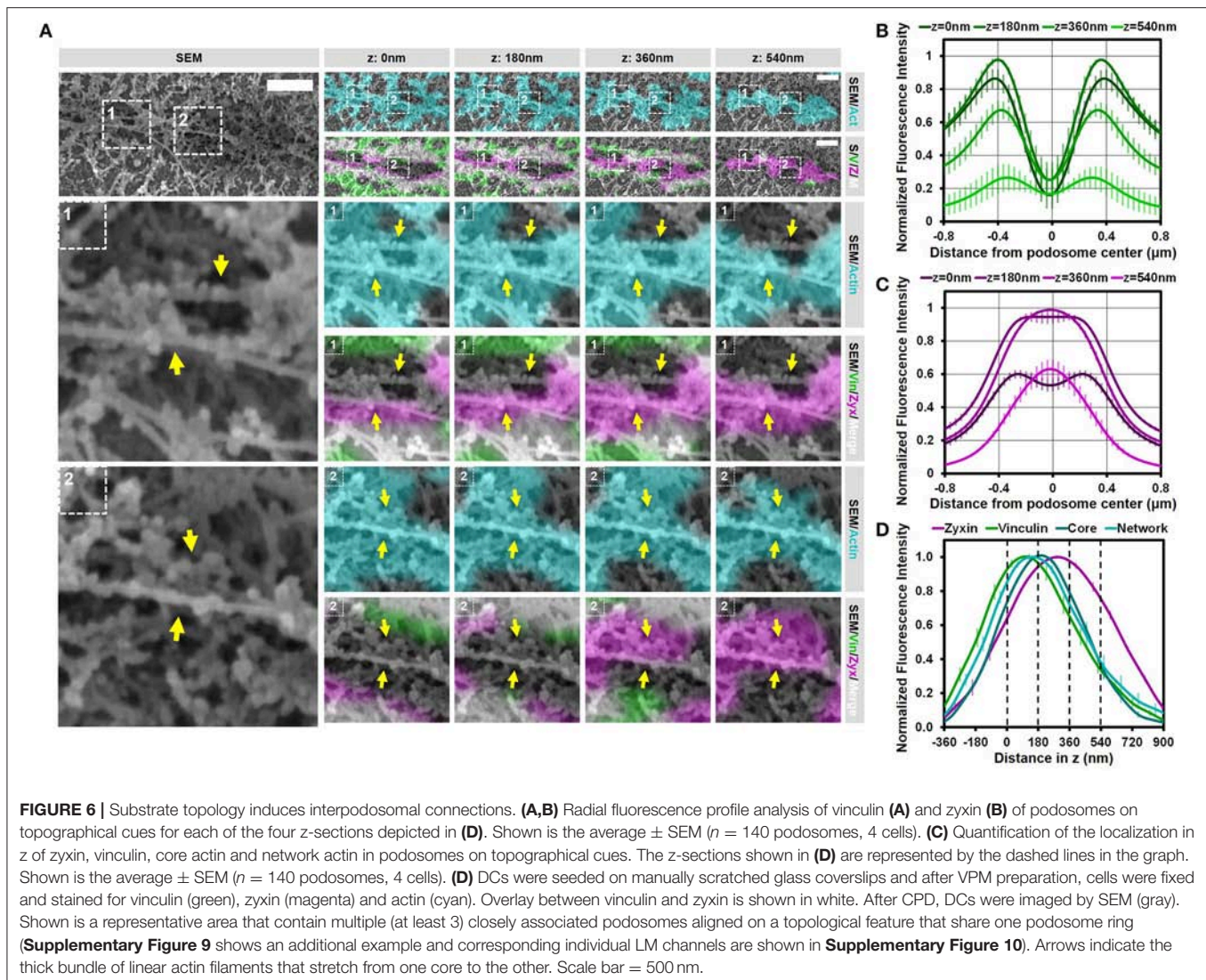
from the podosome center were very similar to those on flat surfaces, demonstrating that, also on topographical cues, zyxin is localized more closely to the podosome core as compared to vinculin. Interestingly, while the intensity profiles as a function of distance in *z* clearly indicated that the proteins were separated in the axial direction similar to the proteins on flat surfaces, the differences were much smaller on topographical cues, something which may be caused by the core protruding in the manually made scratch. Overall, our results indicate that the topographical cues induces only small changes in the localization of the adaptor proteins despite the variety in actin structures induced by the topographical cues.

We next investigated whether the interpodosomal connections observed on a flat surface were also present between podosome cores on topographical cues. Interestingly, we indeed clearly observed linear actin filaments that seem to connect two neighboring cores in our representative examples (**Figure 6A** and **Supplementary Figure 9**, arrows). Quantitative analysis showed that in 41 out of 41 (100%) events, these linear actin filaments were present, suggesting that, also on

topographical cues, these filaments play an important role in regulating the close association of two neighboring podosomes. Moreover, these actin filaments were positive for zyxin but not for vinculin, suggesting that the nature of these filaments on the topographical cues is very similar compared to a flat surface. These filaments may therefore be a general feature of podosomes that are closely associated, and possibly undergo fission or fusion.

Actin Polymerization Essential for the Integrity of Vinculin-Associated Filaments but Not for Zyxin-Associated Interpodosomal Connections

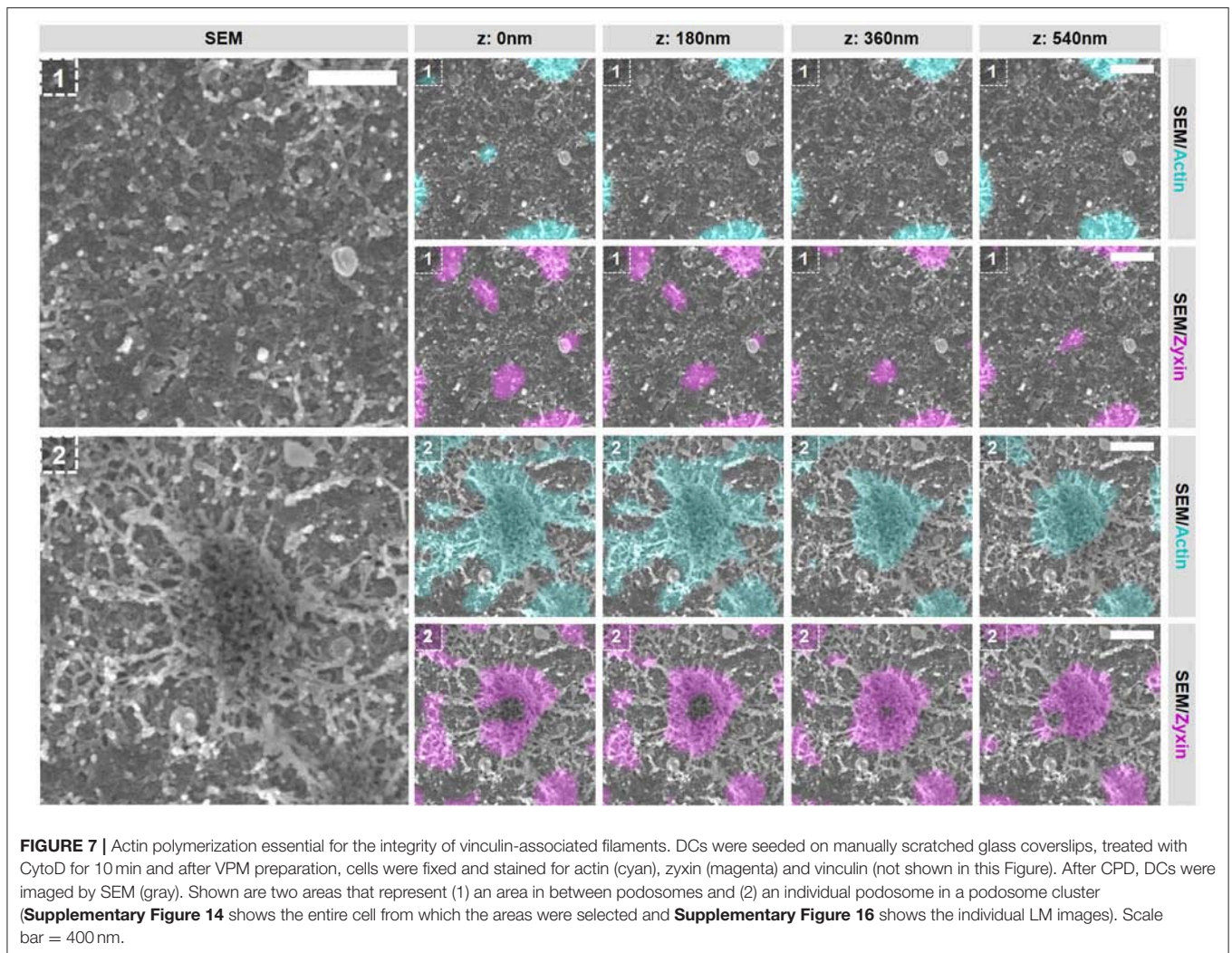
So far, our results strongly suggest that vinculin and zyxin primarily associate to different actin structures within podosome clusters. To investigate whether these different actin structures have a different dynamic regulation, we performed SR-CLEM on cells treated with CytoD, an inhibitor of actin polymerization. We have shown before that inhibition of actin polymerization by CytoD causes the immediate disappearance of vinculin and



the actin filaments from podosome clusters while zyxin and the podosome cores remain present for prolonged times (12). We therefore first examined the localization of vinculin, zyxin and actin in podosomes by SR microscopy after the inhibition of actin polymerization and confirmed these published observations (**Supplementary Figures 12, 13**). It should be noted though that, while zyxin localization is still mostly intact, the ring-shaped localization is less pronounced and the distance between the remaining network and zyxin is decreased after treatment with CytoD (**Supplementary Figure 13**), indicating that minor changes occur in the organization of the podosome core and associated zyxin after inhibition of actin polymerization.

To evaluate the integrity of the actin structures within podosome clusters after the inhibition of actin polymerization, we first selected 2 areas in the podosome cluster representing (1) an area in between podosome cores and (2) an individual podosome within the cluster (**Figure 7** and **Supplementary Figures 14, 16**). Firstly, we observed large

changes in the area in between podosome cores (**Figure 7**, region 1). Whereas comparable areas in non-treated cells are completely covered with actin filaments that do not appear to be associated with podosomes (**Figure 3C**), these have all disappeared after inhibition of actin polymerization. Secondly, we noted that also the large filaments that connect to podosome cores have mostly disappeared (**Figure 7**, region 2). We did observe some actin filaments that remained associated to the core but these are mostly present at the bottom and no longer at the top of podosome cores. Together, these results indicate that a majority of the actin filaments that appeared to be vinculin-associated by our SR-CLEM observations have disappeared after inhibition of actin polymerization and may therefore reflect the most dynamic pool of actin within the podosome cluster. Interestingly, by SEM, we observed that the podosome core itself appeared to be mostly intact (**Figure 7**, region 2), correlating with the prolonged presence of zyxin and suggesting that the core actin is less dynamic.

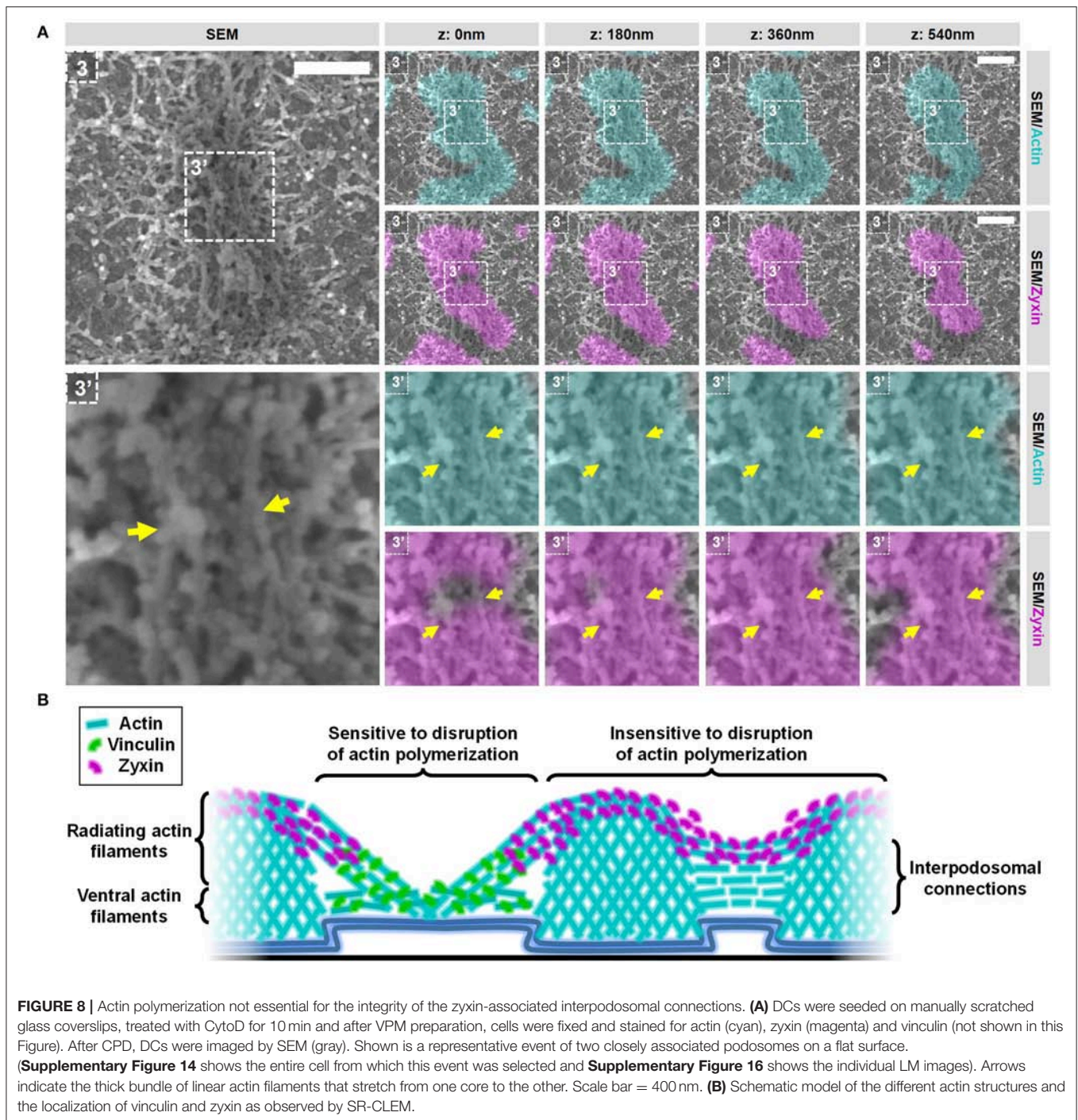


Next, we selected 2 areas representing closely associated podosomes on a flat surface and a topographical cue (**Figure 8** and **Supplementary Figures 15, 16**) to study the integrity of the interpodosomal connections after inhibition of actin polymerization. Strikingly, and in sharp contrast to the vinculin-associated filaments, we still very frequently observed interpodosomal connections between two closely associated podosomes, both on flat surfaces (**Figure 8**, arrows) and on topographical cues (**Supplementary Figure 15**, arrows). Although our initial criteria could not be used in identifying closely associated podosomes on flat surfaces since vinculin was completely absent, we still observed 26 interpodosomal connections in 4 cells on flat surfaces and in 20 out of 22 neighboring podosomes (91%) on topographical cues, which was very much comparable to our observations in untreated cells. Interestingly, these interpodosomal connections did not only appear to be completely intact based on inspection of the SEM image, they were also still associated with zyxin (**Figure 8** and **Supplementary Figure 15**). Together, these data indicate that the interpodosomal connections are resistant to the inhibition

of actin polymerization and may represent a less dynamic pool of actin that is different in nature compared to the vinculin-associated ventral or radiating filaments.

DISCUSSION

We here developed and optimized a correlative multicolor Airyscan super-resolution and SEM imaging pipeline to study the organization of the ventral plasma membrane. With our procedure, we achieve a near perfect overlay of at least three fluorescent channels with a SEM image across entire cells and reveal novel insights into the ultrastructural organization of podosomes in DCs. We show that the actin network at podosome clusters is more dense and complex than previously anticipated. Further, we find that zyxin, classically known as a podosome ring protein, appears to be more associated with the core. Also, we show that closely associated podosomes on flat surfaces as well as topographical cues are connected by thick actin filaments that are associated with zyxin, but not with vinculin. Finally, we show that actin filaments present in the podosome cluster have a differential



sensitivity to inhibition of actin polymerization suggesting that they are dynamically distinct pools of actin.

We show that podosome clusters in DCs contain many different types and layers of actin filaments (**Figure 8B**), which is clearly different from the rest of the cell where the actin network is much thinner. While some of the identified filaments in the podosome cluster clearly associate with the side of podosome cores, others do not, something which has not been

described before. Filaments that do not associate with the side of the core are much smaller and located closer to the plasma membrane. Based on our data, these filaments could either be not associated to podosome cores at all or only at the bottom of podosomes. The complex organization of dense actin filaments around podosomes was already shown for the sealing zone in osteoclasts (9). The sealing zone, however, is a specialized bone resorbing organelle in osteoclasts, and it was unclear

to what extent its actin organization could be compared to podosome clusters in other cell types. We showed before that the enrichment of α M β 2 integrins and talin specifically delineates the podosome cluster in DCs (8). Together with the specialized actin organization presented here, these results clearly demonstrate that the podosome cluster in DCs is a region at the ventral plasma membrane that should be considered as a separate regulatory platform. For future experiments, it would be interesting to focus on unraveling the exact nature of these different filaments and how they contribute to the different podosome functions in DCs such as protrusion and mechanosensing. We expect that the large core-associated actin filaments stabilize podosomes cores and contribute to protrusion while the small actin filaments close the plasma membrane may have a function in vesicle transport within the podosome cluster or the positioning of the podosome cores.

Vinculin and zyxin are two adaptor proteins that are classically associated with the podosome ring (5). Using our SR-CLEM procedure, we here show that zyxin is in fact for the most part associated with the podosome core rather than the ring (**Figure 8B**). This novel finding on the differential organization of vinculin and zyxin further extends our previous findings that zyxin is located more close to the core than vinculin (15) and that zyxin localization in podosomes is not dependent on the integrity of the actin filaments that are located in the ring region (12). Furthermore, our result that zyxin is located in a higher vertical plane than vinculin matches the nanoscale organization of these proteins in focal adhesions (16), indicating structural similarities between the radiating filaments and focal adhesions. Interestingly, the localization of zyxin in podosomes that we reveal here is very reminiscent of podosome cap proteins such as supervillin (22) and LSP-1 (23). We therefore propose that zyxin is present in the podosome cap mainly at site where the filaments radiate from the core. In the cap, zyxin could be involved in the repair of actin filaments that are under high stress due to the protrusive forces of podosomes, as has been shown for strained stress fibers (14). Next step would be to unravel the mutual localization of other podosome components with our SR-CLEM procedure, which may also have been misclassified.

Podosomes continuously split and fuse but the function and mechanism for these events are very poorly understood. One study shows that podosome fission is a means of new podosome assembly at the leading edge of macrophages (19). Furthermore, it was shown that fission and fusion are regulated by microtubules and are therefore thought to be active processes that are tightly regulated (19, 20). Here, we identify a novel actin-based structure that connects two closely associated podosome cores, which are likely in the process of fusion or fission since they share a vinculin ring. This structure has not been observed before, probably due to the density of actin features in LM images. This newly identified structure also connects closely associated podosomes that align along the edges of topographical cues suggesting that this structure may have a general function in interpodosomal communication and podosome mechanosensing. Interestingly, the integrity of this structure appeared to be insensitive for inhibition of actin polymerization suggesting that it represents a slow dynamic pool of actin that is clearly different from actin in the radiating actin filaments, which rapidly lose their

integrity after actin polymerization is blocked. Lastly, the fact that this structure is positive for zyxin suggests that this structure may be under tension in steady state conditions, possibly created by pushing and pulling from the two podosome cores. It would now be particularly interesting to classify these closely associated podosomes by performing VPM preparation immediately after single cell live imaging, to reveal whether this novel structure is particularly important for fusion, fission or both.

In conclusion, we here demonstrate that our novel SR-CLEM imaging pipeline is a valuable tool for revealing the mutual localization of different proteins in complex multimolecular structures. Using our workflow, we were able to reveal novel ultrastructural details of podosomes in DCs which would have been extremely challenging by LM alone. To provide an even more detailed view on the organization of multiple proteins with respect to the actin ultrastructure, effort should now be put in adapting our imaging pipeline for correlating multicolor 3D-STORM with SEM, theoretically feasible with our procedure since the LM is performed under wet conditions. Lastly, we envisage that our workflow can further be used to study the organization of other multimolecular structures at the ventral plasma membrane such as clathrin coated pits, the cortical actin network, focal adhesions and the immune synapse.

ETHICS STATEMENT

All experiments involving human material were carried out after obtaining written informed consent from all subjects as per the Declaration of Helsinki. The study was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center, Commissie Mensgebonden Onderzoek.

AUTHOR CONTRIBUTIONS

BJ optimized the SR-CLEM imaging pipeline, performed all experiments and acquired the images. MW, JF, AC, and KvdD provided input for optimizing the SR-CLEM imaging pipeline. BJ and KvdD optimized image alignment and the LM-SEM merge procedure. BJ, AC, and KvdD designed the study and interpreted the data. AC and KvdD supervised the study. KvdD prepared the figures and wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

The authors thank the Microscopic Imaging Centre of the Radboud Institute for Molecular Life Sciences for use of their microscopy facilities. The authors also thank Norbert Hermesdorf and the Technical Support Group of the Radboud University for assistance in developing the magnetic imaging chamber. This research was supported by intramural funding from the Radboudumc.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01908/full#supplementary-material>

REFERENCES

- De Boer P, Hoogenboom JP, Giepmans BN. Correlated light and electron microscopy: ultrastructure lights up! *Nat Methods* (2015) 12:503–13. doi: 10.1038/nmeth.3400
- Schermelleh L, Heintzmann R, Leonhardt H. A guide to super-resolution fluorescence microscopy. *J Cell Biol*. (2010) 190:165–75. doi: 10.1083/jcb.201002018
- Sochacki KA, Shtengel G, Van Engelenburg SB, Hess HF, Taraska JW. Correlative super-resolution fluorescence and metal-replica transmission electron microscopy. *Nat Methods* (2014) 11:305–8. doi: 10.1038/nmeth.2816
- Kopek BG, Paez-Segala MG, Shtengel G, Sochacki KA, Sun MG, Wang Y, et al. Diverse protocols for correlative super-resolution fluorescence imaging and electron microscopy of chemically fixed samples. *Nat Protoc*. (2017) 12:916–46. doi: 10.1038/nprot.2017.017
- Linder S, Wiesner C, Himmel M. Degrading devices: invadosomes in proteolytic cell invasion. *Annu Rev Cell Dev Biol*. (2011) 27:185–211. doi: 10.1146/annurev-cellbio-092910-154216
- Cougoule C, Lastrucci C, Guet R, Mascarau R, Meunier E, Lugo-Villarino G, et al. Podosomes, but not the maturation status, determine the protease-dependent 3D migration in human dendritic cells. *Front Immunol*. (2018) 9:846. doi: 10.3389/fimmu.2018.00846
- Baranov M, Ter Beest M, Reinieren-Beeren I, Cambi A, Figdor CG, Van Den Bogaart G. Podosomes of dendritic cells facilitate antigen sampling. *J Cell Sci*. (2014) 127:1052–64. doi: 10.1242/jcs.141226
- Van Den Dries K, Schwartz SL, Byars J, Meddens MB, Bolomini-Vittori M, Lidke DS, et al. Dual-color superresolution microscopy reveals nanoscale organization of mechanosensory podosomes. *Mol Biol Cell* (2013) 24:2112–23. doi: 10.1091/mbc.e12-12-0856
- Luxenburg C, Geblinger D, Klein E, Anderson K, Hanein D, Geiger B, et al. The architecture of the adhesive apparatus of cultured osteoclasts: from podosome formation to sealing zone assembly. *PLoS ONE* (2007) 2:e179. doi: 10.1371/journal.pone.0000179
- Schmidt S, Nakchbandi I, Ruppert R, Kawelke N, Hess MW, Pfaller K, et al. Kindlin-3-mediated signaling from multiple integrin classes is required for osteoclast-mediated bone resorption. *J Cell Biol*. (2011) 192:883–97. doi: 10.1083/jcb.201007141
- Luxenburg C, Winograd-Katz S, Addadi L, Geiger B. Involvement of actin polymerization in podosome dynamics. *J Cell Sci*. (2012) 125:1666–72. doi: 10.1242/jcs.075903
- Van Den Dries K, Meddens MB, De Keijzer S, Shekhar S, Subramaniam V, Figdor CG, et al. Interplay between myosin IIA-mediated contractility and actin network integrity orchestrates podosome composition and oscillations. *Nat Commun*. (2013) 4:1412. doi: 10.1038/ncomms2402
- Ziegler WH, Liddington RC, Critchley DR. The structure and regulation of vinculin. *Trends Cell Biol*. (2006) 16:453–60. doi: 10.1016/j.tcb.2006.07.004
- Smith MA, Blankman E, Gardel ML, Luettjohann L, Waterman CM, Beckerle MC. A zyxin-mediated mechanism for actin stress fiber maintenance and repair. *Dev Cell* (2010) 19:365–76. doi: 10.1016/j.devcel.2010.08.008
- Meddens MB, Rieger B, Figdor CG, Cambi A, Van Den Dries K. Automated podosome identification and characterization in fluorescence microscopy images. *Microsc Microanal*. (2013) 19:180–9. doi: 10.1017/S1431927612014018
- Kanchanawong P, Shtengel G, Pasapera AM, Ramko EB, Davidson MW, Hess HF, et al. Nanoscale architecture of integrin-based cell adhesions. *Nature* (2010) 468:580–4. doi: 10.1038/nature09621
- Thurner B, Roder C, Dieckmann D, Heuer M, Kruse M, Glaser A, et al. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J Immunol Methods* (1999) 223:1–15. doi: 10.1016/S0022-1759(98)00208-7
- De Vries IJ, Eggert AA, Scharenborg NM, Vissers JL, Lesterhuis WJ, Boerman OC, et al. Phenotypical and functional characterization of clinical grade dendritic cells. *J Immunother*. (2002) 25:429–38. doi: 10.1097/00002371-200209000-00007
- Evans JG, Correia I, Krasavina O, Watson N, Matsudaira P. Macrophage podosomes assemble at the leading lamella by growth and fragmentation. *J Cell Biol*. (2003) 161:697–705. doi: 10.1083/jcb.200212037
- Kopp P, Lammers R, Aepfelbacher M, Woehlke G, Rudel T, Machuy N, et al. The kinesin KIF1C and microtubule plus ends regulate podosome dynamics in macrophages. *Mol Biol Cell* (2006) 17:2811–23. doi: 10.1091/mbc.e05-11-1010
- Van Den Dries K, Van Helden SE, Te Riet J, Diez-Ahedo R, Manzo C, Oud MM, et al. Geometry sensing by dendritic cells dictates spatial organization and PGE(2)-induced dissolution of podosomes. *Cell Mol Life Sci*. (2012) 69:1889–901. doi: 10.1007/s00018-011-0908-y
- Bhuwania R, Cornfine S, Fang Z, Kruger M, Luna EJ, Linder S. Supravillin couples myosin-dependent contractility to podosomes and enables their turnover. *J Cell Sci*. (2012) 125:2300–14. doi: 10.1242/jcs.100032
- Cervero P, Wiesner C, Bouissou A, Poincloux R, Linder S. Lymphocyte-specific protein 1 regulates mechanosensory oscillation of podosomes and actin isoform-based actomyosin symmetry breaking. *Nat Commun*. (2018) 9:515. doi: 10.1038/s41467-018-02904-x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Cytokines and Soluble Mediators in
Immunology,
a section of the journal
Frontiers in Immunology

Received: 30 May 2018

Accepted: 07 August 2018

Published: 28 August 2018

Citation:

Hammerschmidt SI, Werth K,
Rothe M, Galla M, Permanyer M,
Patzner GE, Bubke A, Frenk DN,
Selich A, Lange L, Schambach A,
Bošnjak B and Förster R (2018)
CRISPR/Cas9 Immunoengineering of
Hoxb8-Immortalized Progenitor Cells
for Revealing CCR7-Mediated
Dendritic Cell Signaling and Migration
Mechanisms *in vivo*.
Front. Immunol. 9:1949.
doi: 10.3389/fimmu.2018.01949

CRISPR/Cas9 Immunoengineering of Hoxb8-Immortalized Progenitor Cells for Revealing CCR7-Mediated Dendritic Cell Signaling and Migration Mechanisms *in vivo*

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To present antigens to cognate T cells, dendritic cells (DCs) exploit the chemokine receptor CCR7 to travel from peripheral tissue via afferent lymphatic vessels to directly enter draining lymph nodes through the floor of the subcapsular sinus. Here, we combined unlimited proliferative capacity of conditionally Hoxb8-immortalized hematopoietic progenitor cells with CRISPR/Cas9 technology to create a powerful experimental system to investigate DC migration and function. Hematopoietic progenitor cells from the bone marrow of Cas9-transgenic mice were conditionally immortalized by lentiviral transduction introducing a doxycycline-regulated form of the transcription factor Hoxb8 (Cas9-Hoxb8 cells). These cells could be stably cultured for weeks in the presence of doxycycline and puromycin, allowing us to introduce additional genetic modifications applying CRISPR/Cas9 technology. Importantly, modified Cas9-Hoxb8 cells retained their potential to differentiate *in vitro* into myeloid cells, and GM-CSF-differentiated Cas9-Hoxb8 cells showed the classical phenotype of GM-CSF-differentiated bone marrow-derived dendritic cells. Following intralymphatic delivery Cas9-Hoxb8 DCs entered the lymph node in a CCR7-dependent manner. Finally, we used two-photon microscopy and imaged Cas9-Hoxb8 DCs that expressed the genetic Ca²⁺ sensor GCaMP6S to visualize in real-time chemokine-induced Ca²⁺ signaling of lymph-derived DCs entering the LN parenchyma. Altogether, our study not only allows mechanistic insights in DC migration *in vivo*, but also provides a platform for the immunoengineering of DCs that, in combination with two-photon imaging, can be exploited to further dissect DC dynamics *in vivo*.

Keywords: CRISPR/Cas9, Hoxb8, immortalization, dendritic cells, migration, CCR7, calcium signaling

INTRODUCTION

A key feature of dendritic cells (DCs) is their capability to migrate and transport antigens from peripheral tissues to secondary immune organs, thus inducing tolerogenic and inflammatory immune responses (1, 2). This migration is mediated mainly by the interaction of the chemokines CCL19 and CCL21 with their receptor, CCR7, expressed on DCs (3). In peripheral tissues, haptotactic gradients of CCL21 secreted by lymphatic endothelial cells attract CCR7⁺ DCs toward lymphatic capillaries (4) where locally released CCL21 regulates their entry into the vessel lumen (5, 6). Within lymph capillaries, DCs actively follow CCL21 chemokine gradients crawling toward collecting vessels, in which they are passively transported by lymph flow toward the lymph node (LN) (7). Lymph delivers transported cells into the LN subcapsular sinus (SCS), a space between LN capsule and cortex, where lymphatic endothelial cells lining the SCS ceiling form CCL21 (and possibly CCL19) gradients crucial for direct DC transmigration through SCS floor toward the T cell zone (8, 9).

Although the CCL19/21-CCR7 axis is indisputably the main axis regulating DC migration, many questions regarding the mechanisms of DC migration and function remain unresolved and hamper development of novel therapeutic and vaccination strategies. For example, it would be crucial to establish the relative importance of several other chemokine receptors and their ligands implicated in the migration of DCs, including CX3CL1–CX3CR1 (10) as well as CXCL12–CXCR4 (11). Furthermore, it would be essential to directly compare deficiency of various receptors implicated in DC migration through tissue, such as C-type lectin receptor CLEC-2 (12), hyaluronan (13), or sphingosine 1-phosphate receptors (14). Moreover, it would be important to visualize contributions of divergent signaling cascades, including genes involved in calcium signaling and cytoskeletal organization, in various stages of DC migration (1, 15). Last of all, these studies would need to be done in complex three-dimensional environments, ideally *ex vivo* or *in vivo* (16).

The recent discovery and application of clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated nuclease (Cas9) technology in eukaryotic cells presented a milestone for genome engineering due to its simplicity (17–20). Single guide RNA (sgRNA) screens in bone marrow (BM) cells from mouse strains that express *Streptococcus pyogenes* Cas9 have already identified genes involved in B cell activation and differentiation (21) and DC activation (22, 23). However, another level of the CRISPR/Cas9 technology can be achieved by its coupling to long-term *in vitro* hematopoietic progenitor cell lines. These hematopoietic precursors, transiently immortalized by retroviral transduction with an estrogen-inducible form of the transcription factor Hoxb8 (24), were recently used for further transduction with lentiviruses coding for Cas9 and guide RNAs (gRNAs) (25, 26). Grajkowska et

al. used CRISPR/Cas9 to target E protein transcription factor TCF4 in either protein coding or enhancer region to decipher mechanisms by which isoform-specific TCF4 expression controls the development of plasmacytoid DCs (25). Leithner et al. used a similar system to target *Itgb2*, coding for integrin $\beta 2$, and *Ccr7* and reported that the knockout cells are impaired in integrin-mediated adhesion to glass surfaces and migration toward CCL19 in 3D collagen gels, respectively (26).

Transduction with Cas9 expressing lentiviruses used in previous studies, however, requires antibiotic selection that is time consuming and might affect differentiation potential of transiently immortalized Hoxb8⁺ hematopoietic progenitor cells (25, 26). To circumvent that problem, we used bone marrow (BM) cells from a Cas9 expressing mouse strain (22) and lentivirally transduced them with an inducible form of the transcription factor *Hoxb8*, creating conditionally immortalized murine hematopoietic cells. These cells could be expanded for weeks in cell culture, providing sufficient time for their genetic engineering by successive transductions with lentiviral vectors encoding for sgRNAs, while at the same time retaining their potential for differentiation into DCs, macrophages or granulocytes. Our lentiviruses also coded for fluorescent proteins, allowing not only for the selection of successfully transduced cells with gene editing, but at the same time also facilitated their tracking *in vivo*. We used Cas9-Hoxb8-derived DCs to track CCR7-mediated DC migration and visualize CCR7-mediated calcium signals while entering LN via afferent lymphatics.

MATERIALS AND METHODS

Animals

Mice were bred at the Central Animal Facility at Hannover Medical School under specific pathogen-free conditions. The following mouse strains were used: C57BL/6J (as donors for the generation of immortalized progenitor cells), C57BL/6N (as recipients for Hoxb8 cell-derived dendritic cells), B6J.129(Cg)-Gt(ROSA)26Sortm1.1(CAG-cas9⁺,-EGFP)Fezh/J (designated here as Cas9 mice), B6-Tg(TcraTcrb)1100Mjb Ptpcr-Pepcb/Boy (OT-I Ly5.1 mice), B6Cg-Tg(TcraTcrb)425Cbn/J (OT-II Ly5.1 mice). All experiments were conducted in accordance with the local animal welfare regulations reviewed and approved by the institutional review board and the “Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES).”

Antibodies and Staining Reagents

Following antibodies and staining reagents were used in this study: Brilliant Violet 510 anti-mouse I-A/I-E (clone M5/114.15.2), PE-Cy7 anti-mouse CD11c (N418), APC rat IgG2c κ Isotype control (RTK4174), PerCP-Streptavidin, PerCP-Cy5.5 anti-mouse CD8 α (53–6.7), PerCP anti-mouse CD4 (RM4-5), APC anti-mouse CD40 (3/23), APC anti-mouse CD80 (16-10A1), APC Armenian Hamster IgG Isotype control (HTK888), FITC anti-mouse MHCII/I-Ab (AF6-120.1), APC rat IgG2b κ Isotype control (RTK4530), PE-Cy7 anti-mouse CD11b (N418) (all from Biolegend), PE anti-mouse CD11b (M1/70), PE anti-mouse TCR V α 2 (B20.1) (all from Invitrogen), eF660 anti-mouse CD11b (M1/70), eF450 anti-mouse CD11b (M1/70),

Abbreviations: BM, bone marrow; cDC, conventional dendritic cell; DC, dendritic cell; Dox, doxycycline; dTom, dTomato; GCaMP6S, GFP-Calmodulin-M13-6 slow; GM-CSF, granulocyte macrophage colony-stimulating factor; LPS, lipopolysaccharide; LN, lymph node; M-CSF, macrophage colony-stimulating factor; pDC, plasmacytoid dendritic cell; pt, post transduction; Puro, puromycin; SCS, subcapsular sinus; sgRNA, single guide RNA.

PE anti-mouse F4/80 (BM8), PE anti-rat IgG2a κ -Isotype control (BR2a), APC anti-mouse CXCR4 (2B11), Alexa Fluor 488 anti-mouse Lyve-1 (ALY7), APC anti-mouse CD117/cKit (ACK α), PE anti-mouse CD135/Flt3 (A2F10), APC anti-mouse CD11c (N418), PE-Cy7 anti-mouse Ly-6A/E/Sca-1 (D7), biotin anti-mouse CD115/M-CSFR (AF598), APC anti-mouse Ly6C (HK1.4), PE-Cy7 anti-mouse CD45.1 (A20), biotin rat IgG2a κ Isotype control (eBR2a), PE rat IgG2b κ Isotype control (eB149/10H5), APC anti-mouse CD86 (GL1), APC rat IgG2a κ Isotype control (eBR2a), PE anti-mouse CD197 (CCR7) (4B12), APC anti-mouse CD197 (CCR7) (4B12), APC-eF780 anti-mouse Ly-6G/GR-1 (RB6-8C5; all from eBioscience), ATT0647 GFP-Booster (ChromoTek), FITC anti-mouse CD317 (PDCA-1; JF05-1C2.4.1) (Miltenyl Biotec), PE anti-mouse CD11c (HL3) (BD Biosciences), PE-Streptavidin and Cy5 anti-mouse B220 (TIB146; both homemade).

Vector Construction and Viral Particle Production

Third generation lentiviral self-inactivating (SIN) vectors co-expressing respective sgRNAs and fluorescent marker genes were based on pLKO_TRC005 (Addgene) and constructed in the following manner. sgRNA transcripts are initiated from human RNA polymerase III promoter U6 (hU6) and expression of the fluorescent marker genes is driven by enhancer/promoter sequences of the spleen focus forming virus (SFFV). To facilitate cloning of gene targeting protospacer sequences into the vector backbone, a 105 bp-BsmBI-stuffer sequence was inserted downstream of hU6 and upstream of the sgRNA scaffold. Nuclear export and stabilization of the vector's mRNA transcripts were enhanced by incorporation of the post-transcriptional regulatory element of the Woodchuck hepatitis virus (PRE) (27, 28). pLKO5.hU6.sgRNA.BsmBI-Stuffer.SFFV.dTomato.PRE was generated by replacing the Tet2 protospacer sequence of pLKO5.hU6.sgRNA.Tet2.SFFV.dTomato.PRE with the BsmBI-Stuffer from pRRL.PPT.hU6.BsmBI-Stuffer.SF.SpCas9.T2A.dTomato.PRE.LoxP using restriction enzymes NdeI and EcoRI. pLKO5.hU6.sgRNA.BsmBI-Stuffer.SFFV.Cerulean.PRE and pLKO5.hU6.sgRNA.BsmBI-Stuffer.SFFV.eYFP.PRE were obtained by removal of dTomato from pLKO5.hU6.sgRNA.BsmBI-Stuffer.dTomato.PRE via AgeI and BsrGI and insertion of AgeI/BsrGI digested Cerulean or EGFP cDNAs from pRRL.PPT.SF.Cerulean.PRE and pRRL.PPT.PGK.eYFP.Tubulin.PRE (both kindly provided by Tobias Maetzig, Hannover Medical School, Hannover, Germany), respectively.

Protospacer sequences were designed with the online tools CRISPR Design (<http://crispr.mit.edu/>) (Zhang Lab, MIT, 2015) or CCTop (<https://crispr.cos.uni-heidelberg.de/>) (29). Generation of pLKO5.hU6.sgRNA.Ccr7.SFFV.dTomato.PRE is described elsewhere (30). Lentiviral vectors expressing sgRNAs targeting *Cxcr4* and *Trpml1* were cloned as follows: designed sequences were ordered as oligonucleotides carrying BsmBI overhangs at their 5'-ends. After phosphorylation and annealing of the respective oligonucleotide pairs, the double-stranded protospacer DNA was incorporated into the

BsmBI site of pLKO5.hU6.sgRNA.BsmBI-Stuffer.dTomato.PRE, pLKO5.hU6.sgRNA.BsmBI-Stuffer.Cerulean.PRE and/or pLKO5.hU6.sgRNA.BsmBI-Stuffer.eYFP.PRE according to Heckl et al. (31). The following gene targeting protospacer sequences were used: *Ccr7*, GTTTGGCGTCTACCTGTGTAA; *Cxcr4*, ACGTTTTCATCCCGGAAGCA; *Trpml1*, TGCCAGCGTACTACCACCG (sgRNA 1) and GGGCTGGTGAGTCCCCACC (sgRNA 3).

The lentiviral SIN vector RRL.PPT.T11.Hoxb8.hPGK.M2.P2A.Puro.pre (LV.T11.Hoxb8.Puro) was generated from construct pRRL.PPT.T11.MCS.PGK.M2.pre. We introduced a codon-optimized mouse Hoxb8 transgene (protein coding sequence see NCBI Reference Sequence: NP_034591.1) under the inducible T11 Tet promoter (32). Codon optimization was performed with the GeneArt algorithm (ThermoFisher). The reverse transactivator M2 is constitutively expressed from the human phosphoglyceratekinase (hPGK) promoter. For selection, we used the puromycin resistance gene 3' of M2, fused by a P2A self-cleaving site (33, 34). Details are available on request.

Lentiviral particles were produced by transient transfection of 293T cells based on the calcium phosphate precipitation method assisted by 25 μ M chloroquine (Calcium Phosphate Transfection Kit and chloroquine from Sigma-Aldrich). 293T packaging cells were co-transfected with 15 μ g pcDNA3.gp.4xCTE (HIV-1 Gag-Pol) (35), 5 μ g pRSV-Rev (kindly provided by T. Hope, Northwestern University, Chicago, IL, USA), 3 μ g K73 pEcoEnv-IRES-puro (36) and 5 μ g sgRNA-expressing vector. Supernatants were harvested 24 and 48 h after transfection and 100-fold concentrated by ultracentrifugation (16–18 h, 13,238 $\times$ g, 4°C). Viral pellets were resuspended in IMDM (Biochrom) supplemented with 10% FBS (PAA Laboratories), 1% penicillin-streptomycin, and 1% glutamine (Gibco) (designated as complete IMDM) and stored at -80°C .

The MLV-based gammaretroviral vector plasmid pRSF91.GCaMP6S.i2.dTomato.pre was generated in multiple steps. First, the GCaMP6F open reading frame of pGP.CMV.GCaMP6F (37) was amplified with primers 5'-ATCCGCTAGCGCTACCGGTCTCAGATCTCG-3' (forward) and 5'-GGTATGGCGGATCTCTAGAGTCGCGG-3' (reverse), thereby introducing 5' the AgeI and 3' the BamHI restriction site. The GCaMP6F amplicon was subcloned into a shuttle vector and verified by sequencing. Next, GCaMP6F was excised via AgeI/BamHI (1,395 bp) and ligated with 346 bp (BamHI/BspmI) and 5,849 bp (BspmI/AgeI) fragments of pRSF91.i2.dTomato.pre to obtain pRSF91.GCaMP6F.i2.dTomato.pre. Subsequently, part of the GCaMP6F cDNA was replaced with relevant sequences of GCaMP6S via a 4-fragment ligation. For this purpose, pGP.CMV.GCaMP6S (37) was cut with NheI and NotI and the 1,321 bp GCaMP6S fragment was ligated with 1,307 bp (NotI/BsrGI), 4,427 bp (BsrGI/MscI) and 535bp (MscI/NheI) fragments from pRSF91.GCaMP6F.i2.dTomato.pre. The resulting vector plasmid pRSF91.GCaMP6S.i2.dTomato.pre was also verified by sequencing.

The production of ecotropic gammaretroviral RSF91.GCaMP6S.i2.dTomato.pre vector particles was conducted as previously described (38, 39). The supernatants were 50-fold

concentrated via ultracentrifugation at $13,238 \times g$ and 4°C for 16–20 h. Viral vector pellets were resuspended in StemSpan medium and stored at -80°C until further usage.

Generation and Cell Culture of Hoxb8 Progenitor Cell Lines

Mouse hematopoietic stem and progenitor cells were isolated from C57BL/6J or Cas9 mice using a lineage cell depletion kit (Miltenyi). A total of 1×10^5 cells were pre-stimulated for 48 h in StemSpan SFEM (Stem Cell Technologies) supplemented with rm-IL-3 (20 ng/ μl), rm-SCF (50 ng/ μl), rh-Flt-3L, rh-IL-11 (each 100 ng/ μl ; all cytokines from PeproTech), and 1% penicillin-streptomycin (PAN Biotech) in a 24-well suspension plate (Sarstedt). Cells were transduced with LV.T11.Hoxb8.Puro particles (2x MOI 2.5) two times on Retronectin (Takara Clontech) coated wells in medium containing 4 mg/ml protamine sulfate (Sigma-Aldrich). For expansion, we transferred cells to 48-well plates and changed the medium to complete IMDM supplemented with cytokines as described above (except rm-SCF: 100 instead of 50 ng/ μl) in the presence or absence of 1 $\mu\text{g}/\text{ml}$ doxycycline (Dox; Takara Clontech) and/or 0.3–1 $\mu\text{g}/\text{ml}$ puromycin (Puro; Invivogen). On day 4 post transduction (pt), cultures were passaged to 6-well plates and the vector copy number (VCN) per cell was determined as previously described (40). Cultures were fed every 2–3 days and diluted on day 8, 15, and 38 pt to 1×10^6 cells in 4 ml of medium. On day 22–26 pt, samples selected with puromycin and doxycycline were frozen with 2×10^6 cells per aliquot in 90% FBS + 10% DMSO (Merck).

For transduction of Cas9-Hoxb8 cells, viral particles (MOI 4.5 of 50x concentrated GCaMP6S encoding particles and 100 μl of 100x concentrated sgRNA encoding particles) were bound to Retronectin (Takara Clontech)-coated 96-well plates (Sarstedt) by centrifugation (30 min–2 h, $2,000 \times g$, 4°C). After removing the supernatant, Cas9-Hoxb8 cells resuspended in complete IMDM medium supplemented with rm-SCF, rh-IL-11, rh-Flt3L, rm-IL-3, Dox, Puro and polybrene (Sigma-Aldrich; 4 $\mu\text{g}/\text{ml}$) were added to the plate, centrifuged (45 min, $700 \times g$, 32°C) and incubated at 37°C . In some cases, cells were incubated 30 min prior to and during transduction with 10 μM cyclosporine A (Sigma-Aldrich). After 24 h, cells were washed and resuspended in fresh complete IMDM medium supplemented with rm-SCF, rh-IL-11, rh-Flt3L, rm-IL-3, Dox, and Puro.

Generation of Macrophages

Macrophages were generated *in vitro* based on a protocol described by Ho and Sly (41). Briefly, bone marrow cells were cultured overnight in complete IMDM. Non-adherent bone marrow cells were collected the next day. Hoxb8 cells or non-adherent bone marrow cells were then transferred to complete IMDM supplemented with 5 ng/ml rm-M-CSF (Immuno Tools) and 150 μM 1-thioglycerol (Sigma-Aldrich). After 6 days of M-CSF culture, Hoxb8 and bone marrow cells, which have not become adherent by then, were removed and the remaining adherent cells were further cultured in the presence of M-CSF and 1-thioglycerol until analysis on day 9.

Generation of DCs

DCs were generated *in vitro* as described previously (3). Briefly, bone marrow cells or Hoxb8 cells were cultured for 9 days in RPMI medium (Gibco) supplemented with 10% FBS (PAA Laboratories), 1% penicillin-streptomycin, 1% glutamine (Gibco), 2-mercaptoethanol (Sigma), and cell culture supernatant from a GM-CSF producing cell line (5% final concentration). On day 8 of culture, aliquots of cells were collected and stained for the expression of markers specific for DC, macrophages or monocytes. For activation, cells were treated with lipopolysaccharide (LPS; 1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) at day 8 of culture for the remaining 16 h. In all cases, DC differentiation and maturation status was assessed by analysis of the CD11c and MHCII expression. For intralymphatic injection, GM-CSF-differentiated cells were selected based on cell size by fluorescence-activated cell sorting to enrich DCs and to remove dead cells and doublets. Sorting yielded a purity of 78–89% CD11c⁺MCHII⁺ cells.

To check for their potential to differentiate into conventional or plasmacytoid DC (cDCs and pDCs, respectively) BM cells or Hoxb8 cells were cultured for 9 days in RPMI medium (Gibco) supplemented with 10% FBS (PAA Laboratories), 1% penicillin-streptomycin, 1% glutamine (Gibco), 2-mercaptoethanol (Sigma), together with cell culture supernatant from a Flt3L producing cell line, as described previously (42). On day 8–9 of culture, cells were harvested and analyzed by flow cytometry.

Dendritic Cell-Induced Proliferation of T Cells *in vitro*

DCs were generated as described above. During the final 16 h of culture, they were incubated in the presence of lipopolysaccharide (LPS; 1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) and chicken ovalbumin grade VI (200 $\mu\text{g}/\text{ml}$, Sigma-Aldrich). After being washed twice, 10^4 DCs were co-cultured in round-bottom 96-well plates with 10^5 eFluor 670-labeled CD8⁺ or CD4⁺ T cells isolated by magnetic cell separation (CD8 α ⁺ or CD4⁺ T Cell Isolation Kit mouse, Miltenyi) from spleens and lymph nodes of OT-I Ly5.1 or OT-II Ly5.1 mice, respectively. After 3 days of co-culture, T cell proliferation was determined by flow cytometry on LSR II (BD) and analyzed with FlowJo (TreeStar) v.7 and v.10.

Transwell Migration Assay

10^5 *in vitro* differentiated DCs were resuspended in 100 μl complete RPMI and loaded in collagen-coated transwells (Corning BV, 5 μm pore size) that were placed in 24-well plates containing 600 μl complete RPMI containing 0, 10, 100 or 200 ng/ml CCL21 (Peprotech). After incubation for 2 h at 37°C 5% CO_2 , migrated cells were collected and a defined number of 6 μm YG Fluoresbrite Microparticles (Polysciences) were added for counting of migrated cells by flow cytometry.

Gene Editing Efficiency Analysis

DNA was isolated from Hoxb8 cells using QIAmp DNA Mini Kit (Qiagen) and sgRNA target sites were amplified by PCR with NEBNext[®] High-Fidelity 2X PCR Master Mix (New England Biolabs). The following primers were used: Ccr7 exon3: 5'-TGTGCTTCTGCCAAGATGAG-3', 5'-

TCAGCCCAAGTCCTTGAAGA-3'; Mcoln1/Trpml1 exon 2: 5'-GGGAGATCAGAAAGGATAACATC-3', 5'-ACTCAT TGCACATGAAGTTCTC-3'; and Mcoln1/Trpml1 exon 4: 5'-ACCATTGCCTTCCGACATCT-3', 5'-GGTGTGCAAGTGA CAAGGTTA-3'. PCR reactions were purified using QIAquick PCR Purification Kit (Qiagen) for Sanger sequencing. The composition and frequency of insertions and deletions (INDELS) was analyzed using ICE software (Synthego; <https://ice.synthego.com/#/>) (43).

Intralymphatic Injection

Intralymphatic transfer of Hoxb8 cell-derived DCs with or without gene modifications was performed as described previously (9). Briefly, 4×10^4 cells of a defined population were injected in 5 μ l of PBS into the afferent lymphatic vessel of the popliteal LN. In comparative studies, a total of 8×10^4 cells of a 1:1 mixture of two populations were injected. Popliteal LNs were subsequently analyzed using either two-photon microscopy or immunohistology (see below).

Two-Photon Microscopy

LNs were explanted immediately after intralymphatic injection and glued into a custom-built perfusion chamber using tissue adhesive (Surgibond). During imaging, LNs were continuously superfused with prewarmed, oxygenated (95% O₂ and 5% CO₂) RPMI medium supplemented with 5 g/l Glucose (Sigma) as described earlier (44). Images were acquired with an upright Olympus BX51 microscope equipped with a W Plan-Apochromat 20x/1.0 DIC objective (Zeiss), TrimScope scanning unit (LaVision Biotech), and Mai Tai Titanium:sapphire pulsed infrared lasers (Spectra-Physics). For excitation of GFP and dTomato the laser was tuned to 920 nm. Time-lapse series were generated on a view field of $300 \times 300 \times 90$ – 100μ m for up to 2 h with 15–17 images acquired per z-stack every 15–17 s. Imaging data was analyzed using Imaris 7.4–8.4 (Bitplane). A median filter was applied on all movies to reduce background noise. Tracking of cells was performed automatically based on the dTomato signal of the cells. Manual corrections were applied where required and dead or dying cells were excluded from the analysis. GCaMP6S mean intensity values (arbitrary units) were exported to Excel (Microsoft). To account for difference in GCaMP6S expression between cells and differences between the location of cells within LNs, normalized GCaMP6S values for each frame were calculated as a difference of GCaMP6S signal and mean GCaMP6S value for the subsequent 3 minutes (12 frames) of the same track. Finally, data points with GCaMP6S normalized value $>1,000$ arbitrary units were considered as Ca²⁺ signals (see also Figure 11B for details).

Immunohistological Analysis

Popliteal LNs were explanted 4 h after intralymphatic injection, fixed overnight in 2% paraformaldehyde (PFA; Carl Roth) plus 30% (vol/vol) sucrose, embedded in Tissue-Tek OCT (Tissue-Tek, Sakura), and frozen on dry ice. 8 μ m thick cryosections were counterstained in TBS/T (Tris-buffered saline + 0.05% Tween 20) at room temperature using anti-Lyve-1 antibody and GFP booster. High-resolution composite images of whole LN sections

were acquired with a Zeiss AxioScan Z1 (Plan-Apochromat objective: 10x/0.45 M27; camera: AxioCam 506 mono) or a Olympus BX61 (UPlanSApo objective: 10x/0.4; camera: F-View II), respectively. All pictures were contrast adjusted. Distribution of cells and distance measurements to the SCS were performed using Imaris and ImageJ as described earlier (8).

Statistical Analysis

All analyses were done with GraphPad Prism (v4 or v7). We calculated *p*-values using the non-parametric Mann–Whitney test or Fisher's exact test for comparison of two groups, or Kruskal–Wallis test with Dunn's test to compare multiple groups. All data are pooled from at least two independent experiments, as indicated below each figure, and *p* < 0.05 was considered statistically significant.

RESULTS

Generation of Cas9-Hoxb8 Cells

In contrast to previous studies, which used an estrogen-regulated form of Hoxb8 (24–26), we generated a doxycycline (Dox)-inducible third generation lentiviral vector for Hoxb8-mediated immortalization of murine hematopoietic cells. For enrichment of transgene positive cells, we also introduced a puromycin (Puro) selection cassette. To verify the vector-dependent immortalization of primitive hematopoietic stem and progenitor cells, we first transduced lineage-negative cells from C57BL/6J mice followed by cell culture in the presence of mSCF, huIL-11, huFlt3L, mIL-3, Dox, and Puro. Cells expressing the Hoxb8 construct were rescued from Puro treatment until day 15 post transduction (pt), whereas non-transduced cells showed low cell numbers and viability (Figure 1A). To allow for further genome modification with CRISPR/Cas9 technology, we isolated lineage-negative cells from Cas9 mice and selected them with the same strategy as described for the C57BL/6J mice. Only gene-modified cells survived longer than 15 days pt (Figure 1B). These cells, which we could stably culture for at least 16 weeks, were used for further experiments and were designated as Cas9-Hoxb8 cells.

Cas9-Hoxb8 cells showed a roundish cell shape (Figure 1C) and expressed c-Kit, while expression of Sca-1 was absent (Figure 1D), indicating that the cells are skewed toward the myeloid rather than lymphoid lineage. In addition, Cas9-Hoxb8 cells partially expressed CD11b, Ly6C and the M-CSF receptor, while CD11c could not be detected on their surface and Flt3 was only marginally expressed (Figure 1D). Low Flt3 expression did not result from receptor internalization as a consequence of culture in the presence of Flt3L, as Flt3L deprivation for 24 h did not result in Flt3 upregulation on the surface of Cas9-Hoxb8 cells (data not shown).

Cas9-Hoxb8 Cells Have the Potential to *in vitro* Differentiate Into Macrophages and Dendritic Cells

To further characterize the Cas9-Hoxb8 cells, we assessed their myeloid potential by withdrawing mSCF, huIL-11, huFlt3L, mIL-3, Dox, and Puro and exposing the cells to established *in*

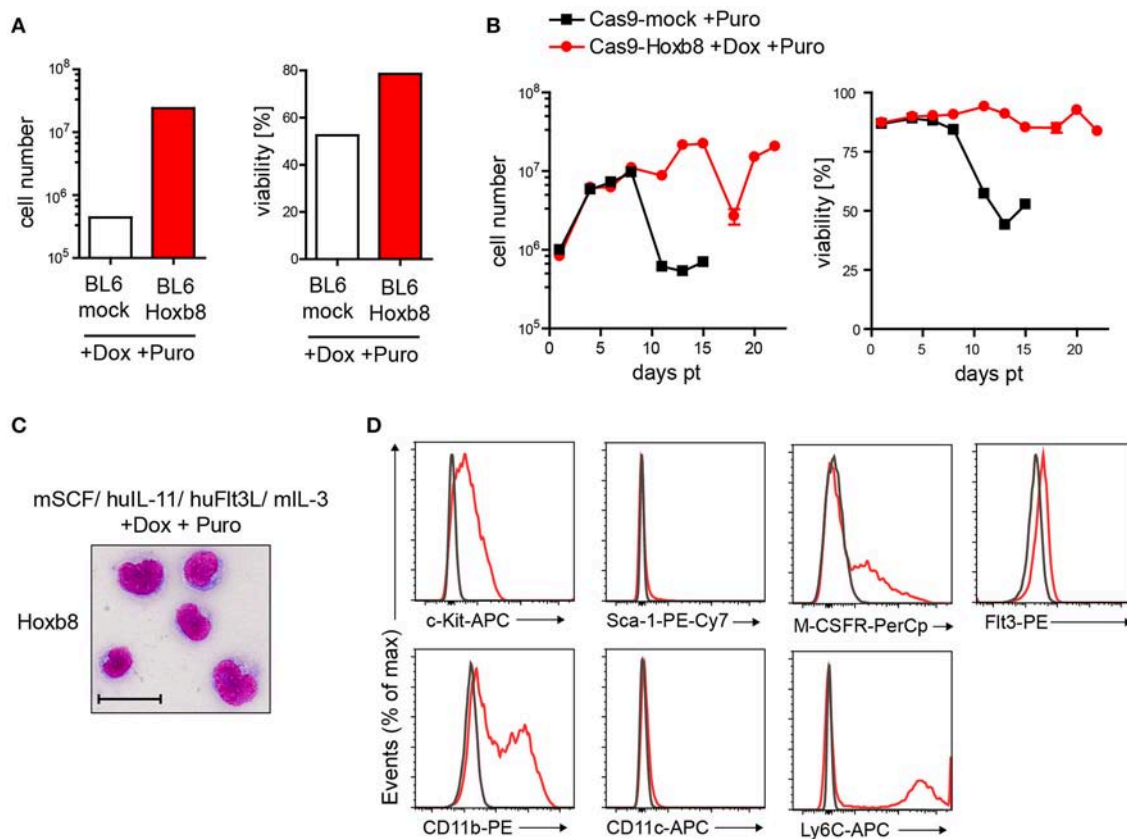


FIGURE 1 | Immunoengineering of conditionally immortalized Hoxb8 cells from murine bone marrow. **(A,B)** Development of cell numbers and viability of lineage-negative BL6 **(A)** or Cas9 **(B)** bone marrow cells after mock infection or transduction with a Hoxb8-encoding lentivirus and subsequent culture in the presence or absence of doxycycline (Dox), and puromycin (Puro). **(A)** Cell numbers and viability from day 15 post transduction (pt) are shown. **(C)** Morphology (bright-field microscopy after cytopsin and Pappenheim staining; scale bar: 25 μ m) and **(D)** flow cytometric analysis of Hoxb8 cells grown in medium with mSCF, huIL-11, huFit3L, mL-3, doxycycline, and puromycin. Data are representative of two independent experiments **(C,D)**.

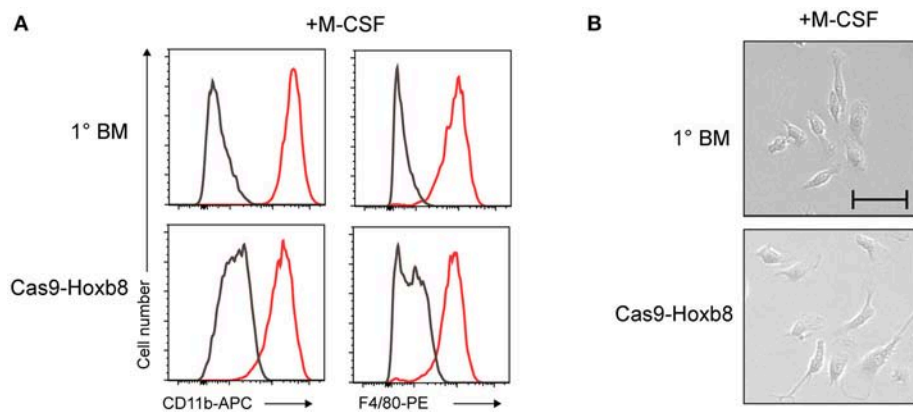


FIGURE 2 | Phenotype of Cas9-Hoxb8 cell-derived macrophages. **(A)** Phenotype and **(B)** morphology (bright-field microscopy; scale bar: 50 μ m) of primary BM cells (1° BM) or Cas9-Hoxb8 cells cultured in the presence of M-CSF for 9 days. Gray curves depict isotype controls. Data are representative of two independent experiments.

vitro myeloid cell differentiation protocols. First, the cells were cultured in the presence of macrophage colony-stimulating factor (M-CSF) according to a macrophage differentiation protocol

described by Ho and Sly (41). On day 9 of M-CSF culture, Cas9-Hoxb8 cells exhibited expression of CD11b as well as F4/80 (**Figure 2A**) and the characteristic adherent morphology

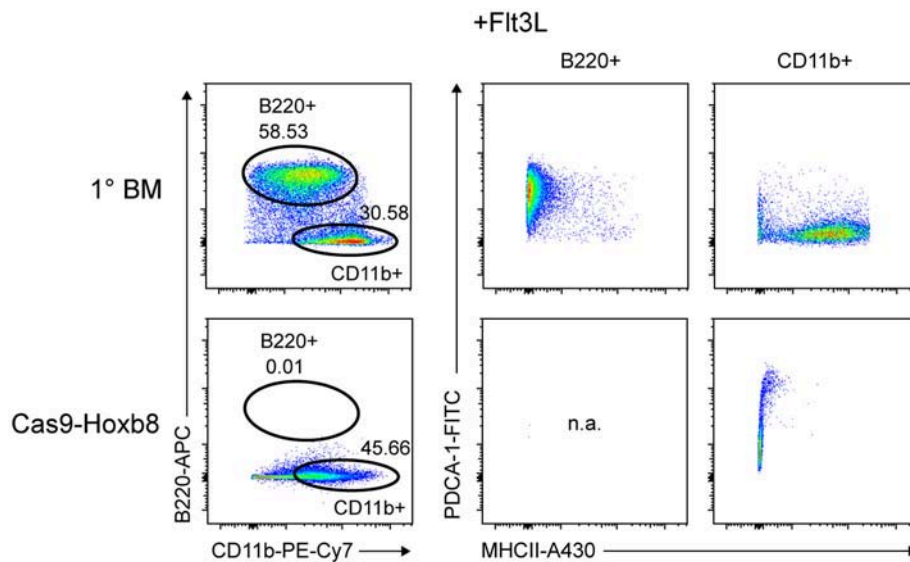


FIGURE 3 | Cas9-Hoxb8 cells do not differentiate into plasmacytoid DCs in the presence of Flt3L. Flow cytometric analysis of primary BM cells (1° BM) or Cas9-Hoxb8 cells cultured for 8 days in the presence of Flt3L. In contrast to primary BM cells, Cas9-Hoxb8 cells failed to acquire a pDC phenotype (CD11b[−]PDCA-1⁺B220⁺). Data are representative for three independent experiments.

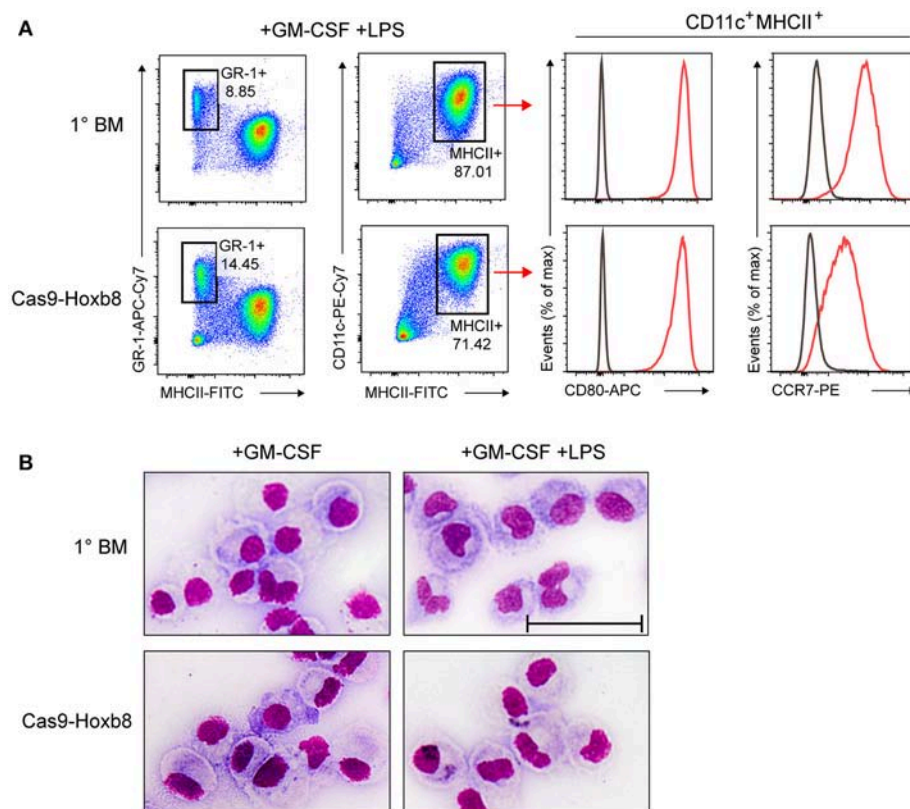


FIGURE 4 | Phenotype of Cas9-Hoxb8 cell-derived DCs after LPS activation. **(A)** Flow cytometry analysis and **(B)** morphology (bright-field microscopy after cytopsin and Papanheim staining; scale bar: 50 μ m) of primary BM cells (1° BM) or Cas9-Hoxb8 cells cultured in the presence of GM-CSF for 9 days followed by overnight treatment with LPS. Gray curves depict isotype controls. Data are representative of two independent experiments.

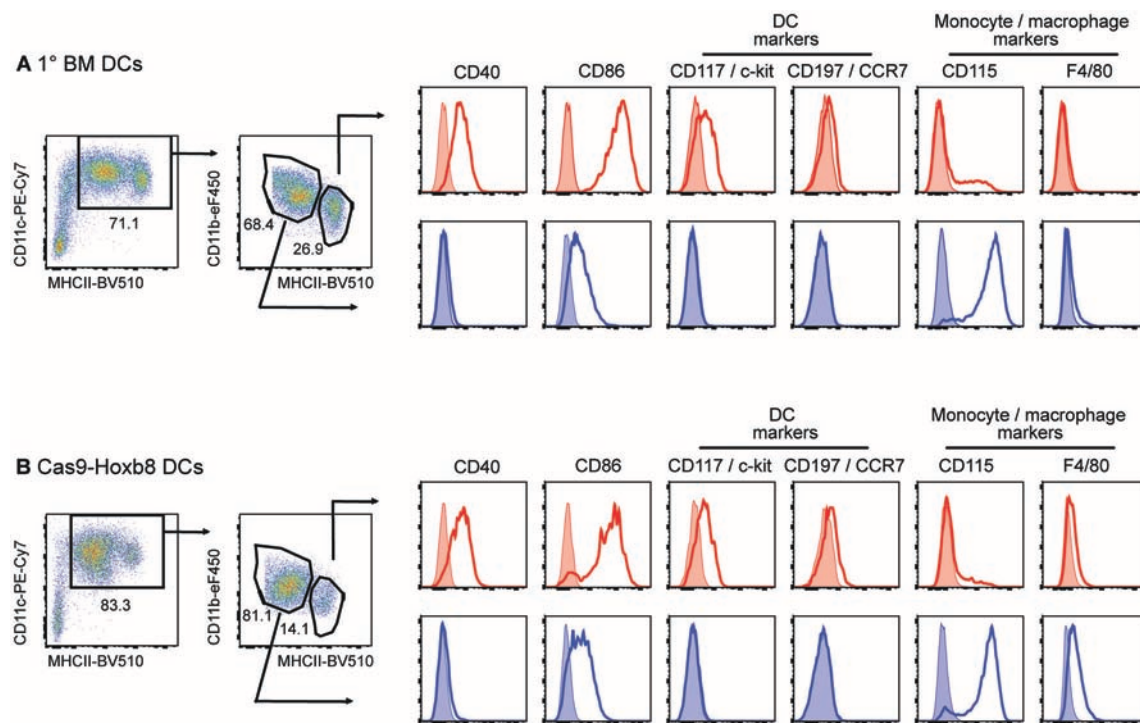


FIGURE 5 | Phenotype of primary bone-marrow derived DCs (1° BM DCs) and Cas9-Hoxb8 DCs in GM-CSF cultures before LPS activation. Flow cytometric analysis of cells cultured for 8 days in the presence of GM-CSF. Without LPS activation, both (A) 1° BM and (B) Cas9-Hoxb8 cells were subdivided on the basis of CD11c, MHCII and CD11b expression. Histograms show surface expression of the markers indicated on MHCII^{hi}CD11b^{lo} (red) and MHCII^{int}CD11b^{hi} (blue) myeloid cell subsets. Data are representative for three independent experiments.

of macrophages (Figure 2B), just like macrophages derived from primary, freshly isolated bone marrow (1° BM) cells, which were treated according to the same protocol. The responsiveness of Cas9-Hoxb8 cells to M-CSF is consistent with their expression of the M-CSF receptor (Figure 1D).

Next, we tested the differentiation potential of Cas9-Hoxb8 cells toward pDCs. Therefore we replaced mSCF, huIL-11, huFlt3L, mIL-3, Dox and Puro by Flt3L. Surprisingly—and in contrast to primary BM cells—Cas9-Hoxb8 cells failed to acquire a pDC phenotype (CD11b⁺PDCA-1⁺B220⁺; Figure 3). This unexpected observation is presumably due to the low expression of Flt3 on Cas9-Hoxb8 cells (Figure 1D) impeding their Flt3L-driven differentiation into pDCs.

Next, we replaced mSCF, huIL-11, huFlt3L, mIL-3, Dox, and Puro with granulocyte-macrophage colony-stimulating factor (GM-CSF), which triggers the *in vitro* generation of dendritic cells and granulocytes. After 9 days of GM-CSF culture, cells were treated with the TLR4 agonist lipopolysaccharide (LPS) to induce DC maturation. GM-CSF-differentiated and LPS-treated Cas9-Hoxb8 cells showed a phenotype very similar to GM-CSF-treated and LPS-matured primary bone marrow cells, characterized by a major population of DCs (CD11c⁺MHCII⁺) and smaller population of granulocytes (GR-1⁺MHCII⁺) (Figure 4A). In addition, GM-CSF-differentiated and LPS-activated Cas9-Hoxb8 cells strongly up-regulated the co-stimulatory molecule CD80 and the chemokine receptor CCR7 (Figure 4A). Interestingly, although CD80 expression on Cas9-Hoxb8 cells was comparable

to the level found on LPS-activated BM-DCs, CCR7 was expressed at slightly lower levels as compared to BM-DCs (Figure 4A). Further, Cas9-Hoxb8 cell-derived DCs acquired the typical morphology of BM-derived DCs (Figure 4B).

Interestingly, before LPS stimulation cells from both primary BM cells and Cas9-Hoxb8 cells express both macrophage and DC markers (Figure 5). This finding is in agreement with recent observations characterizing GM-CSF cultures of primary BM cells as a mixture of DCs and macrophages (45). In contrast, in our hands LPS activation of GM-CSF-differentiated Cas9-Hoxb8 DCs as well as BM-DCs creates population of cells showing homogenous expression of CD86 and CCR7 (Figures 4A, 7A). Since both markers are not expressed on BM-derived macrophages cultured in the presence of GM-CSF (45), we conclude that—similar to Leithner et al. (26)—Cas9-Hoxb8 cells differentiate into a relatively homogenous population of myeloid DCs.

In summary, Cas9-Hoxb8 cells possess the potential to *in vitro* differentiate into macrophages and dendritic cells.

Cas9-Hoxb8 Cell-Derived Dendritic Cells Have the Ability to Induce T Cell Proliferation

Given that GM-CSF-differentiated Cas9-Hoxb8 cell-derived DCs phenotypically resemble their BM-derived counterparts, we compared also their functionality for T cell activation. To that end, we loaded GM-CSF-differentiated Cas9-Hoxb8 as well as

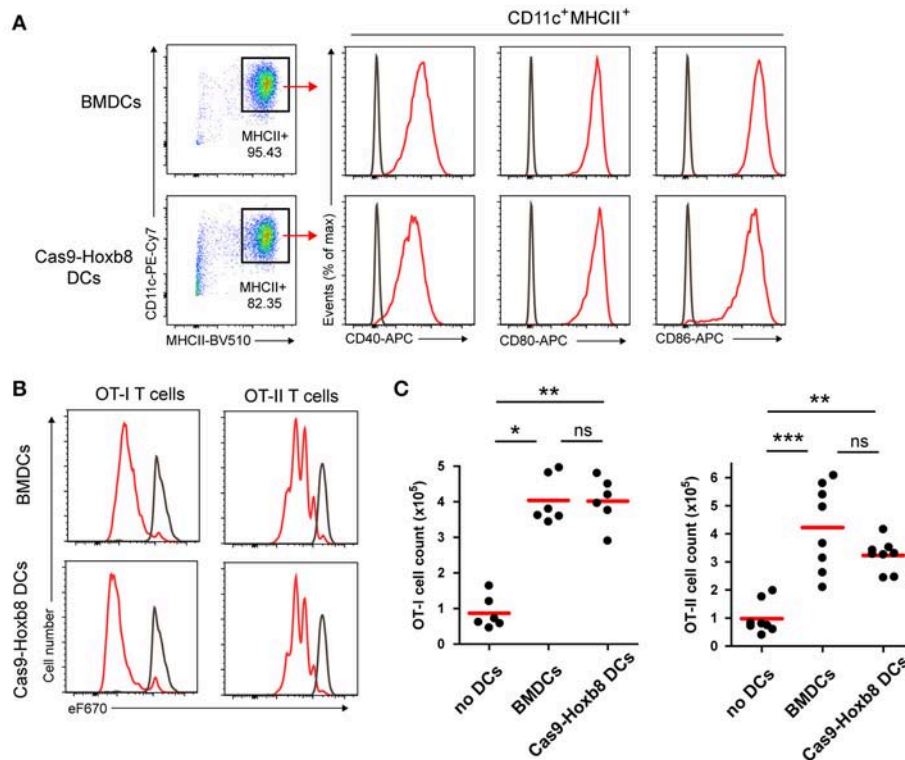


FIGURE 6 | T cell activation potential of Cas9-Hoxb8 cell-derived DCs. **(A)** LPS-induced expression of the costimulatory molecules CD40, CD80, and CD86 on DCs derived from primary BM cells or from Cas9-Hoxb8 cells. Representative plots from three independent experiments. **(B)** Proliferation profiles of eFluor 670 (eF670)-labeled OT-I and OT-II cells cultured for 3 days with Ovalbumin (Ova) loaded, LPS-stimulated BM-derived or Cas9-Hox8 cell-derived DCs. Cells were gated as DAPI[−]CD45.1⁺CD8⁺ (OT-I) or DAPI[−]CD45.1⁺CD4⁺ (OT-II). Gray curves depict control profiles of OT-I and OT-II cells which were cultured in the absence of DCs. Representative plots from three independent experiments. **(C)** Numbers of OT-I and OT-II cells after co-culture for 3 days with ovalbumin loaded, LPS-stimulated BM-derived or Cas9-Hox8 cell-derived DCs. Data are pooled from three (OT-I) or four (OT-II) independent experiments. Red bars, mean; Kruskal–Wallis and Dunn’s multiple comparisons test; ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

BM DCs with ovalbumin during LPS activation and mixed them with MHC class I or MHC class II restricted T cells carrying a transgenic T cell receptor specific for epitopes of ovalbumin. The cells are known as OT1 and OT2 cells, respectively and were stained with a proliferation dye prior use. In line with our previous observations, LPS-matured ovalbumin-loaded Cas9-Hoxb8 and BM-derived DCs showed an equally pronounced up-regulation of the co-stimulatory molecules CD40, CD80, and CD86 (**Figure 6A**). Consequently, Cas9-Hoxb8 cell-derived DCs induced a robust proliferation of CD8⁺ OT-I as well as CD4⁺ OT-II T cells comparable to that induced by BM-derived DCs (**Figures 6B,C**). Thus, Cas9-Hoxb8 cell-derived DCs are equally potent as BM-derived DCs in inducing T cell proliferation.

Cas9-Hoxb8 Cells Provide a Source of Genetically Modified Dendritic Cells for the Study of Dendritic Cell Migration

In addition to T cell activation, migration is another key feature of DCs pivotal for their central role in immunity. For instance, to present antigens to cognate T cells, DCs travel from peripheral tissue to draining lymph nodes via afferent lymphatics in a

CCR7-dependent manner (3, 9). To test if Cas9-Hoxb8 cell-derived DCs rely on the same mechanisms and thus can be exploited as a tool to study DC migration, we used lentiviral-based CRISPR/Cas9 technology to knockout *Ccr7*. Cas9-Hoxb8 cells were transduced with a lentivirus expressing dTomato (dTom) and sgRNA targeting *Ccr7* (30). Successfully transduced cells were purified by fluorescence-activated cell sorting based on dTom expression and subsequently differentiated into DCs in the presence of GM-CSF followed by the treatment with LPS. Flow cytometric analysis confirmed that dTom⁺ DCs completely lacked CCR7 expression despite being fully activated and exhibiting high levels of CD80 (**Figure 7A**). Interestingly, sequence trace decomposition that determines the composition and frequency of insertions and deletions indicated that 4.8% of cells still had intact sgRNA targeting locus (**Figure 7B**). Nevertheless, in contrast to dTom[−] *Ccr7*^{+/+} DCs, these *Ccr7*^{−/−} DCs were not responsive to CCL21 in *in vitro* transwell migration assays (**Figure 7C**). Furthermore, 4h after injection into the afferent lymphatics of popliteal lymph nodes, *Ccr7*^{+/+} DCs populated the lymph node T cell zone, whereas *Ccr7*^{−/−} DCs entered the lymph node parenchyma with delayed kinetics and failed to populate the deep T cell zone (**Figure 7D**), as it was

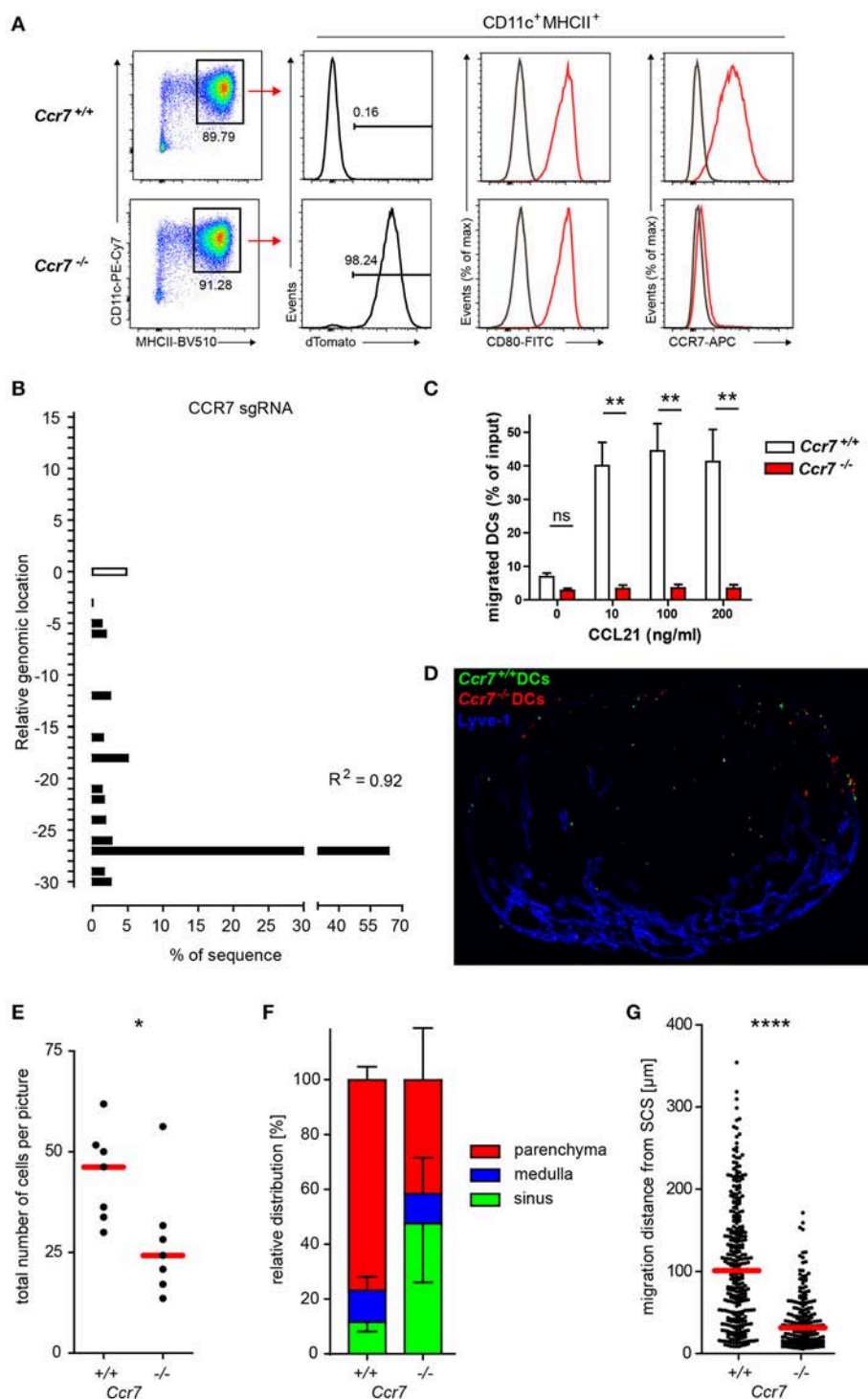


FIGURE 7 | Phenotypic and functional verification of CRISPR/Cas9-mediated knockout of *Ccr7* in Hoxb8 cell-derived DCs. **(A)** Cas9-Hoxb8 cells were transduced with a dTom- and CCR7gRNA-encoding lentivirus. Successfully transduced cells were sorted based on the expression of dTom and subsequently differentiated to mature DCs in the presence of GM-CSF followed by treatment with LPS. *Ccr7*^{+/+} and *Ccr7*^{-/-} DCs were analyzed by flow cytometry for their expression of CD80 and CCR7. Data are representative for four independent experiments. **(B)** Analysis of the composition and frequency of insertions and deletions of *Ccr7*^{-/-} Cas9-Hoxb8 cells. R, Pearson correlation coefficient; R^2 describes how strongly the calculated chromatograph of the indel distribution correlates with the Sanger sequencing results of the sample DNA. **(C)** Chemotactic migration of *Ccr7*^{+/+} and *Ccr7*^{-/-} DCs for 2 h toward medium alone or 10, 100, and 200 ng/ml CCL21. Data are pooled from 3 independent experiments with $n = 8$ in total. Mean + SEM; Kruskal-Wallis and Dunn's multiple comparisons test; ns, not significant;

(Continued)

FIGURE 7 | $^{**}p < 0.01$. **(D)** Microscopy of popliteal lymph nodes obtained 4 h after intralymphatic injection of YFP-expressing *Ccr7*^{+/+} DCs and dTom-expressing *Ccr7*^{-/-} DCs ($5-8 \times 10^4$ cells in 5 μ l PBS; scale bar: 200 μ m). **(E)** Total cell counts and **(F)** relative distribution of *Ccr7*^{+/+} and *Ccr7*^{-/-} DCs 4 h after intralymphatic injection into popliteal LNs of B6 mice. **(G)** Migration distance from the subcapsular sinus (SCS) for the *Ccr7*^{+/+} and *Ccr7*^{-/-} DCs that entered LN parenchyma. Dots represent cell number per LN section **(E)** or individual cells **(G)**. Data are representative for **(D)** or pooled from **(E-G)** two independent experiments with a total of 7 lymph nodes analyzed. Error bars, SD; red bars, median; Mann-Whitney test; $^{*}p < 0.05$; $^{****}p < 0.0001$.

observed in previous studies applying *Ccr7*-deficient BM-derived DCs in a similar setup (9). Quantification showed that there were significantly less *Ccr7*^{-/-} DCs than *Ccr7*^{+/+} DCs per picture taken (**Figure 7E**) and that almost half of *Ccr7*^{-/-} DCs was retained in the SCS, while more than 75% of *Ccr7*^{+/+} DCs penetrated into LN parenchyma (**Figure 7F**). Furthermore, *Ccr7*^{+/+} DCs that penetrated the LN parenchyma from the SCS floor progressed on average almost 4 times further toward the T cell zone than *Ccr7*^{-/-} DCs (**Figure 7G**).

To further validate and expand our approach, we used lentiviral-based CRISPR/Cas9 technology to knockout *Trpml1*, a gene encoding for the ionic channel TRPML1 (transient receptor potential cation channel, mucolipin subfamily, member 1). TRPML1 was recently described to be required for fast and persistent migration of activated DCs, and TRPML1-deficient mature DCs migrated less efficiently to the draining LNs upon injection into the footpad (46). Due to the lack of TRPML1-specific antibodies for TRPML1 detection by flow cytometry, we confirmed *Trpml1* gene editing by sequence trace decomposition and found that two selected *Trpml1*-targeting sgRNAs (sgRNA 1 and 3) induce gene editing in more than 80% of the Cas9-Hoxb8 cells also expressing the fluorescent marker Cerulean encoded by the same lentivirus (**Figure 8A**), suggesting that the large majority, if not all, transduced cells has an edited *Trpml1* gene. Therefore, we used these cells as *Trpml1*^{-/-} Hoxb8-DCs and compared their migration during entry from afferent lymphatics into the lymph nodes via the SCS floor to *Trpml1*^{+/+} DCs (**Figure 8B**). We found slightly less *Trpml1*^{-/-} DCs compared to *Trpml1*^{+/+} DCs within the draining popliteal LN (**Figures 8B,C**). More pronounced was migration impairment of *Trpml1*^{-/-} DCs, preventing them from entry into the LN parenchyma and 4 h after i.l. transfer $41.6 \pm 14.7\%$ of these cells still resided in the LN sinus (**Figures 8B,D**). Furthermore, analysis of those cells that penetrated the LN parenchyma from the SCS floor revealed that, on average, *Trpml1*^{+/+} DCs had progressed more than 2 times further toward the T cell zone than *Trpml1*^{-/-} DCs (**Figure 8E**).

Collectively, these data suggest that Cas9-Hoxb8 cell-derived DCs rely on the same mechanisms for migration like BM-derived DCs and can therefore serve as an excellent tool to dissect DC migration.

The Unlimited Proliferative Capacity of Cas9-Hoxb8 Cells Allows Consecutive Genetic Manipulations

The fact that Cas9-Hoxb8 cells could be maintained for a period of at least 16 weeks in the undifferentiated state while keeping full differentiation capacity offers many experimental benefits. Beside their potential for generating high numbers of knockout cells or

their storage upon freezing for future experiments, the longevity also provides the opportunity to knockout multiple genes by consecutive genetic manipulations. To test this, we transduced dTom⁺ *Ccr7*^{-/-} Cas9-Hoxb8 cells with a lentivirus expressing Cerulean and a sgRNA targeting *Cxcr4*. This second round of transduction was done in the presence of cyclosporine A, as this has been demonstrated to enhance the transduction rate by overcoming a restriction against lentiviruses (47, 48). Transduced cells as well as control Cas9-Hoxb8 cells were subsequently differentiated into mature DCs. Flow cytometric analysis revealed that dTom⁺ Cerulean⁺ DCs lacked CCR7 expression and showed only marginal CXCR4 expression despite being fully activated and exhibiting high levels of CD80 (**Figure 9**).

Cas9-Hoxb8 Cells Also Allow Stable Transduction With Genetically Encoded Calcium Indicator for Tracking of Chemokine-Induced Calcium Signals *in vivo*

To exploit the full potential of the multiple genetic modifications in Cas9-Hoxb8-DCs for investigating DC migration, we decided to retrovirally engineer *Ccr7*^{+/+} and *Ccr7*^{-/-} Cas9-Hoxb8-DCs to express the genetic calcium (Ca²⁺) sensor GCaMP6S (37) together with constitutively expressed dTomato. By this approach we aimed to create DCs proficient or deficient for CCR7 allowing recording Ca²⁺ signaling in real-time during DC entry into the LN parenchyma. Initial calcium flux assays using flow cytometry indicated that CCL21 specifically triggers calcium flux in *Ccr7*^{+/+} but not *Ccr7*^{-/-} Cas9-Hoxb8-DCs (**Figure 10**). We next intralymphatically injected GCaMP6S⁺ *Ccr7*^{+/+} or *Ccr7*^{-/-} Cas9-Hoxb8-DCs and imaged them by two-photon microscopy during the first 2 h after injection. Within this time frame, intralymphatically injected DCs are known to enter the lymph node parenchyma (9). Using this approach, we observed that many GCaMP6S⁺ *Ccr7*^{+/+} Cas9-Hoxb8-DCs exhibit prominent changes in GCaMP6S intensity during the recording period, while only few GCaMP6S⁺ *Ccr7*^{-/-} DCs showed signal alteration above background (**Figure 11A** and **Supplementary Video 1**). To quantify the Ca²⁺ signals, we tracked the injected cells based on their expression of the reporter gene dTomato (not shown) and analyzed mean intensity values of GCaMP6S intensity as described in Materials and Methods. **Figure 11B** illustrates differences in GCaMP6S intensity of representative GCaMP6S⁺ *Ccr7*^{+/+} or *Ccr7*^{-/-} Cas9-Hoxb8-DCs. Quantitative analysis revealed that 39% of injected GCaMP6S⁺ *Ccr7*^{+/+}, but only 17% of GCaMP6S⁺ *Ccr7*^{-/-} Cas9-Hoxb8-DCs had at least one prominent change in GCaMP6S signal, indicating change in intracellular Ca²⁺ concentration (**Figure 11C**). A more detailed

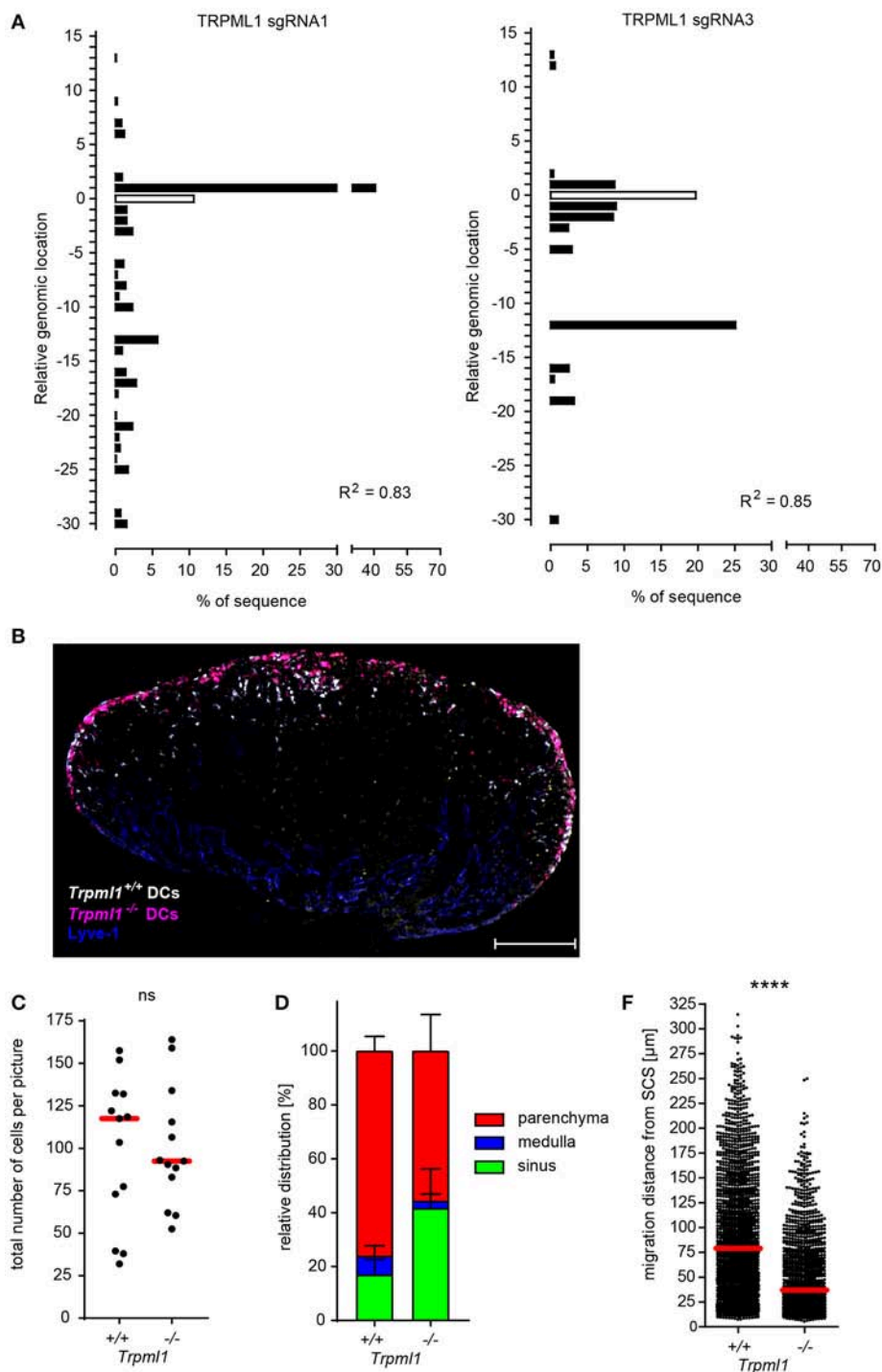


FIGURE 8 | TRPML1 is required for efficient DC migration from the subcapsular sinus into the lymph node parenchyma. **(A)** Analysis of the composition and frequency of insertions and deletions of Cas9-Hoxb8 cells which were transduced with lentiviruses expressing different sgRNAs targeting *Trpml1*. Cas9-Hoxb8 cells which were transduced with lentiviruses expressing either sgRNA 1 or 3 were used to generate *Trpml1*^{-/-} DCs for further experiments. R, Pearson correlation coefficient; R^2 describes how strongly the calculated chromatograph of the indel distribution correlates with the Sanger sequencing results of the sample DNA. **(B)** Microscopy of popliteal lymph nodes obtained 4 h after intralymphatic injection of YFP-expressing *Trpml1*^{+/+} DCs and dTom-expressing *Trpml1*^{-/-} DCs (1:1 mixture; 8×10^4 cells in 5 μ l PBS; scale bar: 200 μ m). Data are representative for three independent experiments with 13 lymph nodes. **(C)** Total cell counts and **(D)** relative distribution of *Trpml1*^{+/+} and *Trpml1*^{-/-} DCs 4 h after intralymphatic injection into popliteal LNs of B6 mice. **(E)** Migration distance from the subcapsular sinus (SCS) for the *Trpml1*^{+/+} and *Trpml1*^{-/-} DCs that entered LN parenchyma. Dots represent average cell number per LN section **(C)** or individual cells **(E)**. Data are pooled from three independent experiments with a total of 13 lymph nodes analyzed. Error bars, SD; red bars, median; Mann-Whitney test; ns, not significant; **** $p < 0.0001$.

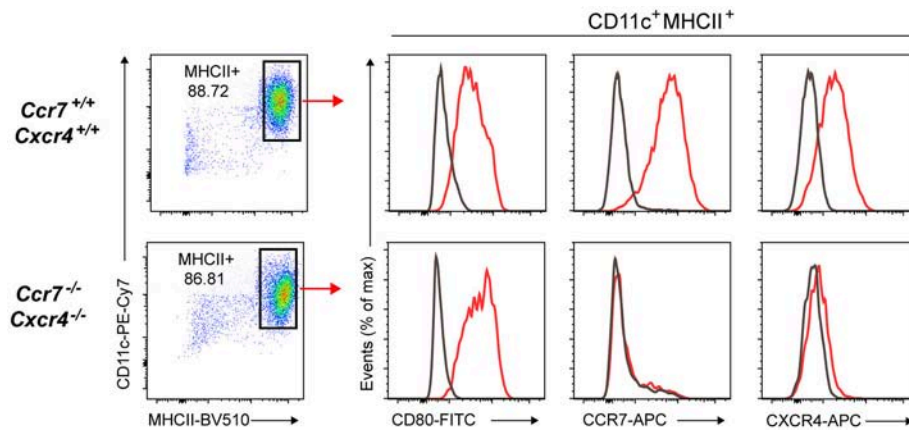


FIGURE 9 | The unlimited proliferative capacity of Cas9-Hoxb8 cells allows the consecutive knockout of multiple genes. *Ccr7*^{-/-} Cas9-Hoxb8 cells were transduced with a Cerulean- and CXCR4-gRNA-encoding lentivirus. Transduced cells as well as control Cas9-Hoxb8 cells were subsequently differentiated to mature DCs in the presence of GM-CSF followed by the treatment with LPS. DCs were analyzed by flow cytometry for their expression of CD80, CCR7, and CXCR4. Gray curves depict isotype controls. Data are representative of two independent experiments.

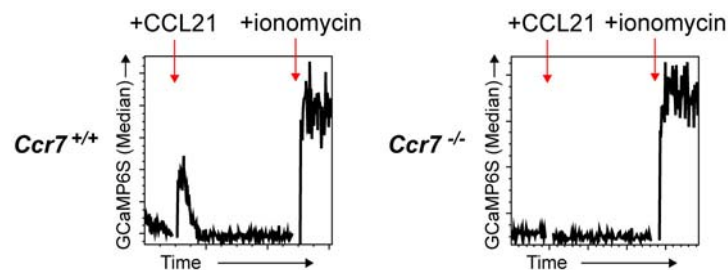


FIGURE 10 | GCaMP6S functions as an efficient and specific sensor for Ca^{2+} flux in Cas9 Hoxb8 cell-derived DCs. *Ccr7*^{+/+} and *Ccr7*^{-/-} Cas9-Hoxb8 cells were transduced with a retrovirus expressing dTom and the Ca^{2+} sensor GCaMP6S. Successfully transduced cells were sorted based on the expression of dTom and subsequently differentiated to mature DCs in the presence of GM-CSF followed by LPS treatment. GCaMP6S intensity was measured by flow cytometry. After recording a baseline for 45 s, cells were treated with 500 ng/ml CCL21 and signals were recorded for additional 240 s. Finally, 1 $\mu\text{g}/\text{ml}$ of ionomycin was added and signals were acquired for another 60 s. Data are representative for three independent experiments.

analysis of cells with changes in GCaMP6S signals indicated that there is no difference in the median number of calcium signals per GCaMP6S⁺ *Ccr7*^{+/+} and *Ccr7*^{-/-} Cas9-Hoxb8-DCs (**Figure 11D**), but that the average signal duration is significantly prolonged in cells expressing CCR7 compared to those were *Ccr7* had been disrupted (**Figure 11E**). Overall, these results suggest that Cas9-Hoxb8-DCs expressing the Ca^{2+} indicator GCaMP6S allow real-time tracking of Ca^{2+} signals during migration and chemokine recognition *in vivo*.

DISCUSSION

In this study, we expanded the benefits of CRISPR/Cas9 technology in hematopoietic progenitor cells transiently immortalized by the transcription factor Hoxb8 which enforces self-renewal and arrests differentiation (24–26, 49, 50). Specifically, we conditionally immortalized hematopoietic progenitor cells from the BM of Cas9-transgenic mice by the

introduction of a Dox-regulated form of Hoxb8 giving rise to Cas9-Hoxb8 cells. These cells resemble common myeloid progenitor cells that can be stably kept in the culture for at least 16 weeks (data not shown) and thus provide superfluous time for multiple genetic modifications by, in our case, consecutive retroviral or lentiviral transduction, as demonstrated for the knockout of *Ccr7*, *Cxcr4*, and *Trpml1*. Moreover, overexpression of the genetically encoded Ca^{2+} sensor GCaMP6S demonstrates that cells with reporter proteins or gene overexpression can also be obtained with high efficacy. Successfully manipulated cells can be purified based on the expression of fluorescent proteins, subsequently expanded, kept in culture and cryopreserved for future projects. Therefore, the Cas9-Hoxb8 cells presented in this study offer a fast track toward genetically modified myeloid cells, circumventing problems associated with low transduction of primary cells as well as time-consuming and costly breeding to obtain multiple-gene knockout mice (depicted on **Figure 12**).

In our hands, Cas9-Hoxb8 cells generated from lineage-depleted BM cell retained macrophage, dendritic cell and

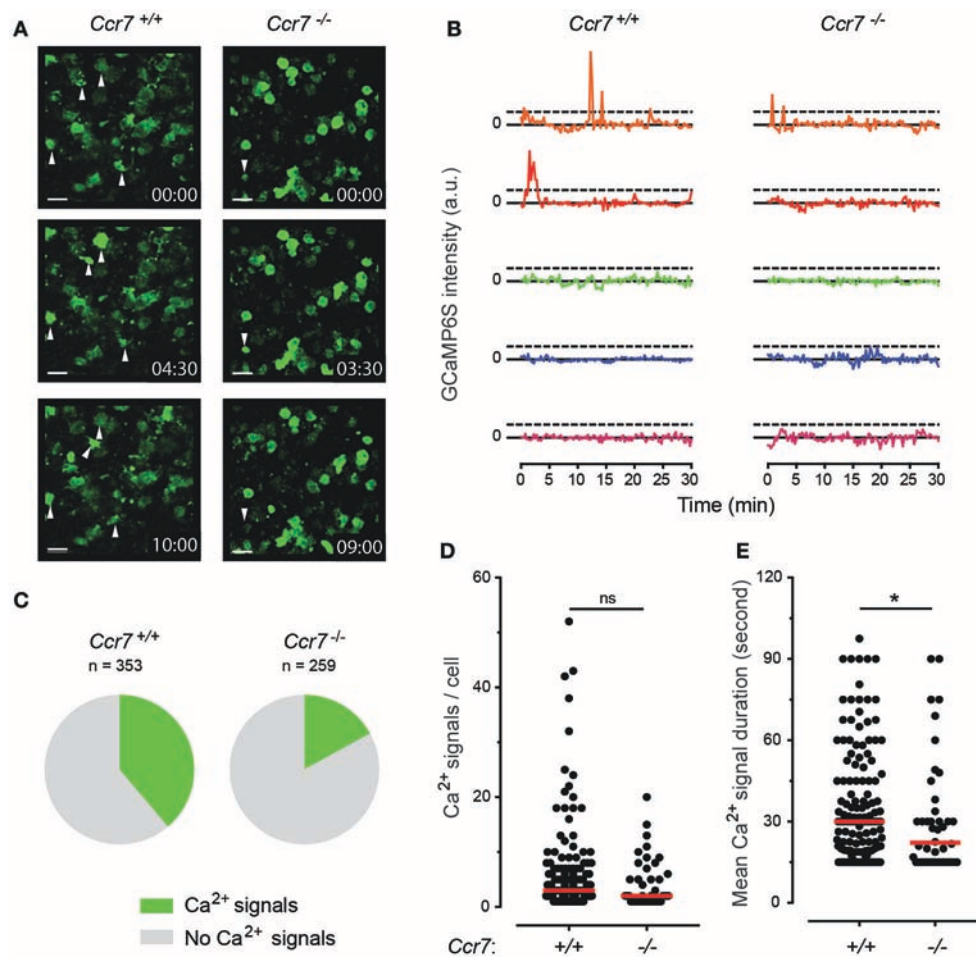


FIGURE 11 | CCR7-induced Ca²⁺ signaling during entry of Cas9-Hoxb8-DCs transduced with Ca²⁺ sensor GCaMP6S into the lymph node. **(A)** *Ex vivo* time-lapse imaging of lymph nodes within 5 min after intralymphatic injection of 4×10^4 *Ccr7*^{+/+} Hoxb8-DCs (left) or *Ccr7*^{-/-} Hoxb8-DCs (right) transduced with Ca²⁺ sensor GCaMP6S. White arrowheads indicate cells with changes in GCaMP6S signal intensity, indicating changes in Ca²⁺ concentration within the cell. For more details see **Supplementary Video 1**. Scale bar represents 15 μ m. **(B)** Changes in GCaMP6S signal intensity for each 5 GCaMP6S⁺ *Ccr7*^{+/+} Cas9-Hoxb8-DCs and GCaMP6S⁺ *Ccr7*^{-/-} Cas9-Hoxb8-DCs. Data are represented as a difference in GCaMP6S signal intensity for each time point to the median value for next 3 min. Horizontal dashed lines depict thresholds [defined as a change in a signal intensity >1,000 arbitrary units (a.u.)] used to detect Ca²⁺ signals. **(C)** Pie-charts indicating the percentage of GCaMP6S⁺ *Ccr7*^{+/+} Cas9-Hoxb8-DCs or GCaMP6S⁺ *Ccr7*^{-/-} Cas9-Hoxb8-DCs with changes in Ca²⁺ signals. There is a significant difference between groups ($p < 0.001$, two-tailed Fisher's exact test). **(D)** Number and **(E)** average duration of Ca²⁺ signals (changes in GCaMP6S intensity) per cell for the tracks with at least one recorded Ca²⁺ signal. In **(D,E)** dots represent individual cells and red line median group value. Asterisk (*) indicates significant difference ($p < 0.05$), while ns indicates no significant difference (Mann-Whitney test). Data are representative **(A,B)** or pool **(C-E)** of 6 independent experiments with total of 7 lymph nodes per cell type.

granulocyte potential. Interestingly, they did not differentiate into cDCs and pDCs in the presence of Flt3L. The non-responsiveness of Cas9-Hoxb8 cells to Flt3L might be linked to their marginal Flt3 expression (**Figure 1D**). In contrast, Redecke and colleagues reported that conditionally immortalized early hematopoietic progenitor cells from crude preparations of BM cells were dependent on Flt3L and could be induced by this factor to differentiate into cDCs and pDCs (24). Moreover, the Hoxb8 cells used in that study even retained B cell and limited T cell potential (24). Likewise, Hoxb8⁺ hematopoietic progenitor cells transduced with Cas9 maintained their potential to differentiate into pDCs, which was exploited to investigate

the role of E protein TCF4 in pDC development (25). Observed differences between these Hoxb8 cells (24, 25) and our Cas9-Hoxb8 cells most likely could be attributed to the differences in the experimental protocols used for immortalization of hematopoietic progenitor cells from BM, such as differences in viral vector design or viral particle tropism leading to the selective infection of progenitors lacking pDC potential. Similarly, the cytokine cocktail, in which the immortalized cells are raised and propagated, presumably shapes their lineage potential. The combination of mSCF, huIL-11, huFlt3L and mIL-3, which we employed here, has been described to support good proliferation of progenitor cells while shifting them toward the myeloid lineage

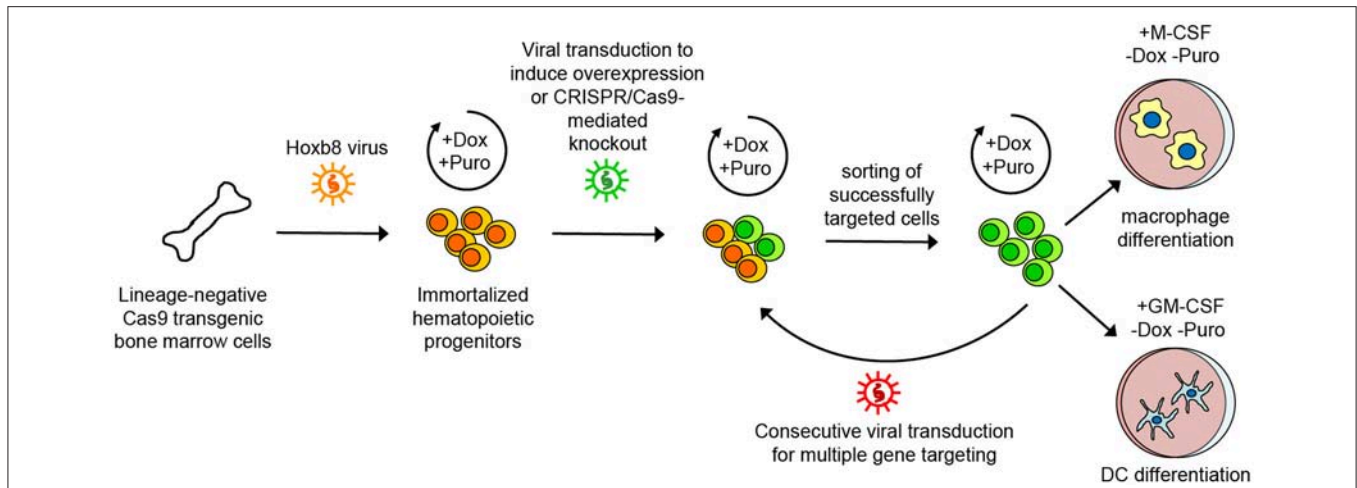


FIGURE 12 | Scheme of generation, maintenance, genetic modification and differentiation of Cas9-Hoxb8 cells. Lineage-negative cells from the bone marrow of Cas9 transgenic mice were conditionally immortalized by lentiviral transduction introducing a doxycycline (Dox)-regulated form of the transcription factor Hoxb8 and a puromycin (Puro) selection cassette. Cas9-Hoxb8 cells could be kept for weeks in a non-differentiated state by culture in the presence of mSCF, huIL-11, huFlt3L, mIL-3, Dox, and Puro. Genetic modifications such as overexpression or CRISPR/Cas9-mediated knockout could be introduced by viral transduction. Successfully targeted cells were selected based on fluorescence-activated cell sorting. The longevity of Cas9-Hoxb8 cells provided the opportunity to knockout multiple genes by consecutive genetic manipulations. For differentiation into macrophages or dendritic cells (DCs), mSCF, huIL-11, huFlt3L, mIL-3, Dox, and Puro were replaced with macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF).

(51) which is in line with the limited lineage potential that we observed in our study. Thus, in the future, further culture protocols employing different combinations of cytokines, such as those described by Lodish Lab (52–55), remain to be tested for a better preservation of the stemness of immortalized cells.

GM-CSF-derived CD11⁺MHCII⁺ DCs from bone marrow have been described to be phenotypically heterogeneous, as they expand from divergent hematopoietic progenitors (45). In this regard, the unlimited proliferative capacity of immortalized progenitor cells might offer new possibilities to obtain a defined population of either only monocyte-derived macrophage or exclusively conventional DC resembling cells from a GM-CSF differentiation culture. This could be possibly achieved by either sorting of defined monocyte or dendritic cell precursor populations from bone marrow as targets for viral transduction with Hoxb8 or, alternatively, by subcloning of cells after Hoxb8-mediated immortalization.

Within the scope of our study, we focused on Cas9-Hoxb8 cells as a source of genetically modified dendritic cells for the investigation of dendritic cell migration. In line with previous observations with Hoxb8-FL cells (24), GM-CSF-differentiated Cas9-Hoxb8 DCs showed the classical phenotype and T cell activation potential of GM-CSF-differentiated BM-derived DCs. Further, they entered the lymph node following intralymphatic injection in a CCR7-dependent manner in a similar fashion as described earlier for BM-derived DCs (9). In the present study, we also addressed the role of the lysosomal ion channel TRPML1 in homing of lymph-delivered DCs. A recent study reported that TRPML1 is required for persistent migration and chemotaxis of activated DCs and *Trpml1*^{-/-} mature DCs were less efficient in migrating to the draining lymph node when

transferred into the footpad of recipient mice (46). To clarify whether *Trpml1*-deficient DCs are impaired in exiting from peripheral tissue or in exiting from the subcapsular sinus toward the deep T cell zone, we intralymphatically injected *Trpml1*-deficient DCs generated from GM-CSF- and LPS-stimulated Cas9-Hoxb8 cells and found that they translocated slower from the SCS to the T cell zone of the LN than *Trpml1*^{+/+} DCs.

Besides being a potent tool for investigation of gene function by their mutation, Cas9-Hoxb8 cells also provide an opportunity to investigate cellular functions by gene overexpression or expression of different reporters. Here, we have focused on chemokine-induced Ca²⁺ signaling by tracking changes in intracellular Ca²⁺ concentration in migrating DC expressing the genetically encoded Ca²⁺ sensor GCaMP6S (37). Our combination of immunoengineering approach and two-photon microscopy allowed us to gain *in vivo* insights into chemokine receptor-induced signaling cascades involved in the entry process of DCs arriving via afferent lymphatics. Intralymphatically administered BM-derived DCs transmigrate through the floor of LN subcapsular sinus in a highly directional way that depends on the interaction of CCR7 with its ligands CCL19 and CCL21 (8, 9). While long lasting Ca²⁺ signals were present in 39% of GCaMP6S⁺ *Ccr7*^{+/+} Cas9-Hoxb8-DCs, they were only observed in 17% of GCaMP6S⁺ *Ccr7*^{-/-} Cas9-Hoxb8-DCs. As Ca²⁺ signals were observed in both, migrating DCs and DCs remaining sessile in the SCS for entire observation period of 2 h, we speculate that Ca²⁺ signals observed in *Ccr7*-deficient cells might arise from the recognition of other chemokines such as CXCL12 that is also present in the subcapsular sinus, or derive independent from any chemokine receptor signaling. As

alterations of intracellular Ca^{2+} concentrations are involved in many DC functions, including their migration and formation of immunological synapses with T cells (15, 56), it will be crucial in future experiments to employ our GCaMP6S⁺ Cas9-Hoxb8 cells to knockout additional genes involved in various aspects of DC function including *Trpm1*, *Cdc42*, or *RhoA*, which are all known to contribute for DC migration (46, 57).

Altogether, the proliferative capacity and gene editing potential of Cas9-Hoxb8 cells represent a potent platform that simultaneously enables multifaceted gene editing and overexpression of genetic reporters in many different cell types, allowing, in combination with immunophysics, almost indefinite possibilities for studies of hematopoietic cell differentiation and immune cell function.

AUTHOR CONTRIBUTIONS

SH, KW, BB, and RF designed the study. MR generated Hoxb8 cells. SH performed viral transduction, *in vitro* differentiation and flow cytometry experiments and analyzed the data. KW performed all intralymphatic injections, two-photon microscopy experiments and analyzed immunohistological data. MR, ASe, MG, AS, LL, and MP designed, cloned and validated viral vectors. AS and MG overviewed viral vector design and production. MG and MP produced viral vector supernatants. DNF performed DC-T cell co-culture experiments and analyzed the data. GEP performed gene editing efficiency analysis. KW, MP, GP, and BB analyzed two-photon microscopy data. AB helped with cell cultures and performed flow cytometry and immunofluorescent staining. BB and RF jointly supervised the project. BB and SH

wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This work was supported by the European Research Council (ERC advanced grant 322645) and by grants of the German Research Foundation (DFG-SFB738-B5 and DFG-Exc-62) to RF. AS, MG, and MR were supported by the German Research Foundation (DFG-EXC-62/REBIRTH). AS, MR, and ASe were supported by R2N, Federal State of Lower Saxony and the German Research Foundation (DFG: RO 5102/1-1). MG was supported by the German Research Foundation (SFB738-C4).

ACKNOWLEDGMENTS

We would like to acknowledge the assistance of the Cell Sorting Core Facility of Hannover Medical School and thank Bettina Weigel, Violetta Dziadek, and Girmay Asgedom for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01949/full#supplementary-material>

Supplementary Video 1 | Ca^{2+} concentration changes in *Ccr7*^{+/+} and *Ccr7*^{-/-} Hoxb8-DCs transduced with Ca^{2+} sensor GCaMP6S during entry into the lymph node. Example two-photon microscopy imaging of GCaMP6S⁺ *Ccr7*^{+/+} and *Ccr7*^{-/-} Hoxb8-DCs (green) in the subcapsular sinus of popliteal LN after intralymphatic injection into the lymph vessel draining to this LN. Scale bar represents 20 μm . Representative of 7 movies from 7 mice for each cell genotype.

REFERENCES

- Worbs T, Hammerschmidt SI, Förster R. Dendritic cell migration in health and disease. *Nat Rev Immunol.* (2017) 17:30–48. doi: 10.1038/nri.2016.116
- Permanyer M, Bošnjak B, Förster R. Dendritic cells, T cells and lymphatics: dialogues in migration and beyond. *Curr Opin Immunol.* (2018) 53:173–9. doi: 10.1016/j.coi.2018.05.004
- Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* (2004) 21:279–88. doi: 10.1016/j.immuni.2004.06.014
- Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DE, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* (2013) 339:328–32. doi: 10.1126/science.1228456
- Vaahromeri K, Brown M, Hauschild R, De Vries I, Leithner AF, Mehling M, et al. Locally triggered release of the chemokine CCL21 promotes dendritic cell transmigration across lymphatic endothelia. *Cell Rep.* (2017) 19:902–9. doi: 10.1016/j.celrep.2017.04.027
- Pflicke H, Sixt M. Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels. *J Exp Med.* (2009) 206:2925–35. doi: 10.1084/jem.20091739
- Russo E, Teixeira A, Vaahromeri K, Willrodt AH, Bloch JS, Nitschké M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep.* (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
- Ulvmar MH, Werth K, Braun A, Kelay P, Hub E, Eller K, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol.* (2014) 15:623–30. doi: 10.1038/ni.2889
- Braun A, Worbs T, Moschovakis GL, Halle S, Hoffmann K, Bölter J, et al. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat Immunol.* (2011) 12:879–87. doi: 10.1038/ni.2085
- Johnson LA, Jackson DG. The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics. *J Cell Sci.* (2013) 126:5259–70. doi: 10.1242/jcs.135343
- Kabashima K, Shiraishi N, Sugita K, Mori T, Onoue A, Kobayashi M, et al. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol.* (2007) 171:1249–57. doi: 10.2353/ajpath.2007.070225
- Acton SE, Astarita JL, Malhotra D, Lukacs-Kornek V, Franz B, Hess PR, et al. Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2. *Immunity* (2012) 37:276–89. doi: 10.1016/j.immuni.2012.05.022
- Johnson LA, Banerji S, Lawrance W, Gileadi U, Prota G, Holder K A, et al. Dendritic cells enter lymph vessels by hyaluronan-mediated docking to the endothelial receptor LYVE-1. *Nat Immunol.* (2017) 18:762–70. doi: 10.1038/ni.3750
- Rathinasamy A, Czeloth N, Pabst O, Förster R, Bernhardt G. The origin and maturity of dendritic cells determine the pattern of sphingosine 1-phosphate receptors expressed and required for efficient migration. *J Immunol.* (2010) 185:4072–81. doi: 10.4049/jimmunol.1000568
- Heuzé ML, Vargas P, Chabaud M, Le Berre M, Liu YJ, Collin O, et al. Migration of dendritic cells: physical principles, molecular mechanisms, and functional implications. *Immunol Rev.* (2013) 256:240–54. doi: 10.1111/imr.12108

16. Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R, Hirsch K, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature* (2008) 453:51–5. doi: 10.1038/nature06887
17. Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* (2012) 482:331–8. doi: 10.1038/nature10886
18. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable dual-RNA – guided DNA endonuclease in adaptive bacterial immunity. *Science* (2012) 337:816–22. doi: 10.1126/science.1225829
19. Cong L, Ran A, Cox D, Shuailiang L, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas system. *Science* (2013) 339:819–24. doi: 10.1126/science.1231143
20. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. *Science* (2013) 339:823–6. doi: 10.1126/science.1232033
21. Chu VT, Graf R, Wirtz T, Weber T, Favret J, Li X, et al. Efficient CRISPR-mediated mutagenesis in primary immune cells using CrispRGold and a C57BL/6 Cas9 transgenic mouse line. *Proc Natl Acad Sci USA*. (2016) 113:12514–9. doi: 10.1073/pnas.1613884113
22. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* (2014) 159:440–55. doi: 10.1016/j.cell.2014.09.014
23. Parnas O, Jovanovic M, Eisenhaure TM, Herbst RH, Dixit A, Ye CJ, et al. A genome-wide CRISPR screen in primary immune cells to dissect regulatory networks. *Cell* (2015) 162:675–86. doi: 10.1016/j.cell.2015.06.059
24. Redecke V, Wu R, Zhou J, Finkelstein D, Chaturvedi V, High AA, et al. Hematopoietic progenitor cell lines with myeloid and lymphoid potential. *Nat Methods* (2013) 10:795–803. doi: 10.1038/nmeth.2510
25. Grajkowska LT, Ceribelli M, Lau CM, Warren ME, Tiniakou I, Nakandakari Higa S, et al. Isoform-specific expression and feedback regulation of E protein TCF4 control dendritic cell lineage specification. *Immunity* (2017) 46:65–77. doi: 10.1016/j.immuni.2016.11.006
26. Leithner A, Renkawitz J, De Vries I, Hauschild R, Häcker H, Sixt M. Fast and efficient genetic engineering of hematopoietic precursor cells for the study of dendritic cell migration. *Eur J Immunol*. (2018) 48:1074–7. doi: 10.1002/eji.201747358
27. Hope T. Improving the post-transcriptional aspects of lentiviral vectors. *Curr Top Microbiol Immunol*. (2002) 261:179–89. doi: 10.1007/978-3-642-56114-6_9
28. Schambach A, Galla M, Maetzig T, Loew R, Baum C. Improving transcriptional termination of self-inactivating gamma-retroviral and lentiviral vectors. *Mol Ther*. (2007) 15:1167–73. doi: 10.1038/sj.mt.6300152
29. Stemmer M, Thumberger T, Del Sol Keyer M, Wittbrodt J, Mateo JL. CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PLoS ONE* (2015) 10:e0124633. doi: 10.1371/journal.pone.0124633
30. Bošnjak B, Permanyer M, Sethi MK, Galla M, Maetzig T, Heinemann D, et al. CRISPR/Cas9 genome editing using gold-nanoparticle-mediated laserporation. *Adv Biosyst*. (2018). doi: 10.1002/adbi.201700184. [Epub ahead of print].
31. Heckl D, Kowalczyk MS, Yudovich D, Belzaira R, Puram R V., McConkey ME, et al. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat Biotechnol*. (2014) 32:941–6. doi: 10.1038/nbt.2951
32. Heinz N, Schambach A, Galla M, Maetzig T, Baum C, Loew R, et al. Retroviral and transposon-based tet-regulated all-in-one vectors with reduced background expression and improved dynamic range. *Hum Gene Ther*. (2011) 22:166–76. doi: 10.1089/hum.2010.099
33. Dreyer AK, Hoffmann D, Lachmann N, Ackermann M, Steinemann D, Timm B, et al. TALEN-mediated functional correction of X-linked chronic granulomatous disease in patient-derived induced pluripotent stem cells. *Biomaterials* (2015) 69:191–200. doi: 10.1016/j.biomaterials.2015.07.057
34. Warlich E, Kuehle J, Cantz T, Brugman MH, Maetzig T, Galla M, et al. Lentiviral vector design and imaging approaches to visualize the early stages of cellular reprogramming. *Mol Ther*. (2011) 19:782–9. doi: 10.1038/mt.2010.314
35. Schambach A, Böhne J, Chandra S, Will E, Margison GP, Williams DA, et al. Equal potency of gammaretroviral and lentiviral SIN vectors for expression of O6-methylguanine-DNA methyltransferase in hematopoietic cells. *Mol Ther*. (2006) 13:391–400. doi: 10.1016/j.yimthe.2005.08.012
36. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther*. (2000) 7:1063–6. doi: 10.1038/sj.gt.3301206
37. Chen TW, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* (2013) 499:295–300. doi: 10.1038/nature12354
38. Halle S, Keyser KA, Stahl FR, Busche A, Marquardt A, Zheng X, et al. *In vivo* killing capacity of cytotoxic T cells is limited and involves dynamic interactions and T cell cooperativity. *Immunity* (2016) 44:233–45. doi: 10.1016/j.immuni.2016.01.010
39. Galla M, Schambach A, Baum C. Retrovirus-based mRNA transfer for transient cell manipulation. *Methods Mol Biol*. (2013) 969:139–61. doi: 10.1007/978-1-62703-260-5_10
40. Rothe M, Rittelmeyer I, Iken M, Rüdrieh U, Schambach A, Glage S, et al. Epidermal growth factor improves lentivirus vector gene transfer into primary mouse hepatocytes. *Gene Ther*. (2012) 19:425–34. doi: 10.1038/gt.2011.117
41. Ho VWH, Sly LM. Derivation and characterization of murine alternatively activated (M2) macrophages. *Methods Mol Biol*. (2009) 531:173–85. doi: 10.1007/978-1-59745-396-7_12
42. Kohli K, Janssen A, Förster R. Plasmacytoid dendritic cells induce tolerance predominantly by cargoing antigen to lymph nodes. *Eur J Immunol*. (2016) 58:7250–7. doi: 10.1002/eji.201646359
43. Hsiao T, Maures T, Waite K, Yang J, Kelso R, Holden K, et al. Inference of CRISPR edits from sanger trace data. *bioRxiv [preprint]*. (2018) doi: 10.1101/251082
44. Halle S, Dujardin HC, Bakocevic N, Fleige H, Danzer H, Willenzon S, et al. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. *J Exp Med*. (2009) 206:2593–601. doi: 10.1084/jem.20091472
45. Helft J, Böttcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c(+)MHCII(+) macrophages and dendritic cells. *Immunity* (2015) 42:1197–211. doi: 10.1016/j.immuni.2015.05.018
46. Bretou M, Sáez PJ, Sanséau D, Maurin M, Lankar D, Chabaud M, et al. Lysosome signaling controls the migration of dendritic cells. *Sci Immunol*. (2017) 2:eaak9573. doi: 10.1126/sciimmunol.aak9573
47. Noser JA, Towers GJ, Sakuma R, Dumont J-M, Collins MKL, Ikeda Y. Cyclosporine increases human immunodeficiency virus type 1 vector transduction of primary mouse cells. *J Virol*. (2006) 80:7769–74. doi: 10.1128/JVI.02427-05
48. Geis FK, Galla M, Hoffmann D, Kuehle J, Zychlinski D, Maetzig T, et al. Potent and reversible lentiviral vector restriction in murine induced pluripotent stem cells. *Retrovirology* (2017) 14:34. doi: 10.1186/s12977-017-0358-1
49. Rosas M, Osorio F, Robinson MJ, Davies LC, Dierkes N, Jones SA, et al. Hoxb8 conditionally immortalised macrophage lines model inflammatory monocytic cells with important similarity to dendritic cells. *Eur J Immunol*. (2011) 41:356–65. doi: 10.1002/eji.201040962
50. Wang GG, Calvo KR, Pasillas MP, Sykes DB, Häcker H, Kamps MP. Quantitative production of macrophages or neutrophils *ex vivo* using conditional Hoxb8. *Nat Methods* (2006) 3:287–93. doi: 10.1038/nmeth865
51. Li Z, Schwieger M, Lange C, Kraunus J, Sun H, van den Akker E, et al. Predictable and efficient retroviral gene transfer into murine bone marrow repopulating cells using a defined vector dose. *Exp Hematol*. (2003) 31:1206–14. doi: 10.1016/j.exphem.2003.08.008
52. Zhang CC, Lodish HF. Murine hematopoietic stem cells change their surface phenotype during *ex vivo* expansion. *Blood* (2005) 105:4314–20. doi: 10.1182/blood-2004-11-4418
53. Zhang CC, Kaba M, Iizuka S, Huynh H, Lodish HF. Angiopoietin-like 5 and IGFBP2 stimulate *ex vivo* expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood* (2008) 111:3415–23. doi: 10.1182/blood-2007-11-122119

54. Zhang CC, Lodish HF. Cytokines regulating hematopoietic stem cell function. *Curr Opin Hematol.* (2008) 15:307–11. doi: 10.1097/MOH.0b013e3283007db5
55. Zhang CC, Kaba M, Ge G, Xie K, Tong W, Hug C, et al. Angiopoietin-like proteins stimulate *ex vivo* expansion of hematopoietic stem cells. *Nat Med.* (2006) 12:240–5. doi: 10.1038/nm1342
56. Shumilina E, Huber SM, Lang F. Ca²⁺ signaling in the regulation of dendritic cell functions. *Am J Physiol Cell Physiol.* (2011) 300:C1205–14. doi: 10.1152/ajpcell.00039.2011
57. Vargas P, Maiuri P, Bretou M, Sáez PJ, Pierobon P, Maurin M, et al. Innate control of actin nucleation determines two distinct migration behaviours in dendritic cells. *Nat Cell Biol.* (2016) 18:43–53. doi: 10.1038/ncb3284

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Lipidomimetic Compounds Act as HIV-1 Entry Inhibitors by Altering Viral Membrane Structure

OPEN ACCESS

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Specialty section:

This article was submitted to
Cytokines and Soluble Mediators in
Immunity,
a section of the journal
Frontiers in Immunology

Received: 29 May 2018

Accepted: 13 August 2018

Published: 04 September 2018

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The envelope of Human Immunodeficiency Virus type 1 (HIV-1) consists of a liquid-ordered membrane enriched in raft lipids and containing the viral glycoproteins. Previous studies demonstrated that changes in viral membrane lipid composition affecting membrane structure or curvature can impair infectivity. Here, we describe novel antiviral compounds that were identified by screening compound libraries based on raft lipid-like scaffolds. Three distinct molecular structures were chosen for mode-of-action studies, a sterol derivative (J391B), a sphingosine derivative (J582C) and a long aliphatic chain derivative (IBS70). All three target the viral membrane and inhibit virus infectivity at the stage of fusion without perturbing virus stability or affecting virion-associated envelope glycoproteins. Their effect did not depend on the expressed envelope glycoproteins or a specific entry route, being equally strong in HIV pseudotypes carrying VSV-G or MLV-Env glycoproteins. Labeling with laurdan, a reporter of membrane order, revealed different membrane structure alterations upon compound treatment of HIV-1, which correlated with loss of infectivity. J582C and IBS70 decreased membrane order in distinctive ways, whereas J391B increased membrane order. The compounds' effects on membrane order were reproduced in liposomes generated from extracted HIV lipids and thus independent both of virion proteins and of membrane leaflet asymmetry. Remarkably, increase of membrane order by J391B required phosphatidylserine, a lipid enriched in the HIV envelope. Counterintuitively, mixtures of two compounds with opposite effects on membrane order, J582C and J391B, did not neutralize each other but synergistically inhibited HIV infection. Thus, altering membrane order, which can occur by different mechanisms, constitutes a novel antiviral mode of action that may be of general relevance for enveloped viruses and difficult to overcome by resistance development.

Keywords: lipidomimetics, HIV-1 envelope, lipid raft modulation, laurdan, membrane order, HIV fusion inhibitors, phosphatidylserine

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is an enveloped retrovirus, which infects CD4-positive human cells. HIV-1 morphogenesis at the plasma membrane of the infected cell is driven by the viral Gag polyprotein whose N-terminal MA (matrix) domain interacts with phosphatidylinositol (4,5) bispophosphate (PI(4,5)P₂) [reviewed in (1)]. Since retroviruses do not encode lipid-synthesizing enzymes, their lipid envelope composition depends on the membrane through which the virus buds (2, 3). However, viral membrane composition may differ from the donor cell membrane if virus assembly occurs at membrane subdomains or involves lipid sorting. Early studies of HIV-1 lipid composition indicated significant differences between the viral membrane and the host cell plasma membranes (4, 5). This observation was later confirmed by more detailed analyses of the entire viral lipidome. The HIV-1 membrane was shown to be significantly enriched in phosphatidylserine (PS), sphingomyelin, hexosylceramide and saturated phosphatidylcholine species when compared to the host cell plasma membrane (6–8). Overall, the HIV-1 lipid composition is typical of lipid rafts (6). Moreover, labeling HIV-1 with the order-sensing dye laurdan revealed a liquid-ordered (l_o) structure of the viral envelope (9).

The intrinsic properties of the viral membrane, as well as its lipid composition, have been shown to be of importance for infectivity. Cholesterol-depleting agents (β -cyclodextrin and statins) (10–13) or cholesterol-binding compounds (amphotericin B methyl ester) (14) as well as inhibition of sphingomyelin biosynthesis (6, 15, 16) strongly reduced HIV-1 infectivity, indicating an important contribution of its raft-like membrane lipid composition and/or structure to viral infection. A similar effect was observed when ceramide levels were increased in the viral membrane (17) or upon addition of a compound (GT11), which leads to higher dihydrosphingomyelin levels (18), an unusual lipid enriched in the viral membrane (6). Based on the concept of inverted cone-shaped lipids as fusion inhibitors (19), synthetic rigid amphipathic fusion inhibitors (RAFIs) have been designed as potential antivirals (20). These compounds insert into the viral membrane and promote positive curvature, thus increasing the energy barrier for fusion. RAFIs were shown to inhibit fusion of several unrelated enveloped viruses.

Here, we performed a screen of lipidomimetic compounds, the majority of which resembling raft lipids, for their capacity to alter the membrane of HIV-1 and interfere with viral infectivity. Several compounds structurally related to cholesterol, sphingosine or aliphatic lipids with long-chain fatty acids inhibited HIV-1 infection at the stage of entry. Similar inhibition was observed when HIV-1 was pseudotyped with heterologous envelope proteins, indicating that the effect was independent of the initial entry pathway and the envelope proteins mediating it. Incorporation of the compounds into the viral membrane inhibited viral membrane fusion, induced changes in viral membrane order and subtle shifts in particle buoyant density. Thus, altering virion membrane structure by lipid-active compounds may be a promising approach for inhibiting HIV.

MATERIALS AND METHODS

Cell Culture and Virus Purification

293T and TZM reporter cells (21) and DFJ8 cells (22) were kept in Dulbecco's modified Eagle's medium (DMEM), MT-4 cells (23) were kept in RPMI 1,640 medium. Both media were supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin, streptomycin, 4 mM glutamine, and 10 mM Hepes. Cell cultures were maintained at 37°C and 5% CO₂. All investigated HIV strains and constructs are listed in **Table S1** in the Supplementary Material. For virus production, MT-4 cells were infected with HIV-1 strain NL4-3 (24), and virus was harvested from cocultures of infected and uninfected cells before cytopathic effects were observed (25). 293T cells were transfected with the proviral plasmid pNL4-3 (24) or with pCHIV (26) by calcium phosphate precipitation. For generation of pseudotyped particles, cells were co-transfected with pNL4-3 carrying a deletion of the envelope gene and a plasmid expressing either the G glycoprotein of Vesicular Stomatitis Virus (VSV) (27) or the envelope proteins of Friend ecotropic Murine Leukemia Virus (MLV) (28) at a molar ratio 1:2. HIV-1 purification was performed essentially as described (25, 29). Briefly, medium was harvested, cleared by filtration, and particles were concentrated by ultracentrifugation through a cushion of 20% (w/w) sucrose. Concentrated HIV-1 was further purified by velocity gradient centrifugation on an Optiprep gradient (Axis-Shield; Oslo, Norway). This step largely removes exosomes and membrane vesicles. The visible virus fraction was collected and concentrated by centrifugation. The final pellet was resuspended in 150 mM NaCl, 10 mM Hepes pH 7.4, rapidly frozen in liquid nitrogen and stored at –80°C. The particle concentration was determined by enzyme-linked immunosorbent assay (ELISA) of p24. Inactivation of infectious HIV-1 was performed by incubating the virus with 5 mM AT-2 (2,2'-dithiodipyridine; aldrithiol-2; Sigma; St. Louis, MO, USA) for 1 h at 37°C with gentle stirring as described (30). Successful inactivation was controlled by culturing inactivated samples for 10 days with highly susceptible C8166 cells. In the case of adeno-associated virus (AAV) purification, standard triple transfection, and caesium chloride (CsCl) density gradient purification procedures were used (31).

Chemistry

The preparation of 3 β -amino-28-methoxylupene is described in (32). J391B (3 α -amino-28-methoxylupene) was synthesized by GVK Bio (Hyderabad, India) following the identical procedure for the β -anomer. The purity was > 98% by high-performance liquid chromatography (HPLC). The synthesis of J582C (Oxazolin 200) was described (33) with a purity of 99.2% by HPLC. IBS70 (STOCK1S-60139) and IBS95 (STOCK3S-53354) were purchased from Interbioscreen Ltd. (<http://www.ibscreen.com>).

Screening

Each compound was screened in duplicate, and each screen was repeated. Compound stock solutions at 2 mM were in glass vials. 100 μ l purified HIV-1_{NL4-3} (250 μ l/well; 0.5–0.75 μ g/ml) was incubated for 30 min at 400 rpm and 37°C on the thermomixer

(Eppendorf) with the compounds at 20 μ M and 1% FCS in a 96-well glass-coated V-bottom plate (LabHut) and then diluted 1:10 into MT-4 cell suspension culture. 180 μ l MT-4 cells were seeded into 96-well plates (CORNING, Poly-D-Lysine surface) at a cell density of 10^5 /well. 20 μ l (25–35 ng p24) virus-compound mixture was added to the cells, mixed by pipetting and incubated at 37°C. 18 h p.i. DNA was extracted and subjected to real-time PCR. DNA was isolated using QIAamp 96 DNA Blood Kit and vacuum extraction according to supplier's instruction. Real time PCR was performed with QuantiTect SYBR Green PCR Master MIX (Qiagen): 20 μ l reaction mix; twin.tec real-time PCR plates 96 (skirted) and optical caps for RT-PCR; 10 μ l master mix plus primers (TIP Molbiol, Berlin) at a concentration of 10 μ M each +8.8 μ l DNA template. Program: 1x 15 min 95°C; 40x 15 s 95°C; 30 s 60°C; 30 s 72°C.

Compound Treatment of Virus Particles and Cells

MT-4 cells were seeded in poly-D-lysine 96-well plates (CORNING, Poly-D-Lysine surface) and TZM cells were seeded in glass 96-well plates (Costar). Stocks of purified HIV-1_{NL43} were incubated with the different compound concentrations or DMSO as solvent control, in glass-coated plates (Costar) for 30 min at 37°C in RPMI or DMEM medium containing 0.1% FCS. Subsequently, 50 μ l virus-compound suspension was diluted into 150 μ l medium containing 0.1% FCS (final p24 amounts 25–35 ng) and used to infect target cells for 2 h. Following 2 h exposure, cells were washed and cultivated for 2 more days in complete DMEM or RPMI media. For pretreatment of cells, compounds at the indicated concentrations or DMSO as solvent control were incubated in glass-coated 96-well plates (250 μ l/well) for 30 min at 37°C in DMEM containing 0.1% FCS. Subsequently, 100 μ l of each compound suspension was added to the target cells for 30 min at 37°C followed by addition of untreated HIV-1 (25–35 ng p24) in 50 μ l of medium with 0.1% FCS. Following 2 h exposure, cells were washed and cultivated for 2 more days in complete DMEM followed by infectivity readout as above. A similar procedure was done for simultaneous virus and compound addition. Cells were pre-washed with media containing 0.1% FCS, and compounds at the indicated concentrations or DMSO as solvent control were incubated in glass-coated 96-well plates (250 μ l/well) for 30 min at 37°C in DMEM containing 0.1% FCS. Afterwards 100 μ l of compounds was added to the cells and immediately followed by adding untreated HIV-1 (25–35 ng p24) in 50 μ l of medium with 0.1% FCS for 2 h. Following a washing step, cells were cultivated for 2 more days in complete DMEM and scored for viral infectivity.

Infectivity and Luciferase Reporter Assay

To determine the effect of the compounds in virus infection, intracellular capsid (CA) staining was performed. MT-4 cells were seeded in poly-D-lysine 96-well plates (CORNING, Poly-D-Lysine surface). Cells were infected, as explained in the previous section, with different amounts of compound-pretreated virus for 2 h, followed by cultivation in medium containing 10% FCS for 2 more days. Subsequently, cells were fixed with 4%

paraformaldehyde and permeabilized for immunostaining. HIV-1 infected cells were identified by automated microscopic readout following staining with a phycoerythrin-conjugated antibody against the viral p24 CA protein (KC57-RD1; Beckman Coulter, Inc. Fullerton, USA). For each well the microscope takes 16 measurements. In case of AAV infection, TZM cells were seeded in glass 96-well plates (Costar). Afterwards virus-compound mixtures in medium containing 0.1% FCS were added to TZM cells for 2 h, followed by cultivation in medium containing 10% FCS for 2 more days. To quantify AAV-infected cells, the encoded mCherry reporter was detected by automated microscopy 48 h after infection. Images were acquired via fluorescence microscopy and then automatically analyzed using proprietary software. The infectivity of compound-treated HIV-1 was determined on TZM-bl reporter cells as described (34) with some modifications. TZM-bl reporter cells contain a luciferase gene under a promoter activated by the viral Tat protein. Upon infection and viral gene expression, the production of viral Tat protein induces luciferase gene expression, which enables the quantification of infection by measuring luciferase activity (35). TZM-bl cells (1.2×10^4 cells/well) were seeded one day before infection in a 96-well plate and were infected with compound-treated virus at desired concentrations as explained above. At 48 h post-infection, cells were lysed and luciferase activity was measured in the lysates as described by the manufacturer using the Promega Steady Glo kit and a microplate luminometer (Luminoskan Ascent; Thermo Labsystems, MA, USA). Uninfected cells were cultivated in the presence of compounds at the identical concentrations used in the infection assays, or in the presence of solvent alone (reference control). Following 2 h compound exposure, cells were washed and cultivated for 2 more days, at which time the cytotoxicity was determined by quantifying the amount of a formazan product metabolized by viable cells from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma) as reported (36). Alternatively, compounds were present for 2 days before the MTT assay.

Entry Assays

Standard HIV fusion assays were performed as described (37). HIV-1 particles carrying a Vpr- β -lactamase (Vpr-BlaM) fusion protein were obtained by co-transfection of 293T cells with pNL4-3 or plasmids for HIV pseudotype production and plasmid pMM310 (38) encoding the Vpr-BlaM fusion protein (5 μ g pMM310: 15 μ g pNL4-3). Particles were harvested, concentrated, and treated with the respective compounds as described above. The virus-compound mixture was added to cells and cells were subsequently washed once with CO₂-independent medium (Invitrogen), 70 μ l of CCF2 β -lactamase loading solution (Invitrogen; prepared according to the manufacturer's instructions) was added and incubation was continued for 17 h at room temperature. Relative fluorescence intensities [excitation wavelength 409 nm, emission wavelengths 447 nm (blue) and 512 nm (green)] were recorded using a TECAN Safire instrument. After subtraction of background from unstained cells at the respective emission

wavelength, the ratio of emission intensities at 447/512 nm was calculated.

Sucrose-Density Equilibrium Gradient Centrifugation and Western Blot Analysis

HIV-1_{NL4-3} or non-infectious HIV-like particles derived by transfection of pCHIV (1–3 µg p24) were incubated with the respective compound, solvent (DMSO; 0.35%) or Triton X-100 (TX-100) (0.5%). Subsequently, the particle suspensions were loaded onto a 20–60% linear sucrose gradient. After ultracentrifugation at 44,000 rpm for 16 h at 4°C in a SW60 rotor, 20 fractions of 200 µl each were carefully collected from top to bottom and the p24 concentration was analyzed as described (39). Their sucrose density was calculated from refractive indices determined with a refractometer (Abbe, Carl Zeiss). Carefully prepared sucrose solutions were used to build the gradient and as standards in the refractometer. For this purpose a sucrose gradient was run placing loading buffer instead of particle suspension on top of the gradient. After ultracentrifugation 20 fractions of 200 µl each were carefully collected from top to bottom and their refractive index was measured. Sucrose density in g/cm³ was calculated from the refractive index of the standards. For stability analysis, purified HIV-1 or HIV-like particles (3 µg p24) were exposed to compounds or solvent (DMSO; 0.35%) for 30 min at 37°C, pelleted through a 20% sucrose cushion by ultracentrifugation (32,000 rpm, 4°C, 2 h) and re-suspended in 25 µl SDS-PAGE sample buffer for subsequent analysis by Western blotting. Briefly, samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride (PVDF) membrane. After incubation with mouse monoclonal antibody against the gp41 trans-membrane glycoprotein 1:2000 [Chessie 8; (40)]; rabbit anti-MA, 1:5000; and sheep anti-p24 (CA), 1:5000, detection was carried out with a LiCoR Odyssey system using as secondary antibodies donkey anti-rabbit 700; donkey anti-sheep 800 and donkey anti-mouse 800, 1:20,000 (LiCoR).

HIV-1 Laurdan Staining and Analysis of Labeled Particles

Optiprep-purified HIV-1 particles were incubated for 10 min at room temperature with 5 µM laurdan (Molecular Probes, Eugene, OR). Labeled HIV-1 particles were subsequently purified by ultracentrifugation as described (9). In the case of compound treatment, laurdan labeled HIV-1 particles were incubated with different amounts of lipidomimetics at 37°C for 30 min under gentle stirring. Subsequently, viral particles were collected by ultracentrifugation for 2 h through a 20% sucrose cushion in a SW60 rotor at 32,000 rpm. Particles were carefully resuspended in 150 mM NaCl, 10 mM Hepes pH 7.4 and analyzed by fluorescence spectroscopy. All fluorescence measurements were made using an SLM Aminco series 2 (Spectronic Instruments, Rochester, NY) spectrofluorimeter as described (9). To quantify changes in the laurdan emission spectrum, generalized polarization (GP) values were calculated:

$GP = (I_B - I_R) / (I_B + I_R)$, where I_B (at 440 nm) and I_R (at 490 nm) correspond to the intensities at the blue and red edges of the emission maxima, respectively (41, 42).

Lipid Extraction, Production of Lipid Vesicles and Intervesicular MPER Lipid Mixing Assay

Lipid extraction was performed as described (6). Large unilamellar vesicles (LUV) were prepared following the extrusion method (43). Laurdan-labeled LUV at a concentration of 30 µM, as described (9), were treated with specified amounts of lipidomimetics in PBS containing 0.1% FCS for 30 min at 37°C under continuous stirring, followed by determination of GP profiles as above. The following lipids were purchased from Avanti lipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA); 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS); 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG); cholesterol (CHO); brain sphingomyelin (SM); 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC); L- α -phosphatidylethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (N-NBD-PE); L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (N-Rho-PE).

Membrane lipid mixing was monitored in an SLM Aminco series 2 (Spectronic Instruments, Rochester, NY) spectrofluorimeter using a resonance energy transfer assay. The assay is based on the dilution of N-NBD-PE and N-Rho-PE as described (44) with some modifications. Briefly, N-Rho-PE acts as a quencher of the N-NBD-PE fluorescence when both are present in a vesicle membrane at sufficient concentration. Upon dilution (e.g., by fusion of labeled with unlabeled vesicles), the fluorescence of N-NBD-PE is dequenched and becomes detectable. Labeled 10 µM POPC:POPS:SM:Chol (18%:15%:33%:33%) LUVs (NBD/Rho LUV) were incubated with different compound concentrations or LPC as fusion inhibition control for 15 min at 25°C with continuous stirring. Afterwards, 40 µM of unlabeled LUV were added for 20 more min. Once the baseline signal stabilized (0% fusion), the system was considered equilibrated. Then, 0.5 µM of membrane-proximal external region (MPER) peptide was added to launch the fusion reaction and incubation continued for 15 min at 25°C. Intervesicular lipid mixing was measured as an increase of fluorescence of N-NBD-PE caused by fusion of the labeled and unlabeled vesicle membranes. Finally, in order to obtain the 100% fusion value, 0.1% of Triton X-100 was added. The fluorescence baseline before addition of MPER was defined as 0% fusion and maximal fluorescence following final detergent addition was defined as 100% of fusion.

Statistics

Experimental groups were compared and significance determined by analysis of variance and Tukey test using SigmaPlot. Data are represented as means with standard deviation ($\pm$ SD).

RESULTS

Compound Screening

Based on prior screening of hydrophobic compounds for a variety of indications where membrane raft disruption was expected to be disease-modulating (45–47) and taking into account published anti-HIV approaches targeting the virus envelope (20, 48), we screened 695 compounds from the proprietary JADO Technologies raft modulator library, combined with selected compounds from commercial libraries of amphiphiles and lipidomimetics. The emphasis was on raft lipid-like scaffolds with 30% of all compounds being sterol-derivatives, 10% ceramide and sphingosine-like molecules, and 20% long (≥ 14 C-atoms) aliphatic main chain- or fatty acid-like (alkyl) phospholipids. Purified HIV-1 (cp. HIV strains and constructs listed in **Table S1** in the Supplementary Material) was incubated with compounds at $20\text{ }\mu\text{M}$ in the presence of 1% serum for 30 min at 37°C and subsequently added to target cells. The infection process was terminated after 18 h and DNA was extracted for real-time PCR analysis of viral reverse transcription products. 214 compounds inhibited infection $> 90\%$ (IC_{90}) at this concentration. After further testing of these compounds at $2\text{ }\mu\text{M}$, 16 hits achieved $> 90\%$ inhibition. Dose-effect relations were recorded for these compounds. Three lipidomimetics were selected from the original 16 hits for mode of action studies, based on the following qualitative criteria: (1) structural diversity, i.e., distinct structural scaffolds similar to different classes of raft lipids; (2) strongest anti-HIV effect during screening and in confirmatory assays; (3) low toxicity in various preliminary assays and cell lines. We chose three structurally distinct compounds (**Figure 1**), a sterol derivative (J391B), a sphingosine derivative (J582C) and a long chain aliphatic lipidomimetic with an unnatural head group (IBS70). A structure that proved inactive in the screen (IBS95), while exhibiting generic characteristics of aliphatic inhibitory compounds, was selected as a negative control.

Lipidomimetics Inhibit HIV-1 Infection

To investigate the effects of these lipidomimetics on HIV-1 infectivity, MT-4 T-cells were infected with the prototypic CXCR4-tropic strain HIV-1_{NL4-3}, which was pretreated or not with the compounds at previously determined concentrations. Infectivity was monitored by microscopic readout after staining for the viral p24 (CA) antigen (**Figure 2A**). Immunofluorescence images randomly taken from infected MT-4 cells showed that the three active compounds at their IC_{90} s, but not the inactive control, inhibited HIV-1 infection to a similar extent as the CXCR4 co-receptor antagonist AMD3100 [$0.5\text{ }\mu\text{M}$; (49)] that served as positive control. Automated microscopic readout allowed quantitation of the inhibitory effects (**Figure 2B**), confirming inhibition of HIV-1 infection at these concentrations. Moreover, virus production was strongly reduced when cells were infected with HIV-1 that had been pretreated with the respective compounds (**Figure 2C**), demonstrating a sustained effect. Compound toxicity was examined by the standard MTT viability assay (36) (**Figure S1**) and by quantifying cell counts upon automated microscopy (**Figure 2B**); no

toxic effects were observed at the relevant concentrations. Concentration-dependent inhibition of HIV-1 replication and potential cytotoxic effects were further investigated by titration experiments (**Figure 3**). The 50% inhibitory concentration (IC_{50}) was $2.5\text{ }\mu\text{M}$ for J391B, $1.7\text{ }\mu\text{M}$ for IBS70, and $4.6\text{ }\mu\text{M}$ for J582C in TZM cells (**Figure 3A**). The maximum tolerated concentrations in TZM cells were $20\text{ }\mu\text{M}$ for J391B, $10\text{ }\mu\text{M}$ for IBS70, and (at least) $100\text{ }\mu\text{M}$ for J582C, and their 50% cytotoxic concentrations (CC_{50}) were $40\text{ }\mu\text{M}$ for J391B, $25\text{ }\mu\text{M}$ for IBS70, and $> 100\text{ }\mu\text{M}$ for J582C (**Figure 3B**). Because the available stock solution of J582C was 10 mM concentrations above $100\text{ }\mu\text{M}$ could not be tested. The resulting selectivity indices were 15.8, 14.2, and > 21 for J391B, IBS70, and J582C, respectively.

Lipidomimetics Target the Virion Membrane

To study the mechanism of inhibition, we first analyzed whether the lipidomimetic compounds acted on the virus or on the host cell. For this purpose, pretreatment of HIV-1 was compared with pretreatment of target cells prior to addition of virus, on the one hand, and to simultaneous addition of virus and compound on the other (**Figure 4**). All three active compounds reduced HIV-1 replication to background levels when virus was pretreated, whereas treating target cells prior to infection or simultaneously adding virus and compound had no effect on HIV-1 infectivity (**Figure 4**). To examine whether pretreatment of cells or simultaneous addition of compound and virus exhibited any antiviral effect, lipidomimetics were applied at 2–5-fold higher concentrations than required for effective pretreatment of the virus (cp. **Figure 2**). These concentrations were still well below the maximal tolerated concentrations in TZM cells (**Figure 3**). In contrast to lipidomimetics, AMD3100, which functions by blocking the CXCR4 co-receptor essential for HIV-1 entry, inhibited viral infection irrespective of the mode of addition. AMD3100 is a hydrophilic compound ($\log P = -0.34$)¹ that reaches the co-receptor from the aqueous phase, whereas the lipidomimetics with $\log P > 4$ are quantitatively taken up by biological membranes and, where present, bind to lipoproteins and other hydrophobic molecules. Thus, the lipidomimetic compounds appeared to exert their effect by inactivating the virus and, therefore, had to be added prior to infection. To determine any potential non-specific effect on a non-enveloped virus, we also tested the lipidomimetic compounds against the parvovirus AAV, applying twice the effective concentrations required for HIV-1. Preincubation of AAV with any of the compounds did not affect infectivity, while heparin blocked AAV infection as expected, since it competes with AAV receptor engagement (50) (**Figure 5**). These results are consistent with an effect of the lipidomimetics on the HIV-1 membrane.

Lipidomimetics Inhibit HIV-1 Entry at the Fusion Step

The mode-of-addition experiments suggested that lipidomimetics act by blocking viral entry through effects on the HIV-1 membrane. To directly address this question,

¹Calculated via chemicalize.com.

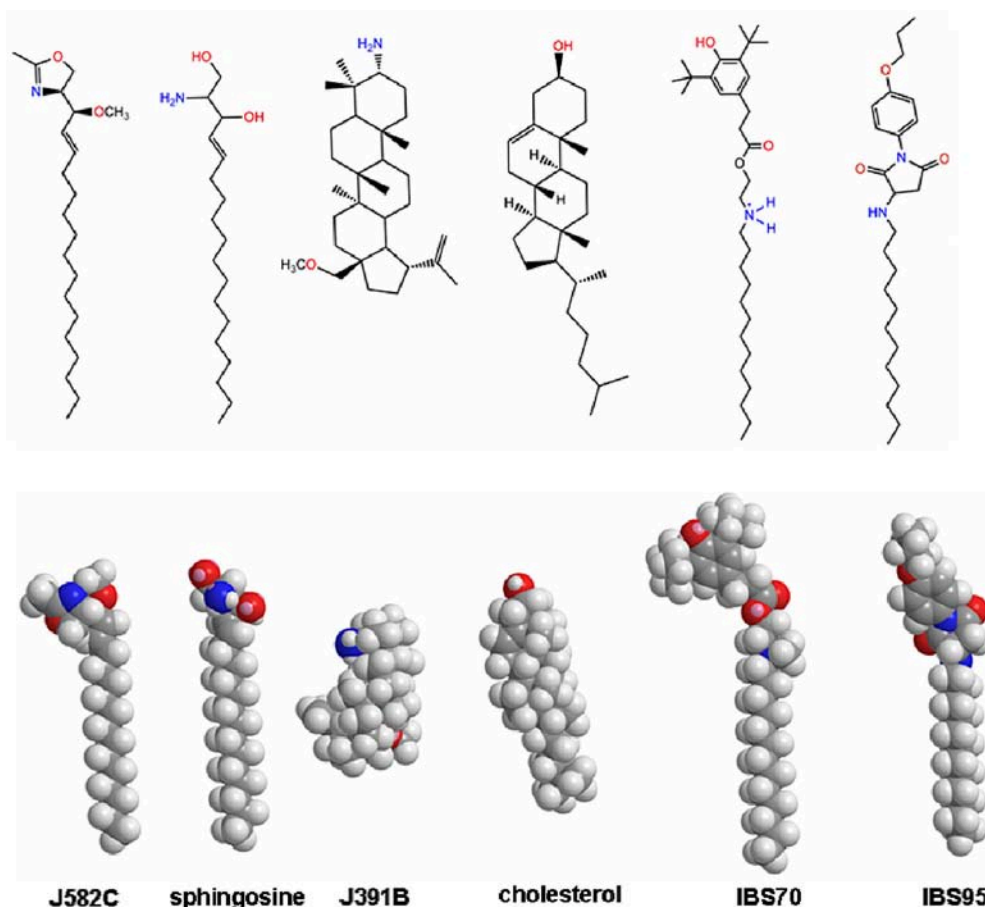


FIGURE 1 | Molecular modeling of the 3D structures of lipidomimetic compounds in comparison to natural lipids. Lowest energy structures in vacuum were computed for relevant compounds and the analogous natural lipids using CS Chem3D Ultra software employing the MM2-force field and the steepest-descent algorithm. Minimum RMS gradient was set to 0.1; minimum and maximum moved to 0.00001 and 1.0, respectively.

we made use of a quantitative HIV-1 entry assay based on incorporation of a Vpr- β -lactamase fusion protein (Vpr-BlaM) into replication-competent HIV-1 particles during virus production (37). Once productive fusion with target cells occurs, β -lactamase is released from the virion into the cytoplasm, where it can cleave a fluorescent substrate, CCF2, loaded immediately after viral infection. Relative fusion activity is quantified as the fluorescence ratio of the cleaved and uncleaved fluorophores. HIV-1 particles pre-incubated with lipidomimetics exhibited a strong reduction of virus entry in this assay, which was also observed for AMD3100, but not for the control compound IBS95 (Figure 6A). To ensure that the infectivity of Vpr-BlaM viruses was comparable with wild-type HIV-1 and to correlate the effects on entry with inhibition of infection, an infection experiment was performed in parallel. No significant difference in infectivity was observed between wild-type HIV-1 and Vpr-BlaM virus, and both viruses were equally affected by lipidomimetics (Figure 6B). Thus, inhibition of HIV-1 infection by lipidomimetics maps to the viral entry step.

To determine whether the effect of the lipidomimetic compounds is dependent on the HIV-1 glycoproteins or

the viral entry route, we performed experiments on HIV-1 pseudotyped with other viral glycoproteins. Pseudotyping is achieved by producing virus particles lacking their cognate viral glycoproteins, but randomly incorporating heterologous glycoproteins synthesized by co-transfecting the same producer cell with an expression vector for the glycoprotein of another virus. Pseudotyping with e.g., the glycoprotein of vesicular stomatitis virus (VSV-G) also changes the viral entry route as VSV enters target cells through a pH-dependent endosomal route (51). In contrast, pseudotyping with Friend ecotropic MLV glycoproteins yields viral fusion and entry at the plasma membrane in a pH-independent manner (52). We therefore produced HIV-1 Vpr-BlaM particles carrying the glycoproteins of either VSV or MLV and tested their capacity to enter target cells after pretreatment with lipidomimetic or control compounds. As background controls, pseudotyped HIV-1 particles were trypsin-treated prior to inoculation. Viral infectivity was determined in parallel for pseudotyped HIV-1 particles treated with lipidomimetic and control compounds as described above. Similar to HIV-1 carrying its cognate envelope proteins, treatment with lipidomimetics

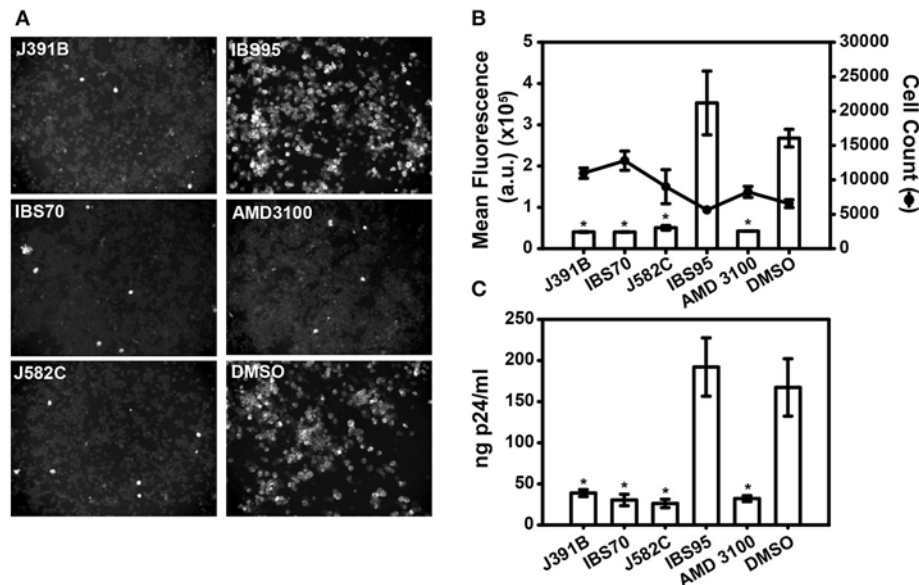


FIGURE 2 | Lipidomimetics affect HIV-1 infectivity. Immunofluorescence microscopy of intracellular p24 in infected MT-4 cells. HIV-1 was pretreated with 6 μ M J391B, 2 μ M IBS70, 20 μ M J582C, 7 μ M IBS95, 1% DMSO, or 0.5 μ M AMD3100 (as indicated in each panel) for 30 min and subsequently added to MT-4 cells for 2 h. Cells were stained 30 h p.i. **(B)** Infectivity with or without compound treatment was automatically quantified from immunofluorescence images as shown in **(A)** by determining the mean fluorescence of infected MT-4 cells. The effect of compound or solvent treatment on cell number was quantified in parallel (right axis). Data represent the mean $\pm$ SD of two replicate experiments with 16 replicas each; *represents a significant ($p < 0.01$) decrease when compared to the DMSO control. **(C)** Infectivity of compound-treated HIV-1 determined by quantitation of progeny virus. HIV-1 was pretreated as described in **(A)** and used to infect MT-4 cells. Released virus at 30 h p.i. was quantitated by p24 ELISA. Data represent the mean $\pm$ SD of two replicate experiments with four replicas each; *represents a significant ($p < 0.01$) decrease when compared to the DMSO control.

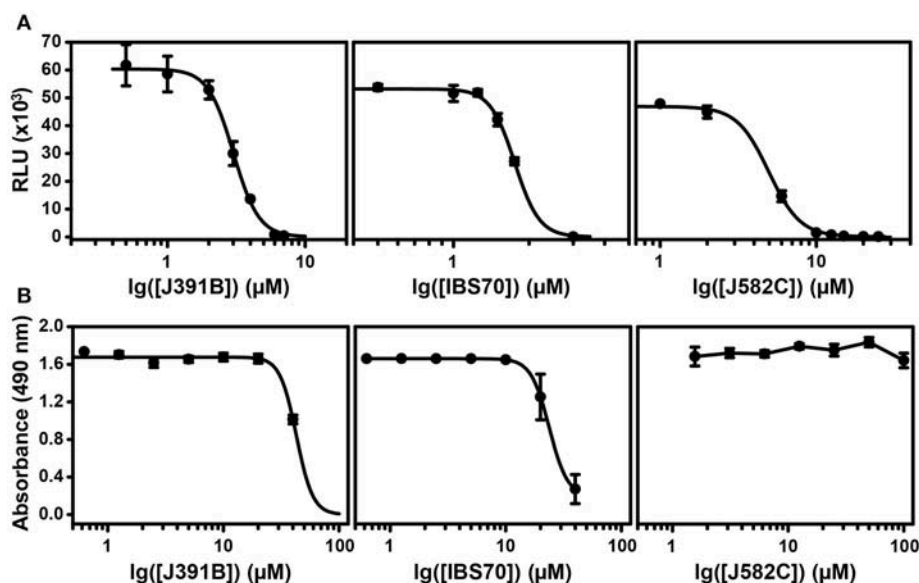


FIGURE 3 | Determination of IC_{50} and CC_{50} of each compound. **(A)** Titration of inhibitory effects. HIV-1 was pretreated with compounds at different concentrations followed by infection of TZM-bl cells. TZM-bl reporter cells were harvested 42–45 h p.i. and luciferase activity induced by newly produced HIV-1 Tat was measured and is shown as relative light units (RLU). RLU values are plotted against compound concentration in a semi-logarithmic way. Data represent the mean $\pm$ SD of four replicate experiments with nine replicas each. **(B)** Determination of the 50% cytotoxic concentration of lipidomimetics. TZM-bl cells were incubated for 48 h in the presence of the compounds as indicated in each panel. The CC_{50} for each compound was calculated from the dose-effect relation; mean values were 40 μ M for J391B, 25 μ M for IBS70 and >100 μ M for J582C. Data represent the mean $\pm$ SD of four replicate experiments with four replicas each.

efficiently and specifically inhibited cell entry by particles pseudotyped either with VSV or with MLV glycoproteins (**Figure 6C**). The effect on entry again correlated with that on virus infection (**Figure 6D**). Accordingly, the antiviral activity of the lipidomimetic compounds is not dependent

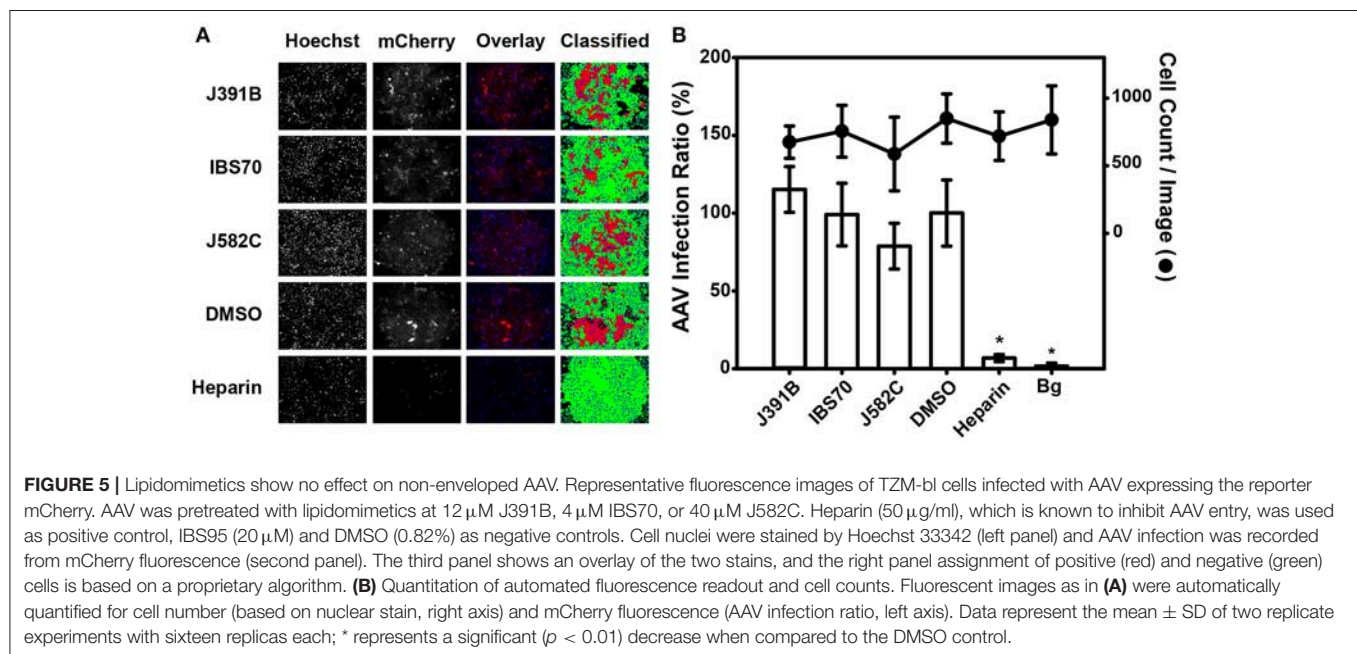
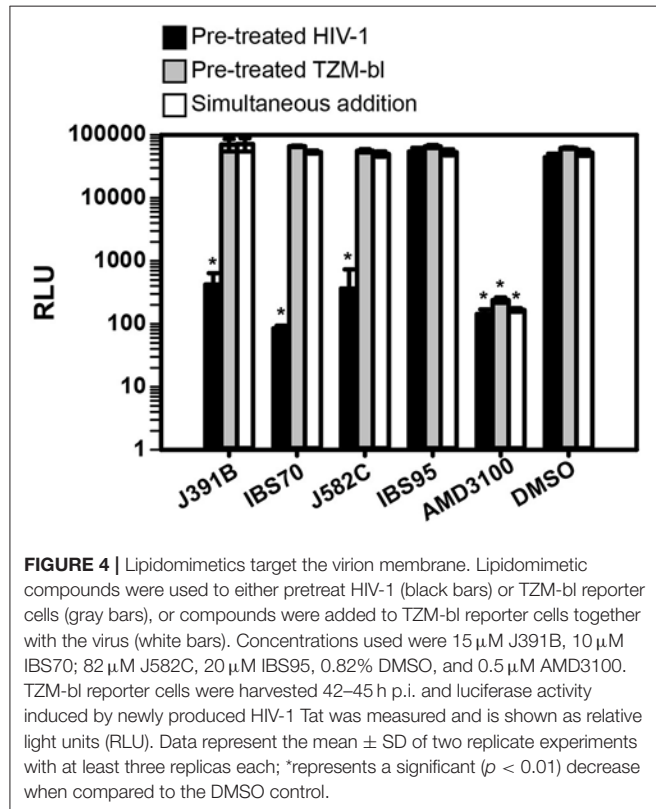
on a specific envelope glycoprotein or on a particular entry pathway.

To further study the mechanism by which lipidomimetics inhibit viral entry, we made use of a liposome-based fusion inhibition assay (44). For this purpose a fusogenic peptide from HIV-1, the gp41 MPER and synthetic LUVs were used (18, 53–55). As a suitable match for the lipidomimetics, LPC was chosen as the positive control compound. LPC is a well-known fusion inhibitor lipid (19) with inverted cone-shaped structure, which affects membrane curvature and therefore inhibits liposomal lipid mixing. Concentration-dependent inhibition of MPER-induced fusion by lipidomimetics was studied (**Figure 7**). The extent of virus and liposome lipid mixing correlated with the compounds' inhibition of virus infectivity (IC_{50}). IBS70 inhibited lipid mixing most effectively, followed by J391B and J582C. Thus, lipidomimetics were capable of inhibiting viral entry at the membrane fusion step.

Effects of Lipidomimetics on HIV-1 Stability and Virion Density

Detergent-like microbicides, e.g., nonoxynol, disrupt membranes non-specifically, thus inactivating HIV-1 with concomitant toxicity (56). To investigate whether virus disruption plays a role in the antiviral activity of the lipidomimetic compounds, purified non-infectious HIV-1-like particles and infectious HIV-1 were treated with compounds at approximately the IC_{90} and subsequently recovered by ultracentrifugation. Western blots detecting capsid, matrix, and envelope proteins and their quantifications revealed no effect of compound treatment on virus recovery and protein composition (**Figures 8A,B** and **Figure S2**).

Previous studies demonstrated shifts in virion density when viral membrane composition was altered by changes in



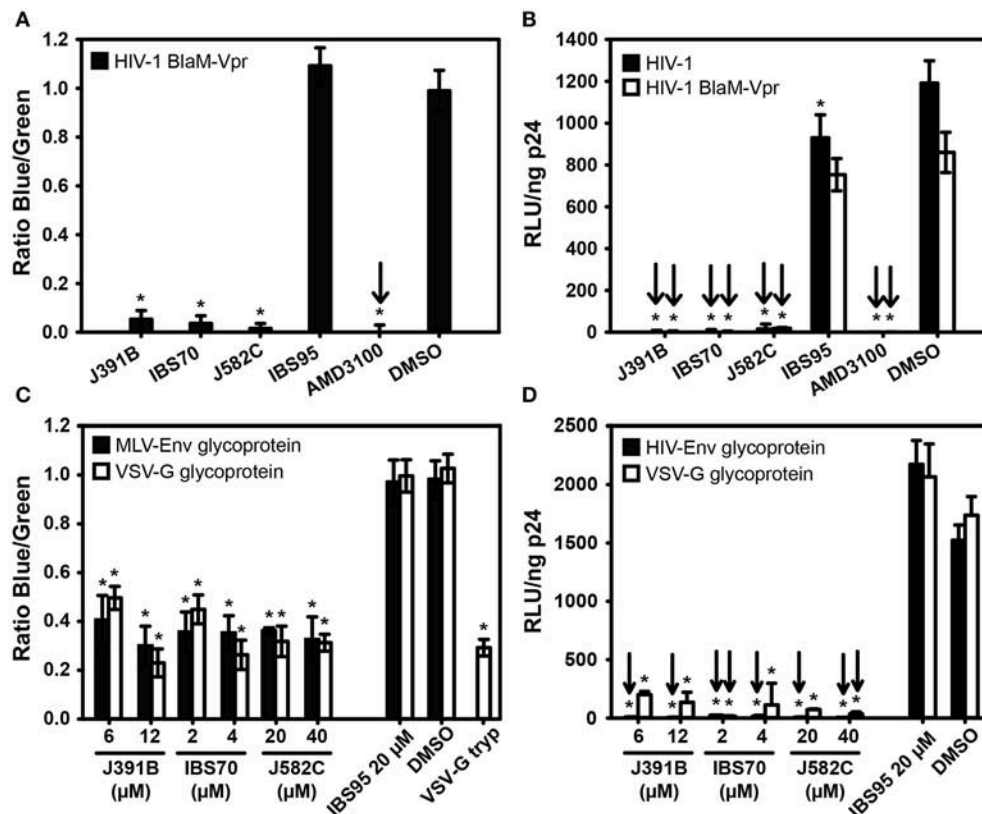


FIGURE 6 | Mapping effects of lipidomimetics to HIV-1 entry. Cytoplasmic entry. HIV-1 carrying BlaM-Vpr was pretreated with 6 μM J391B, 2 μM IBS70, 20 μM J582C, 20 μM IBS95, 0.82% DMSO, or 0.5 μM AMD3100 and added to TZM cells as described in **Figure 2**. Cytoplasmic entry of HIV-1 was analyzed by determining the mean fluorescence for the cleaved (blue) and uncleaved (green) CCF2 substrate after 17 h. The graph shows the ratio of the blue and green fluorescence signal normalized against the DMSO control, which was set to 1. Data represent the mean ± SD of three replicate experiments with nine replicates each; * represents a significant ($p < 0.01$) decrease when compared to the DMSO control. **(B)** Comparison of BlaM-Vpr-carrying and unmodified HIV-1. HIV-1 with (white bars) or without (black bars) BlaM-Vpr was pretreated with compounds as in **(A)** and used to infect TZM-bl reporter cells. Relative infectivity is shown as RLU per ng of p24. Data represent the mean ± SD of two replicate experiments with six (white bars) and nine (black bars) replicates each; * represents a significant ($p < 0.01$) decrease when compared to the DMSO control. **(C)** Influence of viral glycoproteins and entry route on sensitivity to compounds. HIV-1 was pseudotyped with the glycoproteins of Friend ecotropic MLV (black bars) or VSV (white bars). Pseudotyped viruses carrying BlaM-Vpr were pretreated with 6 and 12 μM J391B, 2 and 4 μM IBS70, 20 and 40 μM J582C or control compounds followed by infection of TZM-bl cells (for VSV-G pseudotypes) or DFJ8 cells (for MLV pseudotypes). Cytoplasmic entry was quantified by determining the ratio of blue and green fluorescence normalized against the DMSO control as in **(A)**. VSV-G pseudotyped particles treated with trypsin served as an entry non-competent control. Note that the signal for this control, which determines the background in this experiment, was similar to that of the compound-treated viruses. Data represent the mean ± SD of three replicate experiments with six replicates each; * represents a significant ($p < 0.01$) decrease when compared to the DMSO control. **(D)** HIV-1 carrying its cognate glycoprotein (black bars) or pseudotyped with VSV-G (white bars) and containing BlaM-Vpr was pretreated with compounds as in **(C)** and used to infect TZM-bl cells. Relative infectivity is shown as RLU per ng of p24. Data are the mean ± SD of two replicate experiments with six replicates each; * represents a significant ($p < 0.01$) decrease when compared to the DMSO control.

cholesterol concentration or its replacement by cholesterol analogs (13). In order to monitor potential density shifts following treatment with lipidomimetics, both non-infectious HIV-like particles (**Figure 8C**) and infectious HIV-1 (**Figure S2**) were compound-treated and analyzed by equilibrium centrifugation on continuous sucrose density gradients. HIV-1 protein distribution in gradient fractions was subsequently assayed by quantitative p24 Western blots or ELISA. Purified solvent-treated HIV-1 served as reference with the virus peak in fractions 10–13 at a density ranging from 1.16 to 1.2 g/cm³ (**Figure S3**). In contrast, virus treatment with detergent Triton X-100 led to a complete loss of the virus peak and recovery of soluble p24 antigen in the top fractions of the gradient

(**Figure 8C** and **Figure S2**). Treatment of viral particles with J391B did not influence virus density and resulted in a similar virus yield detected in fractions 10–13 as observed for solvent-treated virus. However, IBS70 elicited a minor increase in virus particle density. As opposed to the other lipidomimetics, a reduction in density to 1.12–1.14 g/cm³ occurred upon exposure of HIV-1 to J582C, a sphingosine mimic (**Figure 8C** and **Figure S2**). Effects on particle density may be due to insertion of the lipidomimetic into the viral membrane. However, changes in virion density were not proportional to antiviral activity. Only a small amount of soluble p24 was released by lipidomimetic-treated virus (**Figure 8C** and **Figure S2**), confirming that the compounds did not act by affecting virion stability.

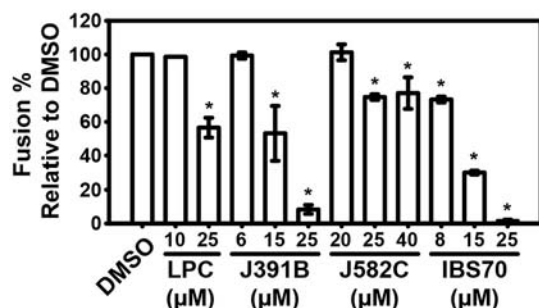


FIGURE 7 | Inhibition of fusion by lipidomimetics. LUVs were composed of POPC:POPS:SM:Chol (18:15%:33:33%) to emulate viral particle membranes. Experiments were performed at 25°C with 0.1% FCS in 150 mM NaCl, 10 mM Hepes pH 7.4 buffer, 10 μM NBD/Rho LUV in 1 ml, incubated for 15 min with compounds or DMSO control (0.82%). Next, 40 μM of unlabeled LUV was added for 20 min. The stable background signal establishes 0% fusion. The fusion reaction was started by adding 0.5 μM MPER fusion peptide. Membrane fusion was measured as the increase of N-NBD-PE fluorescence due to intervesicular lipid mixing and consequent dilution of the fluorophore (NBD) and quencher (Rho). In the absence of MPER there was no fluorescence increase (0% fusion). Finally, TX-100 (0.1%) was added to establish the 100% fusion signal. Data are the mean ± SD of two replicate experiments with three replicates each. *represents a significant ($p < 0.01$) decrease when compared to the DMSO control.

Effect of Lipidomimetics on HIV-1 Membrane Order

Our previous studies had shown that the fluorescent dye laurdan can be used to determine the degree of membrane order in virus particles (9), and we therefore applied laurdan staining to detect potential changes in membrane order upon treatment of HIV-1 with lipidomimetics. Laurdan is homogeneously distributed within the membrane and has an emission maximum around 490 nm for fluid (liquid disordered, l_d) membranes and around 440 nm for condensed membranes (l_o , and gel phase or solid ordered, S_o). The phase state of a membrane can thus be quantified by its GP value, which is defined as the normalized intensity ratio of the two emission channels and provides a relative measure of lipid order (9, 57, 58). GP values between 0.25 and 0.5 indicate l_o structure at the respective temperature, while GP values < 0.25 indicate liquid-disordered (l_d) structure (59).

Prior to compound treatment and laurdan staining HIV-1 was inactivated with AT-2, which covalently modifies the essential zinc fingers in the viral nucleocapsid protein (30) without altering the membrane (9). Inactivated virus was treated or not with lipidomimetics or control compounds and subsequently labeled with laurdan. Particles were recovered by ultracentrifugation, re-suspended in buffer and subjected to fluorescence spectroscopy. Control experiments excluded a direct interaction of lipidomimetics with laurdan (Figure S4). Emission spectra were recorded for treated and untreated viruses at different temperatures and the corresponding GP values were calculated. The charts of GP as a function of temperature of solvent-treated particles (Figure 9, filled circles) exhibited similar shapes and values as previously observed (9). No transition temperature inflection was visible, as expected for membranes

with high cholesterol content (60). Upon treatment with J391B, J582C, and IBS70, HIV-1 particles exhibited a change in GP pattern while treatment with the inactive compound IBS95 was indistinguishable from the solvent control, which was only subject to temperature-induced order decrease (Figure 9A). The cholesterol analogue J391B increased GP values of treated HIV-1 particles, generating greater membrane rigidity and counteracting the effect of temperature increase. Conversely, treatment with IBS70 and, most severely, the sphingosine-analogue J582C induced a decrease in GP values, reflecting enhanced membrane fluidity in the l_d range (GP < 0.25). While the reduction of membrane order by IBS70 was abated with increasing temperature, the disordering activity of J582C was constant and additive to the temperature effect (Figure 9A).

The proteins resident in biological membranes enforce an asymmetric distribution of lipids between the two leaflets, whereas liposomes lacking proteins exhibit a nearly symmetric lipid distribution. To determine whether the effect of the compounds on viral membrane structure is exclusively mediated by viral lipid composition, GP values were determined for LUV composed of HIV-1-derived lipids. To this end, lipids were extracted from purified HIV-1 particles and used to prepare LUV with a diameter of ~100 nm (43). These HIV-derived LUV have the lipid composition of HIV-1 but, according to their physicochemical characteristics, possess a random and presumably symmetric distribution of lipids. HIV-1 lipids-derived LUV were treated or not with the different compounds, labeled with laurdan, and GP was recorded as a function of temperature (Figure 9B). Similar to the results observed for wild-type HIV-1, the control compound IBS95 did not alter LUV structure; IBS70 and J582C caused a temperature-dependent or independent decrease in membrane order, while J391B stabilized membrane order against rising temperature (Figure 9B). The modifications induced by all compounds in LUV were very similar to the ones observed for the complete virus, indicating that their effects were independent of viral or cellular proteins and did not require membrane asymmetry.

Phosphatidylserine-Specific Enhancement of Membrane Order by Steroidal Amine J391B

Further studies with LUVs of different membrane composition revealed an interesting lipid headgroup requirement for the effect of J391B on membrane order. While this compound enhanced membrane order in HIV-1 particles and LUV reconstituted from the complete set of HIV-1 lipids (Figures 9A,B), a slight decrease of membrane order was observed upon J391B treatment of LUV consisting only of cholesterol, sphingomyelin and phosphatidylcholine (the most abundant HIV-1 lipids; Figure 10A). Since J391B is positively charged at neutral pH (Figure 1), apparently the availability of negatively charged lipid head groups determined a switch between J391B reducing, maintaining or increasing membrane order. The HIV-1 lipidome exhibits an enrichment of phosphatidyl serine (PS) compared with the plasma membrane of producer cells (8). LUV composed of the same quaternary mixture as above but including PS

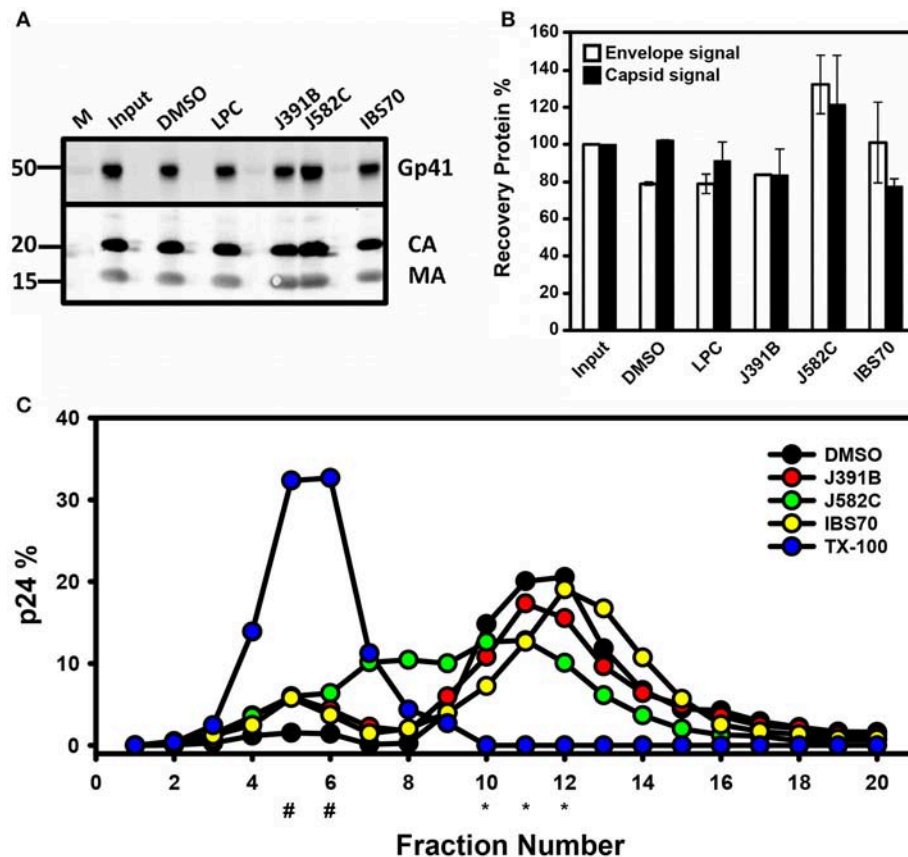


FIGURE 8 | Influence of lipidomimetics on particle stability and density. **(A)** Virus stability. Purified non-infectious HIV-like particles derived by transfection of pCHIV (3 μ g of CA) were treated with 6 μ M J391B, 8 μ M IBS70, 20 μ M J582C, 2.5 μ M LPC, or DMSO (0.35%) for 30 min at 37°C. Subsequently, particles were recovered by ultracentrifugation and analyzed by Western blot using antisera against the HIV-1 trans-membrane glycoprotein gp41 (ENV) (41 kDa), CA (24 kDa), and MA (17 kDa). Input signal refers to control virus before treatment. **(B)** Virus stability. Virus treated and recovered as in **(A)** was analyzed by quantitative Western blot. Measured in LiCoR quantitative system. **(C)** Virus buoyant density. Non-infectious HIV-like particles derived by transfection of pCHIV (3 μ g of CA) were treated with 6 μ M J391B, 8 μ M IBS70, 20 μ M J582C, 0.5% TX-100, or DMSO (0.35%) as in **(A)** and subsequently subjected to equilibrium density gradient centrifugation. Gradient fractions were collected from the top and virus amounts were quantified by Western blot. Data represent the mean of two replicate experiments with five replicas each, # indicates sucrose density fractions where soluble p24 was expected (1.07 g/cm³) and *the fractions where intact virus was expected (1.17–1.2 g/cm³).

substituting for part of the phosphatidyl choline exhibited increased membrane rigidity upon J391B treatment, although the amount of saturated fatty acids had been decreased 2.5 times as a result of the higher level of unsaturated fatty acids in PS (**Figure 10B**). The effect of the compound resembled that on LUV consisting of HIV-1 extracted lipids or on complete virus (**Figure 9**). Replacing PS with other negatively charged lipids, phosphatidyl glycerol or phosphatidic acid, which hardly occur in the HIV envelope, yielded little or no effect on membrane order following J391B treatment (**Figures 10C,D**), indicative of a specific molecular interaction of J391B with PS rather than mere charge neutralization. To explore this molecular interaction in an independent approach HeLa cells were treated with J391B for 30 min and stained with PS-specific annexin V. J391B treatment elicited exposure of PS on the outer leaflet of the plasma membrane (**Figure 11**) whereas the DMSO-treated control gave no signal. This observation indicates that J391B interaction with cell membranes induced PS externalization without apparent signs of apoptosis.

J391B and J582C Exhibit Synergistic Effects on HIV-1 Infectivity

Given that J391B and J582C induced opposing effects on membrane structure, we next asked whether they would antagonize each other. To study their combined effects, the two compounds were mixed at different concentrations, and the infectivity of HIV-1 treated with either compound alone or with the various mixtures was analyzed using a luciferase reporter assay (**Figure 12A**). Again, AMD3100 was employed as the positive and IBS95 as the negative control compound. Titration experiments showed that J391B had no effect on infectivity at a concentration of 1.75 μ M and J582C was inactive at 3.5–4 μ M (**Figure 12A**). Yet a mixture of both compounds at concentrations where either compound alone was inactive strongly inhibited viral infectivity. Thus, J391B and J582C acted synergistically. Enhanced inhibition was not due to toxicity as shown by the parallel MTT test (**Figure 12B**).

We next examined whether the synergistic effect on viral infectivity corresponded to detectable alterations in HIV-1

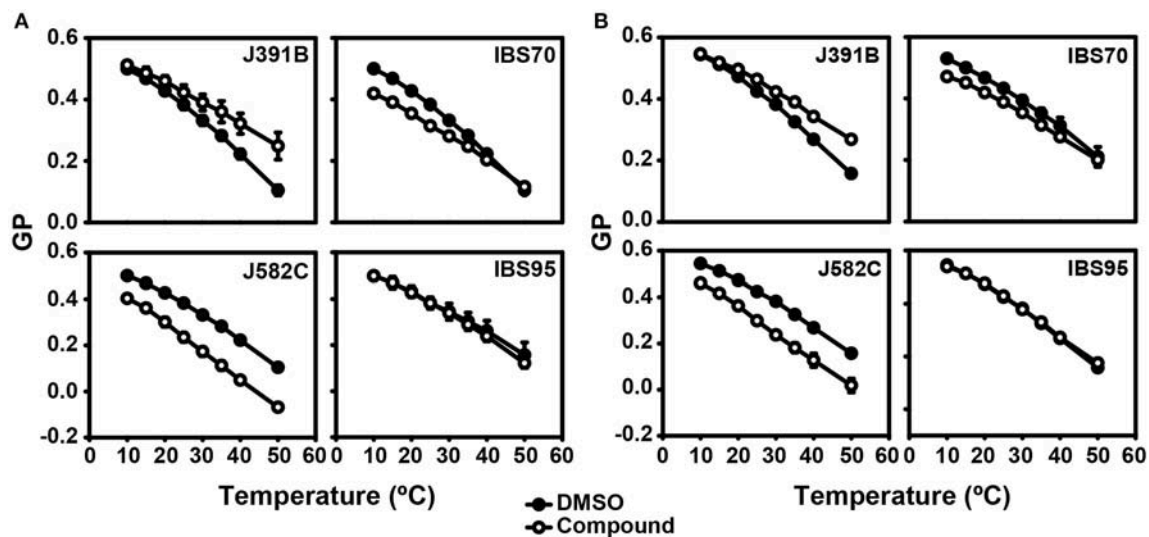


FIGURE 9 | Effect of lipidomimetics on HIV-1 membrane order. **(A)** Comparison of temperature-dependent GP profiles of HIV-1 with (○) or without (●) lipidomimetic treatment. Viruses were stained with 5 μ M laurdan for 20 min, treated with 6 μ M J391B, 8 μ M IBS70, 20 μ M J582C, 7 μ M IBS95, or DMSO (0.35%) for 30 min at 37°C, and analyzed as described in experimental procedures. GP values were calculated from emission spectra recorded at each temperature. Data are the mean $\pm$ SD of three replicate experiments. **(B)** Comparison of temperature-dependent GP profiles of LUV produced from extracted viral lipids with (○) or without (●) compound treatment. Conditions of treatment and analysis were as in **(A)**. Data are the mean $\pm$ SD of three replicate experiments.

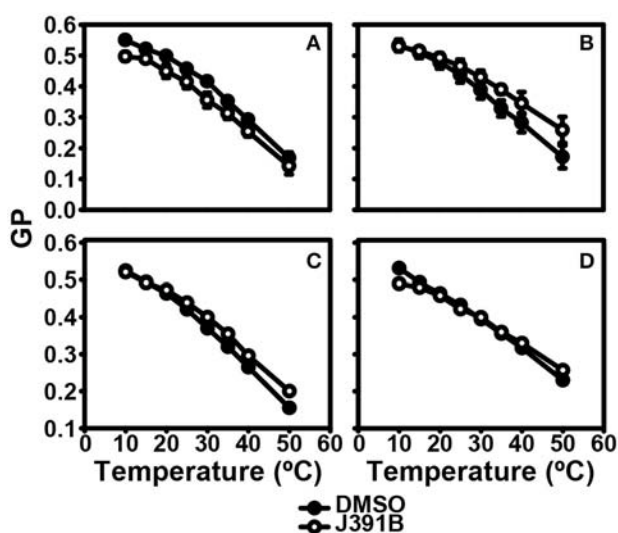


FIGURE 10 | Lipid species requirement for J391B membrane activity. Comparison of temperature-dependent GP profiles of LUVs composed of: **(A)** POPC:DPPC:SM:CHOL (25:16:14:45%). **(B)** POPC:DOPS:DPPC:SM:CHOL (10:15:16:14:45%). **(C)** POPC:DOPG:DPPC:SM:CHOL (10:15:16:14:45%). **(D)** POPC:DOPA:DPPC:SM:CHOL (10:15:16:14:45%) with (○) or without (●) treatment with 6 μ M J391B for 30 h at 37°C. Data represent the mean $\pm$ SD of three replicate experiments.

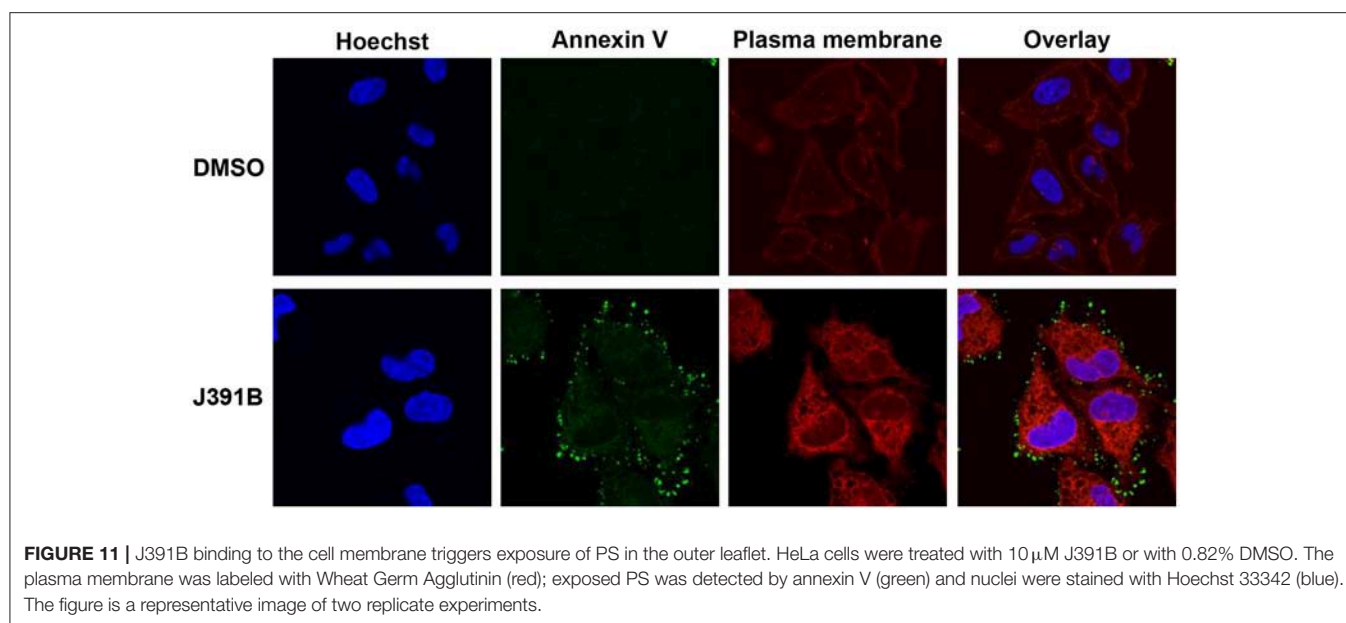
membrane order. HIV-1 particles and LUV composed of HIV-1 lipids were treated with individual or mixed compounds and GP profiles were recorded (**Figure 13A**). Interestingly, the compound mixture delivered a GP pattern close to that

of J582C but with a temperature profile more like the one of J391B. The complex outcome at the level of membrane order was neither an antagonistic nor an additive effect (**Figure 13B**).

DISCUSSION

Targeting the lipid membrane of enveloped viruses is an attractive approach for the development of antivirals applied either systemically or on mucosal surfaces. Detergent-based structures have been developed as topical microbicides (56), but shown to cause toxicity problems upon *in vivo* application (61, 62). Alternative approaches attempted the extraction of key lipids like cholesterol with cyclodextrin derivatives (12) or promoted alteration of membrane fusion properties by e.g., inserting inverted cone-shaped non-lipidic compounds into the viral membrane (20). Here, we screened a library of raft lipid-like lipidomimetics as potential antiviral agents against HIV-1 and identified several compounds, which inhibited viral infection at the entry stage and induced structural alterations in the viral membrane.

A qualitative structure-activity analysis of antiviral vs. inactive compounds from the screen yielded the following results: the hydrophobic anchor of active sterol-like molecules often included a single oxygen atom, attenuating the hydrophobicity of the sterol-like scaffold. There was no preference for the α - or β -sterol configuration. The hydrophobic anchor of active aliphatic lipidomimetics was a single aliphatic chain ≥ 14 –18 C-atoms. Compounds with more than one aliphatic chain were inactive due to their poor diffusibility, despite the presence of serum lipoproteins. Among the inactive compounds, we selected IBS95,



which is similar to the generically active structures but has two ring moieties in its head group.

Head groups of the active compounds often encompassed OH-groups and/or a single aromatic or heterocyclic ring, and presented at least one nitrogen atom, usually positively charged or available for protonation. These observations agree with the findings of (63) who showed that cholesterol derivatives with positively charged head groups disrupt or augment membrane order and, in both cases, interfere with influenza virus infection. It is not unexpected that any modulation of the initial, optimal viral membrane order impairs infectivity, since all physiological processes exhibit concentration and temperature optima, often in the context of protein function. Likewise, biological membranes possess a distinct order determined by lipid species concentrations and temperature, essential for their functions (64). Specifically composed lateral membrane phases underly organellar and viral membrane dynamics (fusion, budding, and fission) and trafficking (65).

Lipidomimetics inhibited HIV-1 infectivity in a concentration-dependent manner with IC_{50} values in the low micromolar range. Activity required prior interaction with the viral membrane, while no effect was seen upon pre-incubation of targets cells with the compounds. Given that these compounds are likely to bind to both virus and cell membranes, this suggests that their interaction with cellular membranes may not influence viral entry or/and that compounds are rapidly removed from cellular but not from viral membranes. Indeed, ATP-dependent transporters (ABC transporters, P glycoprotein) remove foreign amphiphilic compounds from cell membranes (66), and may also work in this manner on the compounds studied here. Further experiments confirmed that the viral membrane is indeed the target: Lipidomimetics inhibited HIV-1 entry independent of the viral envelope glycoprotein and the specific entry pathway, while no effect was observed for the non-enveloped virus AAV. We conclude that the described

lipidomimetics directly target the viral membrane and alter its capacity to fuse with the host cell membrane. This effect suggests potential against a wider spectrum of enveloped viruses, while the lipid-dependent activity differences (see below) may restrict activity to membranes with a defined lipid composition.

Studies of virus stability showed that the compounds did not disrupt particle integrity although J582C caused a shift in buoyant density of the viral particle. Sphingosine-like (J582C) and other long acyl-chain compounds (as IBS70 or IBS95) contain a single hydrocarbon chain and have inverted cone-shape geometry like LPC, generic structures that induce positive curvature and thus inhibit membrane fusion (19). While LPC itself is toxic, less toxic compounds have been shown to inhibit virus entry due to their inverted cone-shape structure (20). These RAFIs consist of a nucleoside coupled to perylene and have a wide antiviral spectrum against enveloped but not naked viruses. A similar broad antiviral spectrum is exhibited by the amphiphilic fusion inhibitor, aryl methylene rhodanine derivative LJ001 (48). Both LJ001 and RAFIs were shown to target membranes though they do not structurally resemble lipids, but the effect of LJ001 is actually mediated by photosensitization (67), unrelated to raft modulation. More relevant, cosalane is a cholestane derivative with an oversized headgroup comprising disalicylmethane with activity against enveloped viruses (68). The cholestane moiety of cosalane is reported to insert into the cell membrane and/or the viral envelope, from where the large disalicylmethane moiety protrudes and blocks the interaction between gp120 and CD4 (69). For the activity of cosalane the membrane raft-targeting property of the lipid anchor (cholestane) appears to be important, however, the mode of action does not involve the disruption of membrane raft domains of the virus envelope. This compound would be defined as a raftophile; it is probably not a raft modulator or disrafter (45, 63).

A number of lipidic HIV inhibitors were previously studied with regard to modifying both the host cell and viral membrane,

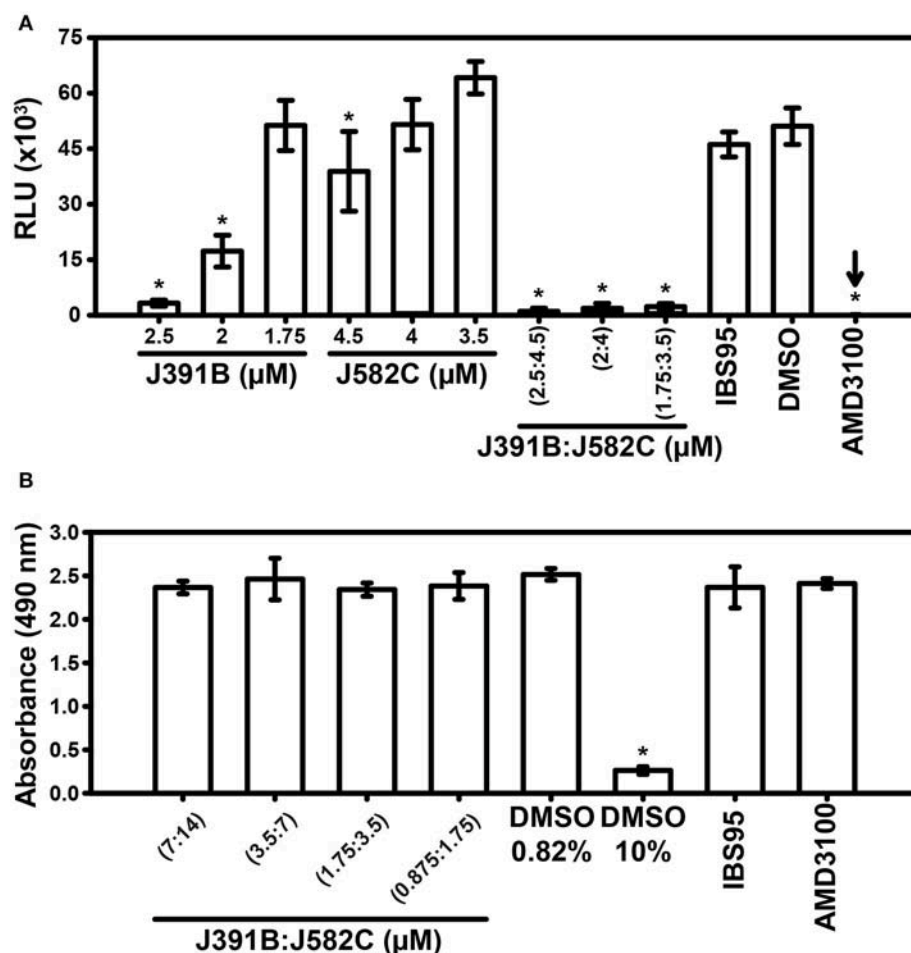


FIGURE 12 | Effect of mixtures of J391B and J582C on HIV-1 infectivity and cytotoxicity. **(A)** HIV-1 was treated with compounds at the indicated concentrations as described in **Figure 2** followed by infection of TZM-bl cells and luciferase assay after 42 h. Data represent the mean $\pm$ SD of three replicate experiments with nine replicates each; * represent a significant decrease ($p < 0.01$) when compared to DMSO. **(B)** MTT cytotoxicity assay for treatment with compound mixtures. The same J391B: J582C (1:2) molar ratio as in **(A)** at expanded concentration range was tested. DMSO (0.82%), IBS95 (7 μ M) and AMD3100 (0.5 μ M) served as controls and 10% DMSO as toxicity control. Data are the mean $\pm$ SD of three replicate experiments with four replicates each; *represent a significant decrease ($P < 0.01$) when compared to the 0.82% DMSO control.

their fluidity and lipid domain structure [reviewed by (70)]. The natural compounds glycyrrhizin and fattiviracin FV-8 possess large, neutral, hydrophilic headgroups, and are structurally far removed from natural raft lipids from which our lipidomimetics originated. In a review of the potential relevance of membrane raft targeting by natural products as an anti-HIV strategy (71), a considerable body of literature on betulinic acid derivatives is cited, structurally related to the lupene derivative J391B, however, none of them carrying a 3-amino group that proved decisive for the specific HIV envelope-stabilizing activity of J391B described here.

In order to study the potential effect of lipidomimetics on membrane structure, we made use of laurdan staining, which allows rapid determination of differences in viral membrane order (9). Diametrically opposed alterations in membrane order were observed for the cholesterol analogue J391B, which increased membrane rigidity and counteracted

temperature-induced melting, and the sphingosine analogue J582C, which increased membrane fluidity independent of temperature. IBS70 also increased the fluidity of the virus envelope, but its effect disappeared at higher temperatures. Membrane rigidification by the steroidal amine J391B only superficially resembled the effect of increasing the proportion of membrane cholesterol, the lipid fundamental to the existence of l_o phases (72, 73). Interestingly and unlike cholesterol, the effect of J391B on membrane order required the presence of PS. This lipid dependence was not caused by an unspecific electrostatic effect as substitution of PS by either phosphatidyl glycerol or phosphatidic acid at a similar concentration in the presence of J391B had little or no effect on membrane order. Surprisingly, introduction of J391B into membranes completely lacking PS and other negatively charged lipid headgroups increased membrane fluidity, similar to J582C and IBS70. We therefore hypothesize that there is a specific electrostatic interaction of J391B with PS,

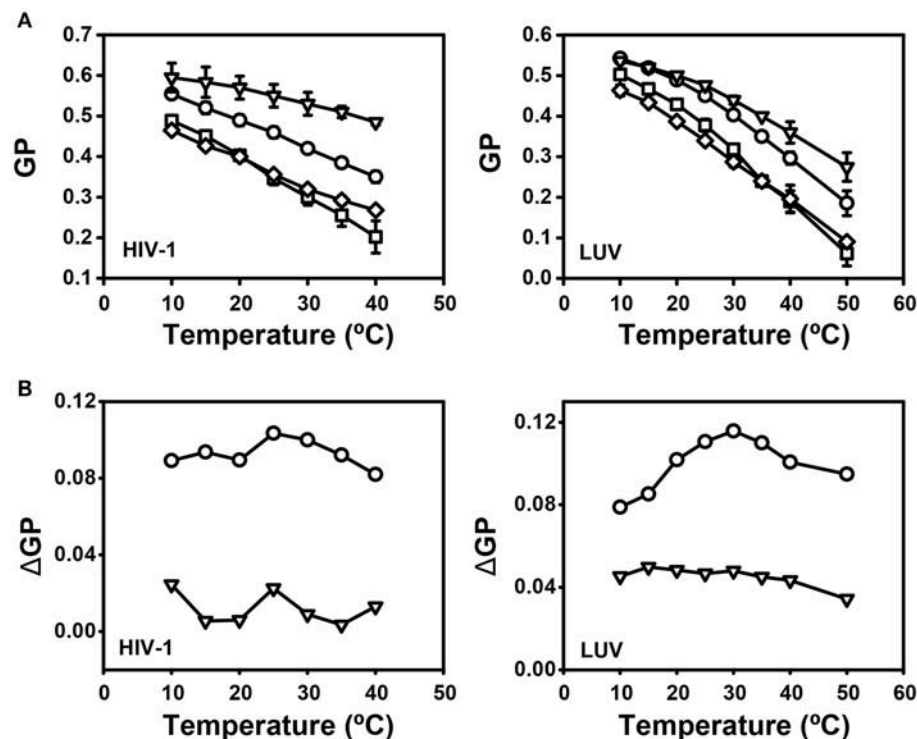


FIGURE 13 | Effect of mixtures of J391B and J582C on HIV-1 membrane structure. **(A)** Comparison of temperature-dependent GP profiles of HIV-1 (left panel) and LUV composed of HIV-1 lipids (right panel). Virions or LUV were treated with 0.82% DMSO (circles), 7.5 μM J391B (triangles), 15 μM J582C (squares) or the mixture of 7.5 μM J391B and 15 μM J582C (diamonds). Data represent the mean ± SD of three replicate experiments. **(B)** Temperature-dependent GP profiles for HIV-1 NL4-3 (left panel) or LUV (right panel) treated with compound mixtures or individual compounds were plotted against DMSO-treated controls. Data are from the experiment shown in **(A)**. In order to address whether the effect of the compound mixture is additive or synergistic, we plotted the GP profile for particles treated with the mixture of J391B and J582C relative to DMSO controls (circles) or against the extrapolated additive plot for particles separately treated with both compounds (triangles).

and this may create more rigid membrane structures. Annexin V staining of cells treated with J391B revealed a rapid exposure of PS on the cell surface without detectable signs of apoptosis (**Figure 11**), indicating that PS flipping from the inner to the outer leaflet is trapped by the compound, which suggests a high binding affinity to PS. The viral membrane is highly enriched in PS compared to the producer cell plasma membrane (8), and this may make HIV-1 a particularly good target for J391B. As a low-molecular weight, membrane-inserting PS ligand, J391B is novel and structurally unrelated to the three hitherto described (non-lipidomimetic) PS binders (74).

Intriguingly, upon HIV engagement of its receptors, flipping of PS to the outer leaflet of the host cell target membrane has been reported to be important for HIV fusion. Zaitseva et al. (75) showed that HIV binding and formation of the pre-fusion Env-CD4-coreceptor complex leads to surface expression of PS. First, the complex triggers a Ca^{2+} signal, which in turn activates lipid scramblase TMEM16F that externalizes PS, which is then essential for the next step of fusion, gp41 restructuring and hemifusion. It will be interesting to determine if virion PS is also important for fusion and whether its function is inhibited by bound J391B. PS in the outer leaflet of the viral membrane has already been shown to be important for cell entry of other enveloped viruses (76), underlining the

relevance of testing J391B activity against these viruses in the future.

Treatment of HIV-1 with the sphingosine-like compound J582C led to decreased membrane order and a concomitant decrease in virus density. Laurdan directly senses the abundance of water molecules within the membrane, which inversely correlates with membrane order (42). Conceivably, insertion of J582C into the viral envelope may cause membrane swelling by allowing more water to penetrate. Indeed, the bulkier, uncharged head group of J582C comprises three hydrogen-bond acceptors, as opposed to the small positively charged head group of sphingosine with its three hydrogen-bond donors (**Figure 1**). Natural sphingosine has a completely different behavior compared to the uncharged compound J582C. The positive charge of sphingosine appears crucial for its membrane activity [reviewed in (77)]. Sphingosine rigidifies the bilayer lipid acyl chains, as a result membrane permeabilization can occur due to the coexistence of domains of different fluidities (77, 78). On the other hand, J582C-induced positive membrane curvature in combination with water incorporation would tend to swell the membrane, explaining the observed decrease in particle density.

Based on the observed opposing effects of J391B and J582C on HIV-1 membrane order, experiments were performed with a mixture of both to investigate their potential antagonism.

Counterintuitively, J391B and J582C synergistically inhibited HIV-1 infectivity, associated with an increased membrane fluidity apparently dominated by J582C, combined with a flatter temperature-dependent GP profile reminiscent of J391B alone. Dominant membrane order enhancement by the specific interaction between J391B and PS appears to be abrogated in the presence of J582C, yet the mixture of both distinct raft modulators appears to create a greater obstacle to fusion than each compound individually. Thus, in addition to the impact on global viral membrane order, as reported by the laurdan assay, membrane lipid mechanics at a smaller scale (as required for fusion) are a target of lipidomimetics, which seem to act as molecular “spanners in the works” of fusion. Further studies will be required to identify the precise mechanism of this synergistic effect.

We are aware that efforts toward selective drug delivery are the precondition to optimizing anti-HIV lipidomimetics, since their hydrophobicity facilitates indiscriminate absorption by cell membranes, followed by either uptake into the cell or expulsion via transporters and re-loading onto lipoproteins (66). Selective drug delivery may, for example, target natural, highly specialized HIV infection pathways. Appropriately engineered lipidomimetic-loaded ganglioside-containing vesicles may be a promising approach of interfering with primary mucosal infection. After targeting siglec-1-expressing mature dendritic cells, which are not productively HIV-infected, the vesicles would inactivate HIV encountered in the same intracellular sac-like compartment (79, 80).

Very little is known about the role of membrane order and fluidity regarding virus pathogenicity and how to modulate the physicochemical properties of the virus envelope to achieve a desired inhibitory phenotype. Studying lipid-modulating compounds like the ones described here provides a glimpse of this fascinating subject and may pave the way for future studies.

AUTHOR CONTRIBUTIONS

BG, CS, and ML performed experimental infectivity and entry capacity data acquisition and analysis; JN-G and ML did lipid

mixing assays, particle stability and density measurements; ML conducted laurdan experiments and cytotoxicity assays and identified J391B lipid requirement as well as the effect of compound mixtures. SA, H-JK, and CZ designed, synthesized, and analyzed the compounds; KS, H-JK, CS, and GJ defined screening strategy and selected compound libraries; CS, CB, MG, and BB performed compound screening. KB purified and tested the infectivity of AAV particles; FC performed molecular modeling analyses and phosphatidylserine exposure experiments; CS, ML, and H-GK designed the experiments and prepared the manuscript; ML, CS, JN-G, and H-GK revised and all authors approved the final manuscript.

FUNDING

This project was supported by BMBF BioChancePLUS3, Projektnummer 0313827 and by TRR83 (project 14) and by MICINN BFU-2015-68981-P and Grupos Consolidados IT838-13 GV. JN-G is supported by a FI predoctoral fellowship from the Basque Government. H-GK is investigator of the CellNetworks Cluster of Excellence (EXC81).

ACKNOWLEDGMENTS

We are grateful to V. Bosch, N. Landau, and D. Grimm for providing the plasmids Δ EnvpNL4-3, pMM310, and the AAV vector, respectively. The following reagents were obtained through the NIH AIDS Reagent Program (Division of NIAID, NIH): Chessie 8 from Dr. George Lewis and MPER peptide, 23-mer. We thank Georg Schlechtingen and Tobias Braxmeier for critical reading of the manuscript, Ines Kästner for efficient project management and Hans-Joachim Schalk for participation in the screening effort.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01983/full#supplementary-material>

REFERENCES

- Lorizate M, Kräusslich HG. Role of lipids in virus replication. *Cold Spring Harb Perspect Biol.* (2011) 3:a004820. doi: 10.1101/cshperspect.a004820
- Griffiths G, Rottier P. Cell biology of viruses that assemble along the biosynthetic pathway. *Semin Cell Biol.* (1992) 3:367–81. doi: 10.1016/1043-4682(92)90022-N
- van Genderen IL, Godeke GJ, Rottier PJ, van Meer G. The phospholipid composition of enveloped viruses depends on the intracellular membrane through which they bud. *Biochem Soc Trans.* (1995) 23:523–6. doi: 10.1042/bst0230523
- Aloia RC, Jensen FC, Curtain CC, Mobley PW, Gordon LM. Lipid composition and fluidity of the human immunodeficiency virus. *Proc Natl Acad Sci USA.* (1988) 85:900–4. doi: 10.1073/pnas.85.3.900
- Aloia RC, Tian H, Jensen FC. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. *Proc Natl Acad Sci USA.* (1993) 90:5181–5. doi: 10.1073/pnas.90.11.5181
- Brügger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Kräusslich HG. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci USA.* (2006) 103:2641–6. doi: 10.1073/pnas.0511136103
- Chan R, Uchil PD, Jin J, Shui G, Ott DE, Mothes W, et al. Retroviruses human immunodeficiency virus and murine leukemia virus are enriched in phosphoinositides. *J Virol.* (2008) 82:11228–38. doi: 10.1128/JVI.00981-08
- Lorizate M, Sachsenheimer T, Glass B, Habermann A, Gerl MJ, Kräusslich HG, et al. Comparative lipidomics analysis of HIV-1 particles and their producer cell membrane in different cell lines. *Cell Microbiol.* (2013) 15:292–304. doi: 10.1111/cmi.12101
- Lorizate M, Brügger B, Akiyama H, Glass B, Müller B, Anderlüh G, et al. Probing HIV-1 membrane liquid order by Laurdan staining reveals producer cell-dependent differences. *J Biol Chem.* (2009) 284:22238–47. doi: 10.1074/jbc.M109.029256
- Mañes S, del Real G, Lacalle RA, Lucas P, Gómez-Moutón C, Sánchez-Palomino S, et al. Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. *EMBO Rep.* (2000) 1:190–6. doi: 10.1093/embo-reports/kvd025

11. Liao Z, Cimasky LM, Hampton R, Nguyen DH, Hildreth JE. Lipid rafts and HIV pathogenesis: host membrane cholesterol is required for infection by HIV type 1. *AIDS Res Hum Retroviruses* (2001) 17:1009–19. doi: 10.1089/088922201300343690
12. Liao Z, Graham DR, Hildreth JE. Lipid rafts and HIV pathogenesis: virion-associated cholesterol is required for fusion and infection of susceptible cells. *AIDS Res Hum Retroviruses* (2003) 19:675–87. doi: 10.1089/0889222032280900
13. Campbell SM, Crowe SM, Mak J. Virion-associated cholesterol is critical for the maintenance of HIV-1 structure and infectivity. *AIDS* (2002) 16:2253–61. doi: 10.1097/00002030-200211220-00004
14. Waheed AA, Ablan SD, Mankowski MK, Cummins JE, Ptak RG, Schaffner CP, et al. Inhibition of HIV-1 replication by amphotericin B methyl ester: selection for resistant variants. *J Biol Chem.* (2006) 281:28699–711. doi: 10.1074/jbc.M603609200
15. Mizrahi Y, Lev M, Harish Z, Sundaram SK, Rubinstein A. L-cycloserine, an inhibitor of sphingolipid biosynthesis, inhibits HIV-1 cytopathic effects, replication, and infectivity. *J Acquir Immune Defic Syndr Hum Retrovir.* (1996) 11:137–41. doi: 10.1097/00042560-199602010-00004
16. Tamma SL, Sundaram SK, Lev M, Coico RF. Inhibition of sphingolipid synthesis down-modulates CD4 expression by peripheral blood T lymphocytes and T lymphoma cells. *Biochem Biophys Res Commun.* (1996) 220:916–21. doi: 10.1006/bbrc.1996.0506
17. Finnegan CM, Rawat SS, Puri A, Wang JM, Ruscetti FW, Blumenthal R. Ceramide, a target for antiretroviral therapy. *Proc Natl Acad Sci USA.* (2004) 101:15452–7. doi: 10.1073/pnas.0402874101
18. Vieira CR, Munoz-Olaya JM, Sot J, Jimenez-Baranda S, Izquierdo-Useros N, Abad JL, et al. Dihydrosphingomyelin impairs HIV-1 infection by rigidifying liquid-ordered membrane domains. *Chem Biol.* (2010) 17:766–75. doi: 10.1016/j.chembiol.2010.05.023
19. Chernomordik L, Chanturiya A, Green J, Zimmerberg J. The hemifusion intermediate and its conversion to complete fusion: regulation by membrane composition. *Biophys J.* (1995) 69:922–9. doi: 10.1016/S0006-3495(95)79966-0
20. St Vincent MR, Colpitts CC, Ustinov AV, Muqadas M, Joyce MA, Barsby NL, et al. Rigid amphipathic fusion inhibitors, small molecule antiviral compounds against enveloped viruses. *Proc Natl Acad Sci USA.* (2010) 107:17339–44. doi: 10.1073/pnas.1010026107
21. Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, et al. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother.* (2002) 46:1896–905. doi: 10.1128/AAC.46.6.1896-1905.2002
22. Barsov EV, Payne WS, Hughes SH. Adaptation of chimeric retroviruses *in vitro* and *in vivo*: isolation of avian retroviral vectors with extended host range. *J Virol.* (2001) 75:4973–83. doi: 10.1128/JVI.75.11.4973-4983.2001
23. Harada S, Koyanagi Y, Yamamoto N. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. *Science* (1985) 229:563–6. doi: 10.1126/science.2992081
24. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, et al. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol.* (1986) 59:284–91.
25. Welker R, Hohenberg H, Tessmer U, Huckhagel C, Kräusslich HG. Biochemical and structural analysis of isolated mature cores of human immunodeficiency virus type 1. *J Virol.* (2000) 74:1168–77. doi: 10.1128/JVI.74.3.1168-1177.2000
26. Lampe M, Briggs JA, Endress T, Glass B, Riegelsberger S, Kräusslich HG, et al. Double-labelled HIV-1 particles for study of virus-cell interaction. *Virology* (2007) 360:92–104. doi: 10.1016/j.virol.2006.10.005
27. Emi N, Friedmann T, Yee JK. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *J Virol.* (1991) 65:1202–7.
28. Sherer NM, Lehmann MJ, Jimenez-Soto LF, Ingmundson A, Horner SM, Cicchetti G, et al. Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. *Traffic* (2003) 4:785–801. doi: 10.1034/j.1600-0854.2003.00135.x
29. Dettenhofer M, Yu XF. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. *J Virol.* (1999) 73:1460–7.
30. Rossio JL, Esser MT, Suryanarayana K, Schneider DK, Bess JW Jr, Vasquez GM, et al. Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. *J Virol.* (1998) 72:7992–8001.
31. Grimm D. Production methods for gene transfer vectors based on adeno-associated virus serotypes. *Methods.* (2002) 28:146–57. doi: 10.1016/S1046-2023(02)00219-0
32. Knölker H-J, Agarwal S, Schlechtingen G, Braxmeier T, Schroeder C, Jennings G. Steroid Sapogenin, Androstane and Triterpenoid Sapogenin Derivatives for the Treatment and Prevention of Infectious Diseases. PCT Int. Appl. WO 2009/090063.
33. Zankl C. Stereoselektive Synthese von Sphingolipiden zur Inhibierung der Degranulation von Mastzellen. Dissertation. Dresden: Technische Universität Dresden (2009).
34. Müller B, Daecke J, Fackler OT, Dittmar MT, Zentgraf H, Kräusslich HG. Construction and characterization of a fluorescently labeled infectious human immunodeficiency virus type 1 derivative. *J Virol.* (2004) 78:10803–13. doi: 10.1128/JVI.78.19.10803-10813.2004
35. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol.* (1998) 72:2855–64.
36. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* (1983) 65:55–63. doi: 10.1016/0022-1759(83)90303-4
37. Cavois M, De Noronha C, Greene WC. A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat Biotechnol.* (2002) 20:1151–4. doi: 10.1038/nbt745
38. Münk C, Brandt SM, Lucero G, Landau NR. A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc Natl Acad Sci USA.* (2002) 99:13843–8. doi: 10.1073/pnas.212400099
39. Geuenich S, Goffinet C, Venzke S, Nolkemper S, Baumann I, Plinkert P, et al. Aqueous extracts from peppermint, sage and lemon balm leaves display potent anti-HIV-1 activity by increasing the virion density. *Retrovirology* (2008) 5:27. doi: 10.1186/1742-4690-5-27
40. Abacioglu YH, Fouts TR, Laman JD, Claassen E, Pincus SH, Moore JR, et al. Epitope mapping and topology of baculovirus-expressed HIV-1 gp160 determined with a panel of murine monoclonal antibodies. *AIDS Res Hum Retroviruses* (1994) 10:371–81. doi: 10.1089/aid.1994.10.371
41. Parasassi T, De Stasio G, d'Ubaldo A, Gratton E. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys J.* (1990) 57:1179–86.
42. Parasassi T, De Stasio G, Ravagnan G, Rusch RM, Gratton E. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. *Biophys J.* (1991) 60:179–89. doi: 10.1016/S0006-3495(91)82041-0
43. Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta* (1985) 812:55–65. doi: 10.1016/0005-2736(85)90521-8
44. Struck DK, Hoekstra D, Pagano RE. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* (1981) 20:4093–9. doi: 10.1021/bi00517a023
45. Rajendran L, Knölker H-J, Simons K. Subcellular targeting strategies for drug design and delivery. *Nat Rev Drug Discov.* (2010) 9:29–42. doi: 10.1038/nrd2897
46. Batista J, Friedrichson T, Schlechtingen G, Braxmeier T, Jennings G, Bajorath J. Computational screening for membrane-directed inhibitors of mast cell activation. *Eur J Med Chem.* (2010) 45:2700–4. doi: 10.1016/j.ejmech.2010.01.061
47. Batista J, Schlechtingen G, Friedrichson T, Braxmeier T, Bajorath J. Lipid-like sulfoxides and amine oxides as inhibitors of mast cell activation. *Eur J Med Chem.* (2011) 46:2147–51. doi: 10.1016/j.ejmech.2011.02.068
48. Wolf MC, Freiberg AN, Zhang T, Akyol-Ataman Z, Grock A, Hong PW, et al. A broad-spectrum antiviral targeting entry of enveloped viruses. *Proc Natl Acad Sci USA.* (2010) 107:3157–62. doi: 10.1073/pnas.0909587107
49. Donzella GA, Schols D, Lin SW, Esté JA, Nagashima KA, Maddon PJ, et al. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med.* (1998) 4:72–7. doi: 10.1038/nm0198-072

50. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol.* (1998) 72:1438–45.
51. Roche S, Albertini AA, Lepault J, Bressanelli S, Gaudin Y. Structures of vesicular stomatitis virus glycoprotein: membrane fusion revisited. *Cell Mol Life Sci.* (2008) 65:1716–28. doi: 10.1007/s00018-008-7534-3
52. McClure MO, Sommerfelt MA, Marsh M, Weiss RA. The pH independence of mammalian retrovirus infection. *J Gen Virol.* (1990) 71:767–73. doi: 10.1099/0022-1317-71-4-767
53. Suárez T, Nir S, Goñi FM, Saéz-Cirión A, Nieva JL. The pre-transmembrane region of the human immunodeficiency virus type-1 glycoprotein: a novel fusogenic sequence. *FEBS Lett.* (2000) 477:145–9. doi: 10.1016/S0014-5793(00)01785-3
54. Sáez-Cirión A, Nir S, Lorizate M, Agirre A, Cruz A, Pérez-Gil J, et al. Sphingomyelin and cholesterol promote HIV-1 gp41 pretransmembrane sequence surface aggregation and membrane restructuring. *J Biol Chem.* (2002) 277:21776–85. doi: 10.1074/jbc.M202255200
55. Moreno MR, Giudici M, Villalán J. The membranotropic regions of the endo and ecto domains of HIV gp41 envelope glycoprotein. *Biochim Biophys Acta* (2006) 1758:111–23. doi: 10.1016/j.bbame.2006.01.007
56. Malkovsky M, Newell A, Dalgleish AG. Inactivation of HIV by nonoxynol-9. *Lancet* (1988) 1:645. doi: 10.1016/S0140-6736(88)91440-7
57. Bagatolli LA. To see or not to see: lateral organization of biological membranes and fluorescence microscopy. *Biochim Biophys Acta* (2006) 1758:1541–56. doi: 10.1016/j.bbame.2006.05.019
58. Kaiser HJ, Lingwood D, Levental I, Sampaio JL, Kalvodova L, Rajendran L, et al. Order of lipid phases in model and plasma membranes. *Proc Natl Acad Sci USA.* (2009) 106:16645–50. doi: 10.1073/pnas.0908987106
59. Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, et al. Lipid rafts reconstituted in model membranes. *Biophys J.* (2001) 80:1417–28. doi: 10.1016/S0006-3495(01)76114-0
60. Parasassi T, Di Stefano M, Loiero M, Ravagnan G, Gratton E. Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence. *Biophys J.* (1994) 66:120–32. doi: 10.1016/S0006-3495(94)80763-5
61. Hillier SL, Moench T, Shattock R, Black R, Reichelderfer P, Veronese F. *In vitro* and *in vivo*: the story of nonoxynol 9. *J Acquir Immune Defic Syndr.* (2005) 39:1–8. doi: 10.1097/01.qai.0000159671.25950.74
62. Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying V, Rees H, et al. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. *Lancet* (2002) 360:971–7. doi: 10.1016/S0140-6736(02)11079-8
63. Agarwal S, Schroeder C, Schlechtingen G, Braxmeier T, Jennings G, Knölker H-J. Evaluation of steroidal amines as lipid raft modulators and potential anti-influenza agents. *Bioorg Med Chem Lett.* (2013) 23:5165–9. doi: 10.1016/j.bmc.2013.07.015
64. Kaiser HJ, Surma MA, Mayer F, Levental I, Grzybek M, Klemm RW, et al. Molecular convergence of bacterial and eukaryotic surface order. *J Biol Chem.* (2011) 286:40631–7. doi: 10.1074/jbc.M111.276444
65. Diaz-Rohrer B, Levental KR, Levental I. Rafting through traffic: membrane domains in cellular logistics. *Biochim Biophys Acta* (2014) 1838:3003–13. doi: 10.1016/j.bbame.2014.07.029
66. The International Transporter Consortium, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. Membrane transporters in drug development. *Nat Rev Drug Discov.* (2010) 9:215–36. doi: 10.1038/nrd3028
67. Vigant F, Lee J, Hollmann A, Tanner LB, Akyol Ataman Z, Yun T, et al. A mechanistic paradigm for broad-spectrum antivirals that target virus-cell fusion. *PLoS Pathog.* (2013) 9:e1003297. doi: 10.1371/journal.ppat.1003297
68. Zhan P, Li Z, Liu X. Cosalane and its analogues: a unique class of anti-HIV agents. *Mini Rev Med Chem.* (2010) 10:966–76. doi: 10.2174/138955710792007222
69. Cushman M, Golebiewski WM, McMahon JB, Buckheit RWJr, Clanton DJ, Weislow O, et al. Design, synthesis, and biological evaluation of cosalane, a novel anti-HIV agent which inhibits multiple features of virus reproduction. *J Med Chem.* (1994) 37:3040–50. doi: 10.1021/jm00045a008
70. Dumas F, Haanappel E. Lipids in infectious diseases - the case of AIDS and tuberculosis. *Biochim Biophys Acta* (2017) 1859(9 Pt B):1636–47. doi: 10.1016/j.bbame.2017.05.007
71. Verma SP. HIV: a raft-targeting approach for prevention and therapy using plant-derived compounds (review). *Curr Drug Targets* (2009) 10:51–9. doi: 10.2174/138945009787122851
72. Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem.* (2000) 275:17221–4. doi: 10.1074/jbc.R000005200
73. Simons K, Ikonen E. How cells handle cholesterol. *Science* (2000) 290:1721–6. doi: 10.1126/science.290.5497.1721
74. Hanshaw RG, Smith BD. New reagents for phosphatidylserine recognition and detection of apoptosis. *Bioorg Med Chem.* (2005) 13:5035–42. doi: 10.1016/j.bmc.2005.04.071
75. Zaitseva E, Zaitsev E, Melikov K, Arakelyan A, Marin M, Villasmil R, et al. Fusion Stage of HIV-1 entry depends on virus-induced cell surface exposure of phosphatidylserine. *Cell Host Microbe* (2017) 22:99–110. doi: 10.1016/j.chom.2017.06.012
76. Morizono K, Chen IS. Role of phosphatidylserine receptors in enveloped virus infection. *J Virol.* (2014) 88:4275–90. doi: 10.1128/JVI.03287-13
77. Goñi FM, Alonso A. Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. *Biochim Biophys Acta* (2006) 1758:1902–21. doi: 10.1016/j.bbame.2006.09.011
78. Contreras FX, Sot J, Alonso A, Goni FM. Sphingosine increases the permeability of model and cell membranes. *Biophys J.* (2006) 90:4085–92. doi: 10.1529/biophysj.105.076471
79. Izquierdo-Useros N, Lorizate M, Contreras FX, Rodriguez-Plata MT, Glass B, Erkizia I, et al. Sialyllactose in viral membrane gangliosides is a novel molecular recognition pattern for mature dendritic cell capture of HIV-1. *PLoS Biol.* (2012a) 10:e1001315. doi: 10.1371/journal.pbio.1001315
80. Izquierdo-Useros N, Lorizate M, Puertas MC, Rodriguez-Plata MT, Zangger N, Erikson E, et al. Siglec-1 is a novel dendritic cell receptor that mediates HIV-1 Trans-infection through recognition of viral membrane gangliosides. *PLoS Biol.* (2012b) 10:e1001448. doi: 10.1371/journal.pbio.1001448

Conflict of Interest Statement: CB, MG, GJ, BB, SA, CZ, and CS were employed by JADO during the study. This company is no longer active, and the authors have no current affiliation to it.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Citation: Nieto-Garai JA, Glass B, Bunn C, Giese M, Jennings G, Brankatschk B, Agarwal S, Börner K, Contreras FX, Knölker H-J, Zankl C, Simons K, Schroeder C, Lorizate M and Kräusslich H-G (2018) Lipidomimetic Compounds Act as HIV-1 Entry Inhibitors by Altering Viral Membrane Structure. *Front. Immunol.* 9:1983. doi: 10.3389/fimmu.2018.01983

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T Cells on Engineered Substrates: The Impact of TCR Clustering Is Enhanced by LFA-1 Engagement

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 06 June 2018

Accepted: 23 August 2018

Published: 18 September 2018

Citation:

Benard E, Nunès JA, Limozin L and
Sengupta K (2018) T Cells on
Engineered Substrates: The Impact of
TCR Clustering Is Enhanced by LFA-1
Engagement. *Front. Immunol.* 9:2085.
doi: 10.3389/fimmu.2018.02085

We created APC-mimetic synthetic substrates to study the impact of ligand clustering on T cell activation and spreading. The substrates exhibit antibodies directed against the TCR-complex in the form of a patterned array of sub micrometric dots surrounded by a fluid supported lipid bilayer (SLB) which may itself be functionalized with another bio-molecule. We show that for T cell adhesion mediated by T cell receptor (TCR) alone, in the patterned, but not in the corresponding homogeneous controls, the TCR, ZAP-70 and actin are present in the form of clusters or patches that co-localize with the ligand-dots. However, global cell scale parameters like cell area and actin distribution are only weakly impacted by ligand clustering. In presence of ICAM-1 - the ligand of the T cell integrin LFA-1 - on the SLB, the TCR is still clustered due to the patterning of its ligands, but now global parameters are also impacted. The actin organization changes to a peripheral ring, resembling the classical actin distribution seen on homogeneous substrates, the patterned membrane topography disappears and the membrane is flat, whereas the cell area increases significantly. These observations taken together point to a possible pivotal role for LFA-1 in amplifying the effect of TCR-clustering. No such effect is evident for co-engagement of CD28, affected *via* its ligand B7.2. Unlike on ICAM-1, on B7.2 cell spreading and actin organization are similar for homogeneous and patterned substrates. However, TCR and ZAP-70 clusters are still formed in the patterned case. These results indicate complementary role for LFA-1 and CD28 in the regulation and putative coupling of TCR micro-clusters to actin. The engineered substrates presented here clearly have the potential to act as platform for fundamental research in immune cell biology, as well as translational analyses in immunotherapy, for example to screen molecules for their role in T cell adhesion/activation.

Keywords: surface bio-engineering, protein nano-patterning, TCR micro-clusters, T cell adhesion, cell spreading, ZAP-70 clusters, actin organization, co-stimulation

1. INTRODUCTION

The interaction of T cells with antigen presenting cells (APCs) plays a central role in adaptive immunity, one of whose salient features is the duality of exquisite sensitivity and strict discrimination in the context of recognition of antigen by T cells through the T cell receptor (TCR). To achieve this, the T cell membrane carries a variety of additional adhesive, co-stimulatory and

inhibitory molecules to complement the basic TCR mediated interaction. Following encounter with its antigen, a key step toward correct activation of the T cell is the reorganization of its membrane and its cytoskeleton. Concomitantly, the cell spreads on the APC, forming the so-called immunological synapse (1, 2). The extent of spreading in T cells is a marker of their activation and eventual proliferation (3).

A very fruitful approach to dissect adhesion and membrane/cytoskeleton reorganization has been to replace the APC with a synthetic antigen presenting substrate (APS). The early APS were, typically, supported bilayers carrying ligands for the TCR/CD3 complex either lipids coupled pMHC (1) or biotinylated anti-CD3 (4), and ligands for the integrin LFA-1 often in the form of a GPI-anchored protein or connected to NTA functionalized lipids (1, 4–6). In the last decade, such substrates have been designed to carry, in addition, a host of other ligands against co-receptors such as CD28 to dissect specific aspects of T cell response.

Seminal work from Dustin and Saito labs underlined the importance of TCR-clusters in initiation of T cell activation (4, 7, 8). Segregation of TCR into clusters is also at the heart of the kinetic segregation model for T cell activation (9–11), where the membrane topography plays an important role. The pair TCR/pMHC being much shorter than LFA-1/ICAM-1 (Intercellular adhesion molecule 1) pair, the TCR clusters exclude the longer LFA-1 / ICAM-1 as well as the long phosphatases like CD45. The absence of CD45 permits the phosphorylation of the ITAM-motifs associated with the TCR/CD3 domain, thus initiating T cell activation.

Simultaneously with activation and adhesion, actin polymerization is triggered at the cell edge to promote spreading (12). The actin is organized roughly as a ring (13), whose fine structure was revealed recently (14–16). It was shown that the peripheral nature of the actin ring is more pronounced for stronger pMHC ligands as compared to weaker ones where the distribution is more homogeneous (14). The TCR as well as LFA-1 get connected to the actin cytoskeleton: the molecular species connecting integrin LFA-1 to actin are well known (17–19), those connecting TCR to actin are yet to be fully identified (17). TCR-clusters that already exclude both LFA-1 and long sugars, are carried by the actin retrograde flow toward the center where they eventually form the central supramolecular activation cluster (cSMAC) (4, 20).

To address the question of the impact of ligand/receptor segregation or clustering two approaches have emerged genetic modification to vary relevant molecular lengths (21, 22), or manipulation of the clustering itself sometimes called spatial mutation (23), for example *via* the use of designed supported lipid bilayers (SLBs) that are patterned with micron size corals which do not allow diffusion of molecules across their fence (20, 24), thus revealing the importance of ligands diffusion for the formation of a stable immunological synapse.

In parallel to the use of SLBs as APS, several groups used protein coated glass instead (13, 25, 26). Using this approach,

Irvin and Doh explored the consequences of micro-clustering of TCR and/or LFA-1, focusing on the formation of cSMAC (25). They showed that T-cells can be fully activated when focal spots of immobilized TCR ligand are at the center of the interacting surface but not if they are patterned differently. Later it was shown that T-cells were able to produce IL-12 when anti-CD3 dots are surrounded by CD28 (co-stimulation molecule that binds to B7.1 or B7.2) whereas when both were co-localized they did not (27). These studies emphasized the importance of the organization of the ligands on APC-side for the formation of the immunological synapse and the activation of the T-cells. The importance of force at the synapse is more and more recognized as central (28). A recent study reported complementary roles of TCR and LFA-1 on cytoskeletal growth and contractility using micro-patterning showing that LFA-1 adhesion enhances actomyosin forces, which in turn modulate actin assembly downstream of the TCR (29).

In previous work using sub-micron sized patterns of TCR-ligands, we showed that T cells respond globally to average density of TCR-ligands, rather than details of the pattern (30), a result consistent with those obtained with nano-patterns, where the ligand spacing and density were independently controlled (31, 32). However, we could additionally show that on patterned substrates, at the local dot-scale, TCR and ZAP-70 are gathered into clusters that overlap with dots of TCR-ligands.

In many of the examples above, micro and nano patterning of ligands was used to manipulate T cell behavior in order to reveal the importance of TCR clustering. More recently, it has been shown that the natural ligands of TCR, the pMHC¹, may in fact be presented as nano-clusters on target cells (33, 34). To this extent, nano-patterned substrates also mimic one aspect of the *in vivo* situation.

Here, as in our previous work (5, 30), the ligand of choice is anti-CD3 which provides sufficient adhesion to the substrate with TCR/CD3 complex alone, in absence of ICAM-1 something not possible if pMHC was used since the TCR-pMHC bond is not strong enough to sustain adhesion. At the same time, it should be pointed out that this is a legitimate approach since anti-CD3 is known to elicit the same signaling pathways as pMHC ligation (35) and the CD3 domain mediates T-cell mechanotransduction (36). We use a combination of colloidal bead lithography and metal sputtering to fabricate sub-micron sized ligand clusters on glass (37, 38). These clusters are then surrounded by supported lipid bilayers, optionally functionalized with ICAM-1 or B7.2² to form substrates that mimic APCs. This approach allowed us to simultaneously observe global adhesion as well as local membrane/actin reorganization using high resolution optical microscopy.

¹So far the reports concern pMHC-II.

²CD28 interacts with two major ligands, B7.1 and B7.2 with no significant differences in the functions of CD28/B7.1 versus CD28/B7.2. For this study, we used B7.2 as it is generally the first B7 molecule encountered, due to its constitutive expression on numerous antigen presenting cells.

2. MATERIALS AND METHODS

2.1. Substrates Preparation

2.1.1. Protein Nano-Pattern

The details of the fabrication process for making the patterned substrates was published previously (30, 38, 39). Briefly, hydrophilic glass coverslips (thickness = 170 microns, Assistant, Karl Hecht KG, Germany, 24 x 24 mm) were obtained by cleaning by ultrasonication in aqueous solution of a detergent (Hellmanex, Sigma, France), followed by a thorough rinsing in ultrapure water. Self-assembly of colloidal beads was used to create the primary mask. Monodisperse silica colloidal beads (Corpuscular, USA), 2 μ m diameter, were washed 10 times with ultra-pure water before utilization. The concentration of the beads suspension needs to be optimized in order to avoid multilayer of beads during the deposition. Moreover in order to have an optimal mask, size standard beads was used. A cleaned glass slide was placed on the platform with an angle of about 15° and a calibrated volume of the colloidal suspension was allowed to spread on the slide. After complete evaporation, a large area covering most of the slide of a very ordered array of beads is generated.

A thin and controlled layer of aluminum was deposited on the glass slide through the beads using a radio frequency (RF) magnetron sputtering technique from an aluminum target with 1% silicon (Kurt J. Lesker Company, purity 99.99%). The geometry of the sputtering system is off-axis and the mean free path is 10 mm in the operating pressure range. The samples were placed at a distance of 105 mm onto a rotating table (3–5 rpm). After aluminum deposition, the colloidal beads were rinsed away by ultra-sonication in ultra-pure water to reveal the secondary mask which is the aluminum layer displaying an ordered array of empty nano-holes. The slides were then placed in a chamber containing 3-aminopropyltriethoxysilane (APTES) (Sigma-Aldrich, France) in vapor phase at about 60°C for 1 hour. Next, Bovine Serum Albumin conjugated with biotin (BSA-Biotin, Sigma, France) was incubated at the concentration of 25 μ g/ml for 30 min. Finally, the layer of aluminum was removed by incubation in PBS + Sodium hydroxide (NaOH), pH $\geq$ 11 for at least 4 hours until complete dissolution at room temperature. At this stage the coverslide was covered with uniform nano-dots of functional BSA-Biotin surrounded by bare glass (39).

2.1.2. Preparation of the SLB

The bare glass separating the BSA-biotin dots was filled with a supported lipid bilayer using Langmuir-Blodgett technique [see, for example (5) or (40) for details]. Lipids (Avanti Polar Lipids, USA) were received either dried or already solubilized in chloroform. Dry lipids were dissolved in clean chloroform (99.9%, Sigma, France) before use. Supported lipid bilayers are composed of either pure DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), or DOPC+5% NTA-lipids (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)). In order to verify the presence and check the quality of the SLB, 0.01 % of Dansyl PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (ammonium salt)) was systematically added to the mixture.

2.1.3. Functionalization

Substrates were further functionalized by incubation with 2 μ g/ml Neutravidin Fluorescein isothiocyanate conjugated (Sigma Aldrich, France) or Neutravidin Dylight650 conjugated, (Life technology, France), (both henceforth called NaV) for 30 min, followed by incubation in anti-CD3 at 2 μ g/ml (multinotylated UCHT1, eBioscience, France) alone or with ICAM-1 His-Tag (ICAM1 Recombinant Human Protein, hIGG1-Fc.His Tag, ThermoFisher, France) or with B7-2 His-Tag (B7-2/CD86 Recombinant Human Protein, R&D systems, France) at 5 μ g/ml for 30 min.

2.1.4. Homogeneous Controls

For homogeneous substrates, the supported lipid bilayer was deposited directly on cleaned glass slides. Two types were used. For negative controls, the SLB composition is the same as described previously and for homogeneous controls, the SLBs were additionally doped with 0.01% cap-biotin (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt)) in order to have the same protein composition as on patterned substrates.

2.2. Cell Culture, Fixation and Labeling

Jurkat T cells (clone E6-1, ATCC) were cultured in complete RPMI 1640 medium (Life Technologies, France) containing red phenol and L-glutamine supplemented with 1 % glutaMAX (Life Technologies, France) and 10% Fetal Bovine Serum (Life Technologies, France). Cells were in exponential growth phase at the time of experiment. The functionalized glass coverslips formed the bottom of a custom made chamber which was filled with PBS+0,1%BSA buffer. 200 μ l of the medium containing cells was added. The cells were allowed to sediment on to the substrate and were incubated for 30 min at 37°C and 5% CO₂. Cells were then fixed by incubation in 2% pre-warmed paraformaldehyde for 30 min at 37 °C, followed by extensive rinsing with PBS. The cells were blocked with 1% BSA overnight and immunostained by incubation with 5 μ g/ml of FITC fluorescent Anti-V β 8 TCR (BD Biosciences, USA) which is directed against the beta chain of the T-cell receptor, or with 20 μ g/ml of Alexa Fluor 488 -phalloidin (dissolved in methanol, ThermoFischer, France) which labeled filamentous actin or with Alexa Fluor 647 Mouse Anti-ZAP70 (PY319)/Syk (PY352) (BD biosciences, France) which labeled the kinase ZAP-70 during 30 min. Samples were rinsed extensively before imaging.

2.3. Microscopy

Total internal reflection microscopy (TIRFM) and reflection interference contrast microscopy (RICM) were performed using an inverted microscope (AxioObserver, Zeiss, Germany), equipped with an EM-CCD camera (iXon, Andor, UK). Acquisition was performed using Andor iQ, or Zeiss ZEN software. TIRF and RICM images were taken with a 100X 1.45 NA oil or a custom 100X 1.46 NA oil antilex objective (Zeiss). For TIRF exposure time was 100 ms, and fluorescence filter sets adapted to the fluorophores were used. For RICM exposure time was 200 ms.

2.3.1. Image Analysis

Image analysis was performed using macros written in-house in ImageJ/Fiji (41) and IgorPro (Wavemetrics, USA).

Supported lipid bilayer: The presence and the fluidity of the SLB were systematically verified in epi-fluorescence. Quantitative measurements of the fluidity were performed on randomly selected substrates using continuous photobleaching (CPB) (40, 42). Briefly, the fluorescent SLB is continuously illuminated and observed in epi-mode through a partially closed diaphragm, such that the exposed fluorophores are irreversibly bleached. If the SLB is fluid, unbleached fluorophores enter and exit the observation area, resulting in a luminous ring along the edge of the diaphragm. Quantification of the radial profile of this ring yields the lipid diffusion constant.

Note that the SLB fluidity acts as quality check here in our experience fluid bilayers are less prone to defect-formation and phase separation. The proteins however may diffuse with a different diffusion constant than the lipids, the former being usually much slower. In fact, anti-CD3 does not diffuse at all on the SLBs prepared using Langmuir-Blodgett (5). We verified, by measurement of a partial recovery after bleaching, that his-tagged proteins (like ICAM-1 and B7.2 used here) do diffuse, but with a diffusion constant of $\ll 0.001 \mu\text{m}^2$ - too slow to be quantified by CPB.

Nano-dots: The nano-dot were characterized using an automated algorithm in terms of diameter size and fluorescence intensity. The spacing between each dots is set by the diameter of the beads used. For each field of fluorescent nano-dot array, each dot was segmented and a median dot was constructed. The size of the dots was characterized in terms of full-width at half maximum (FWHM) of the radial profile of fluorescence of the median dot. The intensity inside (I_{max}) and outside (I_{min}) of the nano-dots corresponding respectively to the value of the intensity of the peak and the value of the baseline intensity on the radial profile. The fluorescence intensity is an important parameter as it is proportional to the protein density. In order to convert fluorescence intensity value of NaV to a estimate density, a calibration was done. For this a special substrate with very low surface density of fluorescent molecules was prepared. The number of fluorescent molecules/ μm^2 was calculated, by assuming that fluorescent dots correspond to one molecule. Then for a corresponding intensity value, the density is known. Therefore, by comparison of the emitted light intensity of experimental substrates, the density of NaV can be determined [also see (5)].

Cell adhesion area: Cell adhesion was characterized based on the RISM images in terms of adhesion area. Cell contour was determined from RISM images using a spatial variance filter with a radius of 4 pixels followed by a thresholding. Then the function "Particle analysis" of ImageJ was applied to identify the edge of the cell providing an accurate measurement of the contact area (5, 43). For each condition, a large number of experimental data was available but these had to be additionally vetted to exclude outliers which exhibit atypical behavior probably due to undetected differences in the substrates whose preparation is complex. To systematize this, in addition to discarding substrates

for which quantification of the protein density was not available, we also compared data from each sample to an aggregate data-set for the same condition and rejected the data-sets whose p-values showed them to be significantly different ($p < 0.0001$).

Uniform and textured adhesion: Casual inspection of the cells RISM images on patterns reveals that two major cell adhesion morphologies are possible (Figure S1). In RISM images, dark areas correspond to tight adhesion and gray areas to the background. Bright pixels however may either arise from the proximal membrane of the cell close to the substrate (typically up to about 800 nm) but not tightly adhered, or sometimes from internal organelles of the cell. The presence of multiple dark patches whose size and spacing matches that of the underlying ligand-dots, points to a cell membrane being textured due to the patterning of the substrate. Here we adapted the convention that presence of at least 7 dark patches (intensity $\ll$ background), whose size and spacing are compatible with the pattern signify textured adhesion, all others are considered uniform adhesion.

TCR-clusters: The fluorescent images of TCR were prepared in ImageJ by doing a segmentation of the cells using the corresponding RISM image, then a intensity thresholding algorithm was used to segment the clusters [The algorithm as a plugin was kindly provided by Dr. Rajat Varma ((6)). The algorithm uses an initial intensity thresholding using the mean intensity under the cell but outside the clusters. Then different parameters were defined : an upper cutoff for cluster size (here 10 pixels), a step value for convergence (here 0.05) and a step value to determine how much to trim each cluster (here 0.8). The algorithm outputs the size of the clusters which was directly used to construct the size histograms [see also (30)]. We verified that the output of this algorithm is robust to small (10–25%) changes in the input parameters and the choice of intensity thresholding.

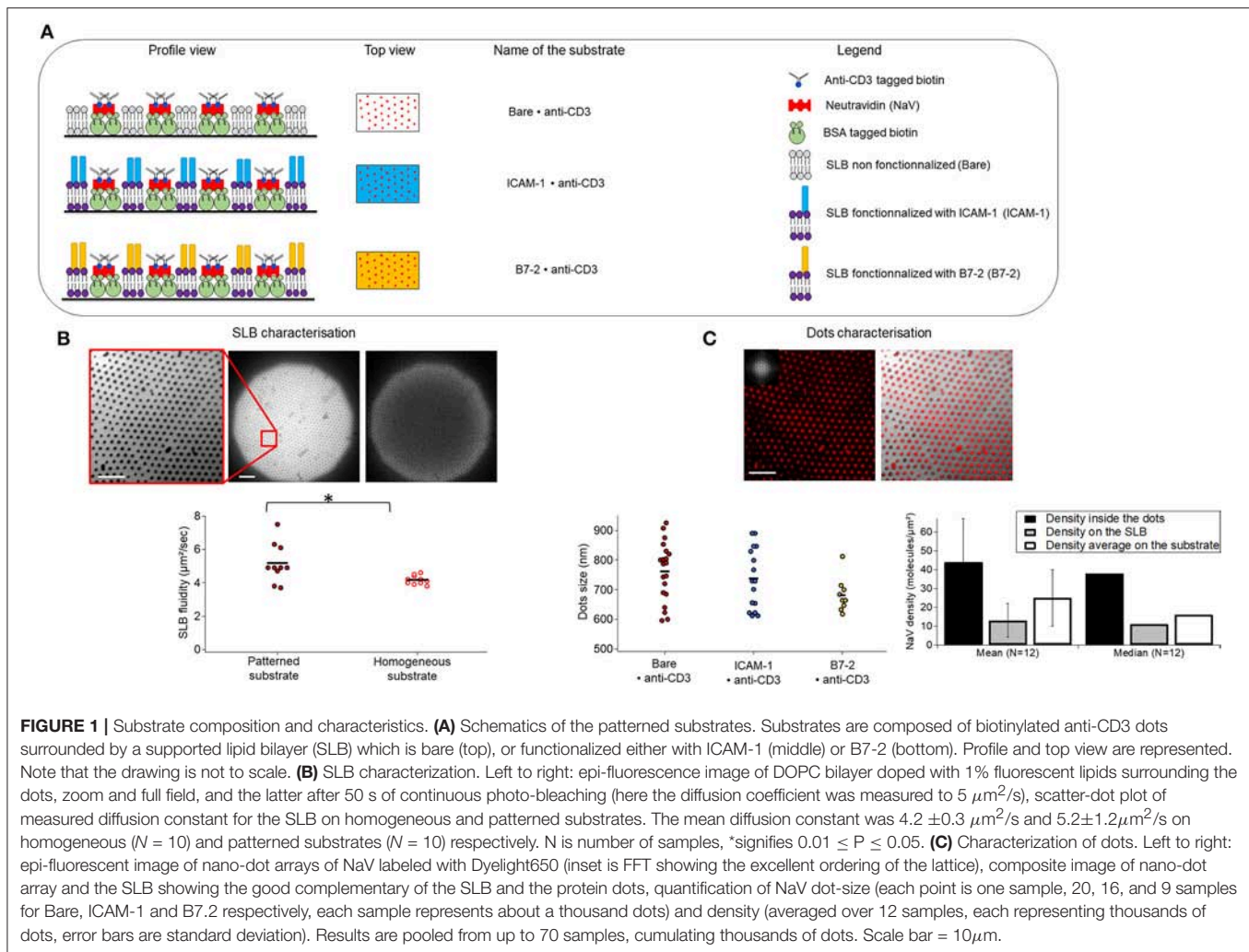
F-Actin clearance: In order to determine the organization of the actin (homogeneous or peripheral), fluorescence intensity within a circle of $1.2 \mu\text{m}$ at the center (I_{Center}) and in the rest (I_{RestCell}) of the cell is measured and then the F-actin clearance is calculated using $\frac{I_{\text{Center}}}{I_{\text{RestCell}}}$.

Statistical test and errors: Error-bars are standard deviations unless otherwise stated. The data were compared pair-wise using 2 tailed Wilcoxon-Mann-Whitney rank test, performed using the in-built function in IgorPro (Wavemetrics, USA). p -values were used to determine significance levels. ***signifies $P \leq 0.0000001$, **signifies $P \leq 0.0001$, * signifies $P \leq 0.01$ and NS (or absence of any *) signifies > 0.01 . In addition, within populations deemed to be different, we quantify the size of the difference (effect-size) through the difference in the medians.

3. RESULTS

3.1. The Substrates

The APC mimetic substrates were prepared using nano-sphere lithography and aluminum sputtering as discussed above. The basic patterned substrate consists of anti-CD3 dots, arranged in a hexagonal pattern and surrounded by a SLB, which can be optionally functionalized with either ICAM-1 or B7.2



(Figure 1A). For ease of reference, we adopt the following nomenclature: anti-CD3 clusters embedded in bare/ICAM-1/B7.2-functionalized SLB are called Bare/ICAM-1/B7.2•anti-CD3. For each type of ligand, patterned substrates were compared to equivalent homogeneous substrates, which are SLBs on which the anti-CD3, conjugated *via* biotin/neutravidin is immobile (5) but the ICAM-1/B7.2, conjugated *via* his-tag/NTA is mobile. The corresponding homogeneous substrates are called Bare/ICAM-1/B7.2 + anti-CD3. The distance between the dots (pitch) is set by the choice of the beads for lithography, here $2 \mu\text{m}$. The fluidity of the SLB was quantified using continuous photo-bleaching and was found to vary from about 4 to $8 \mu\text{m}^2/\text{s}$ (Figure 1B). The dot size is fixed by shadow effects during sputtering (38). The dot size and the density of ligands inside or outside the dots was quantified by analysis of epi-fluorescence images of the neutravidin using self-written routines that allowed us to easily analyze thousands of dots (Figure 1C). At about 700–800 nm, the size of the ligand-dots happen to roughly match the typical size of signaling micro-clusters reported in literature (6). The average density of ligands inside a dot was estimated to be about 40 molecules/ μm^2

and outside at about 10 molecules/ μm^2 , yielding a good contrast. The average ligand density under a cell was about 20 molecules/ μm^2 .

The variation of the density from substrate to substrate as well as within each substrate could potentially impact the results. Since data from a large number of substrates are pooled, it was necessary to verify that the density of the ligands does not impact cell spreading area in the studied range. Indeed as can be seen in Figures S2A–C, this is the case for both patterned and homogeneous substrates, with or without additional functionalization with ICAM-1/B7-2.

Jurkat T-cells were allowed to interact with these APC mimetic substrates for 30 minutes and were fixed. They were then imaged in RICM, and/or TIRF-M. The cell response was quantified through the analyses of the cell spreading area (also called adhesion area), the T-cell membrane molecular distribution (TCR and ZAP-70), and the actin organization. Five parameters are discussed: the cell spreading area, which is a measure of cell activation in T cells (3), TCR/ZAP-70 clustering and membrane topography which together is thought to be essential

for activation (9) and actin architecture which plays an important role in molecular transport (17) as well as mechanosensing (36).

3.2. Cell Spreading and Actin Organization on Patterned and Homogeneous Anti-CD3

For anti-CD3 dots embedded in bare SLB (Bare●anti-CD3), casual inspection of RISM images reveals that on Bare●anti-CD3 two major cell adhesion morphologies are possible—either uniform or textured (**Figure 2A**). In the first case, true for about 75–80% of the cells, the cell membrane adheres uniformly to the underlying substrate; and in the second case, true for about 20–25% of the cells, the membrane is textured (**Figure 2, Figure S1**). The cell spreading area for the two types of adhesion ($184 \pm 78 \mu\text{m}^2$ for textured adhesion and $214 \pm 97 \mu\text{m}^2$ for uniform adhesion) is not statistically different (**Figure 2B**). Averaging over all cells on Bare●anti-CD3, spreading area is $207 \pm 95 \mu\text{m}^2$ (average $\pm$ s.d, from 8 experiments, totaling 109 cells, SEM=9 and median = $170 \mu\text{m}^2$), to be compared to spreading area for homogeneously partitioned anti-CD3 grafted on an SLB ($148 \pm 57 \mu\text{m}^2$, SEM = 7, median = $130 \mu\text{m}^2$). The two distributions are statistically different and the effect-size is $40 \mu\text{m}^2$ (see **Tables S1, S2** and **Figure S3**).

The spreading of a T cell is driven by actin polymerization (5, 44) and therefore the adhesion and extent of spreading can be expected to be intimately linked with actin organization. In the classical case of T cells spreading on SLBs functionalized homogeneously with anti-CD3 and ICAM-1, at full spreading, the actin forms a ring along the periphery of the cell (5, 44, 45). In the present case of Bare●anti-CD3 however, the actin is either homogeneous (75–80% of the cells) or appears as dots that clearly coincide with the pattern (20–25%). Quantification of the extent of actin clearance from the center shows that cells on Bare●anti-CD3 and Bare + anti-CD3 are similar in terms of actin clearance (**Figure 2C, Figure S4**).

3.3. Cell Spreading and Actin Organization When LFA-1 Integrins or CD28 Co-receptors Are Engaged

For the case of anti-CD3 clusters embedded in ICAM-1 functionalized SLB (ICAM●anti-CD3), RISM images reveal that the membrane adheres uniformly to the substrate for all cells and the substrate patterning has no discernible impact on the membrane roughness (**Figure 3A**). Indeed, the RISM images of cells on ICAM●anti-CD3 are not qualitatively different from those on ICAM + anti-CD3. The cell area however, is significantly larger (**Figure 3B, Tables S1, S2**). Averaging over all cells on ICAM●anti-CD3, spreading area is $298 \pm 153 \mu\text{m}^2$ (average $\pm$ s.d, from 12 experiments, totaling 171 cells, SEM = 9 and median = $259 \mu\text{m}^2$), to be compared to spreading area for homogeneously partitioned anti-CD3 grafted on an ICAM-1 bearing SLB (from 7 experiments and 164 cells, $160 \pm 68 \mu\text{m}^2$, SEM = 5, median = $147 \mu\text{m}^2$). The effect-size quantified by difference in median is $111 \mu\text{m}^2$, much higher than in absence of ICAM-1. A comparison between the patterned substrates with and without ICAM-1 (Bare●anti-CD3 and ICAM●anti-CD3), also reveals a significant difference, with the effect-size being 70

μm^2 . We conclude that the presence of ICAM-1 enhances the impact of the clustering of the TCR.

Visual inspection as well as quantification of actin clearance show that the actin organization for cells on ICAM●anti-CD3 is clearly peripheral whereas on ICAM + anti-CD3, a range of behavior from fairly homogeneous to peripheral is seen (**Figure 3C**). As anticipated, ICAM-1 alone, in the form of dots or not, does not induce cell spreading (**Figure S5**).

In presence of B7.2, comparing B7.2●anti-CD3 and B7 + anti-CD3, no statistical difference is detected either in terms of adhesion area or in terms of actin clearance (**Figure 4**). In both cases, the adhesion is homogeneous, and actin is peripheral.

3.4. TCR and ZAP-70 Organization

The co-localization of TCR with anti-CD3 dots occurs in all cases where anti-CD3 is in form of dots (**Figure 5A**). Whereas on homogeneous anti-CD3, with or without additional presence of ICAM-1 or B7.2, the anti-CD3 is distributed all over the surface and the TCR get bound and immobilized uniformly, on dot anti-CD3 (Bare●anti-CD3 with or without ICAM-1/B7.2) a pool of diffusive and non-bound TCR molecules may be able to diffuse over the SLB and co-localize with the anti-CD3 dots (**Figure 5A**). On ICAM-1●anti-CD3 and B7.2●anti-CD3, the ICAM-1 or B7.2 can diffuse, and do not hinder the diffusion of TCR. As is seen in **Figure 5B**, the coincidence of the TCR clusters with the dots is near perfect even though there is no correlation between the ligand density in the dots and the TCR density (reflected by the fact that faint dots may harbor bright clusters and vice versa). **Figure 5C** shows [after (6)] the distribution of cluster size *via* the fraction of clusters present for each size (also see **Table S3A**). In each case, an enrichment of large clusters (size greater than, or of the order of ligand-dot size) is evident in the patterned substrate with respect to the homogeneous one. Finally, at the cell scale, the TCR clusters are distributed uniformly, without the formation of an evident cSMAC (**Figure 5D**). This is quantified *via* the TCR parameter, which was found to be >3 for T cells on SLBs with mobile ligands (5, 30).

ZAP-70 clusters mostly follow the pattern on Bare●anti-CD3 whereas on ICAM-1●anti-CD3 the overlap is relatively poor (**Figures 6A,B, Figure S6**). Moreover, in presence of ICAM-1 or B7.2, the proportion of ZAP-70 molecules homogeneously distributed over the cell membrane in the form of small clusters (much smaller than ligand-dot size) is higher (**Figure 6C, Table S3B**). Note the much more pronounced co-localization of the TCR clusters (**Figure 5**) with the underlying pattern (see also **Figure S6** for quantification). These observations are consistent with the plot of cluster size distribution (**Figure 6C**). Like TCR, no centralization of ZAP-70 was detected (**Figure 6D**).

4. DISCUSSION

Here we presented single cell experiments to explore the link between formation of sub-micron scale TCR clusters and the response at both the local cluster-scale and the global cell-scale. In agreement with our previous work (30, 37), where a passive polymer (PLL-PEG, poly L lysine-polyethylene glycol), rather than a SLB covered the area between the anti-CD3 dots, we

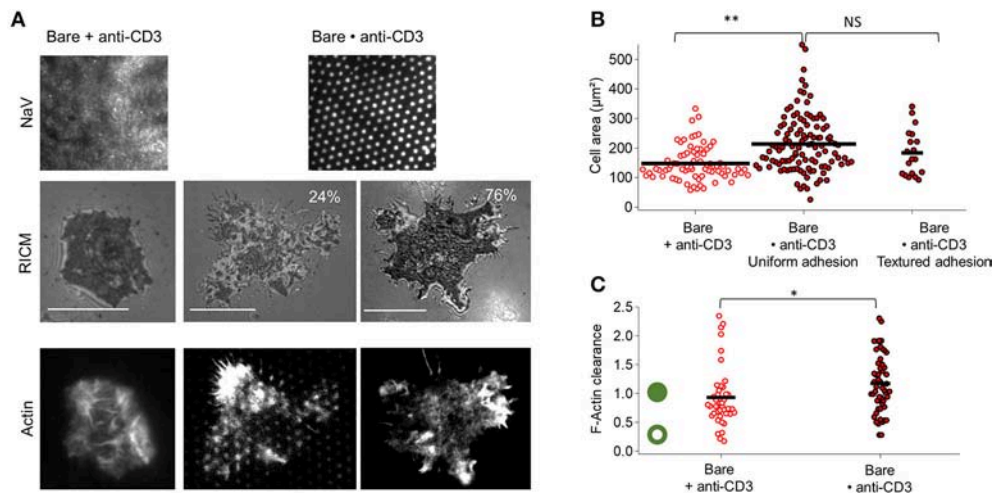


FIGURE 2 | Impact of anti-CD3 clustering on cell adhesion and actin organization. Cells were allowed to interact with homogeneous and patterned substrates, and were fixed and labeled with fluorescent phalloidin. **(A)** T-cell adhesion and actin organization. Top to bottom: epi-fluorescent images of the underlying protein (NaV), RICM images, and TIRFM images of the actin-labeled cells. For patterned substrates, two types of adhesion are shown: 'patterned' adhesion seen in about quarter of the cases and 'uniform' adhesion seen in the rest of the cases. Scale bar = $10\mu\text{m}$. **(B)** Scatter-dot plot of cell spreading area measured from RICM images ($N = 4$, $n = 75$ for homogeneous and $N = 8$, $n = 109$ for patterned). **(C)** Scatter-dot plot of the F-actin clearance calculated from TIRFM images ($N = 2$, $n = 41$ for homogeneous and $N = 5$, $n = 52$ for patterned). Green ring/disc schematizes actin architecture for corresponding clearance parameter. Bar is at mean value. N is number of samples and n is total number of cells.

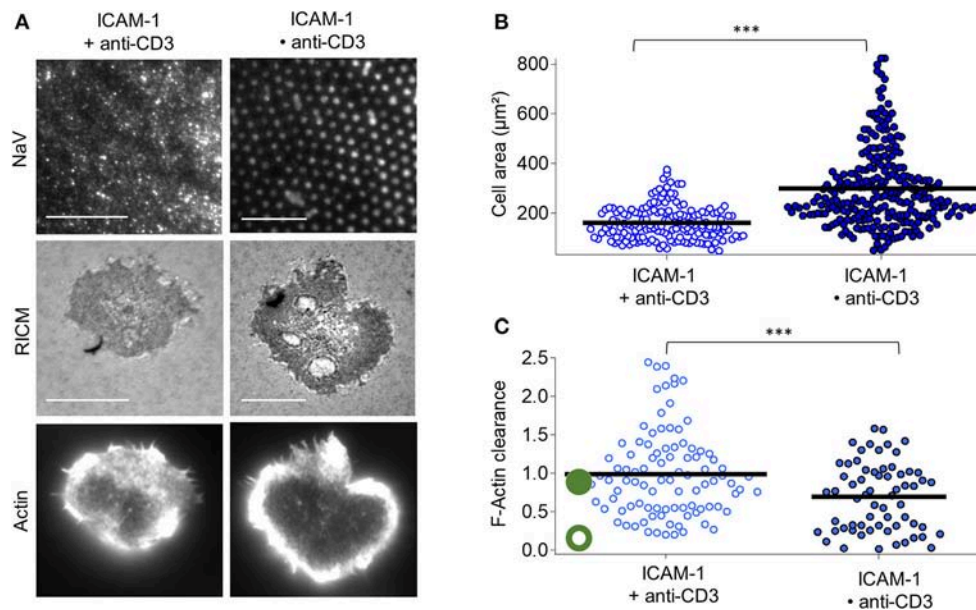


FIGURE 3 | Impact of anti-CD3 clustering on cell adhesion and actin organization in presence of ICAM-1. Cells were allowed to interact with homogeneous and patterned substrates, and were fixed and labeled with fluorescent phalloidin. **(A)** T-cell adhesion and actin organization. Top to bottom: epi-fluorescent images of the underlying protein (NaV), RICM, and TIRFM images of the actin-labeled cells. Scale bar = $10\mu\text{m}$. **(B)** Scatter-dot plot of cell spreading area measured from RICM images ($N = 7$, $n = 164$ for homogeneous and $N = 12$, $n = 271$ for patterned). **(C)** Scatter-dot plot of the F-actin clearance calculated from TIRFM images ($N = 4$, $n = 97$ for homogeneous and $N = 4$, $n = 64$ for patterned). Bar is mean value.

showed that when ligands of TCR-complex are immobilized to form of dots or clusters, the TCR themselves form corresponding clusters. The importance of micro-clusters of TCR (4, 7, 8) is now well established and traditionally, it was considered that a SLB

containing mobile ligands is necessary to generate micro-clusters on artificial APCs (4). However, unlike in a continuous SLB, the patterned substrates do not allow the centralization of the TCR, the micro-clusters are arrested on top of the pattern, somewhat

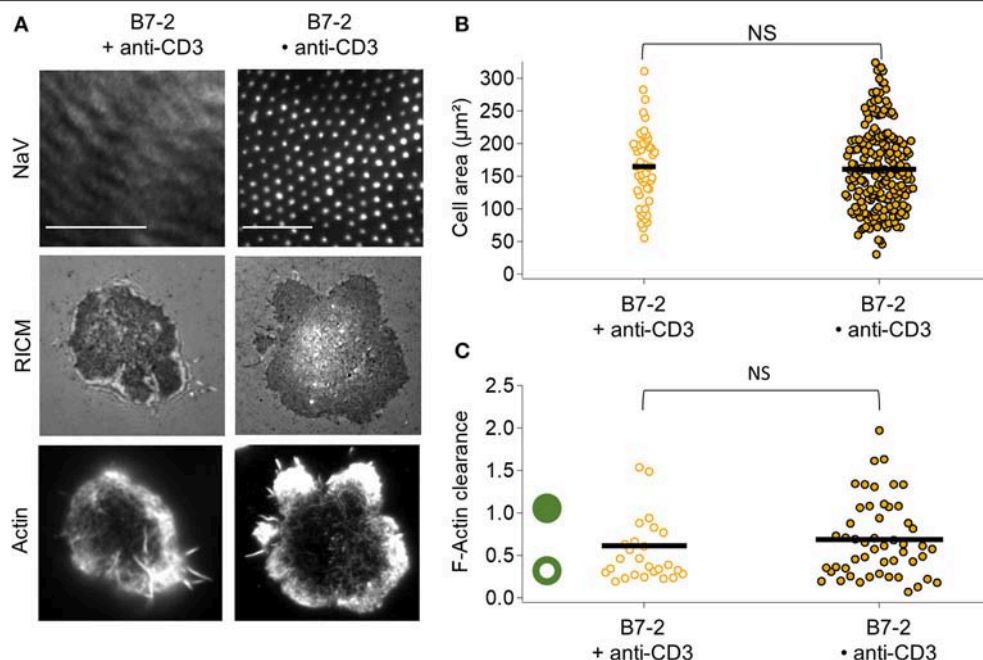


FIGURE 4 | Impact of anti-CD3 clustering on cell adhesion and actin organization in presence of B7-2. Cells were allowed to interact with homogeneous and patterned substrates, and were fixed and labeled with fluorescent phalloidin. **(A)** T-cell adhesion and actin organization. Top to bottom: epi-fluorescent images of the underlying protein (NaV), RICM, and TIRFM images of the labeled cells. Scale bar = $10\mu\text{m}$. **(B)** Scatter-dot plot of cell spreading area measured from RICM images ($N = 2$, $n = 50$ for homogeneous and $N = 11$, $n = 203$ for patterned). **(C)** Scatter-dot plot of the F-actin clearance calculated from TIRFM images ($N = 2$, $n = 26$ for homogeneous and $N = 4$, $n = 51$ for patterned). Bar is median value. NS signifies $P \geq 0.05$.

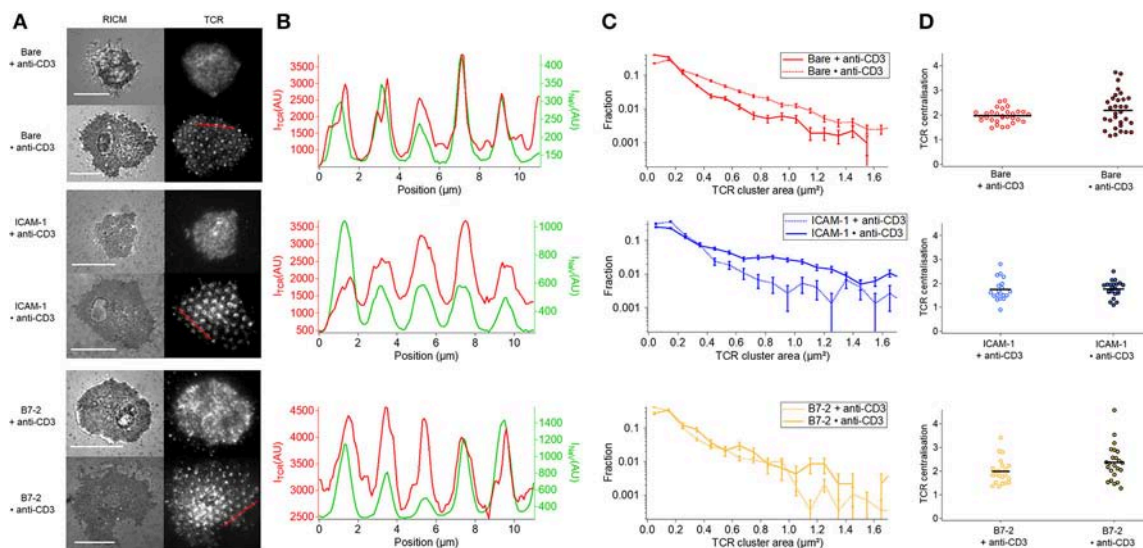
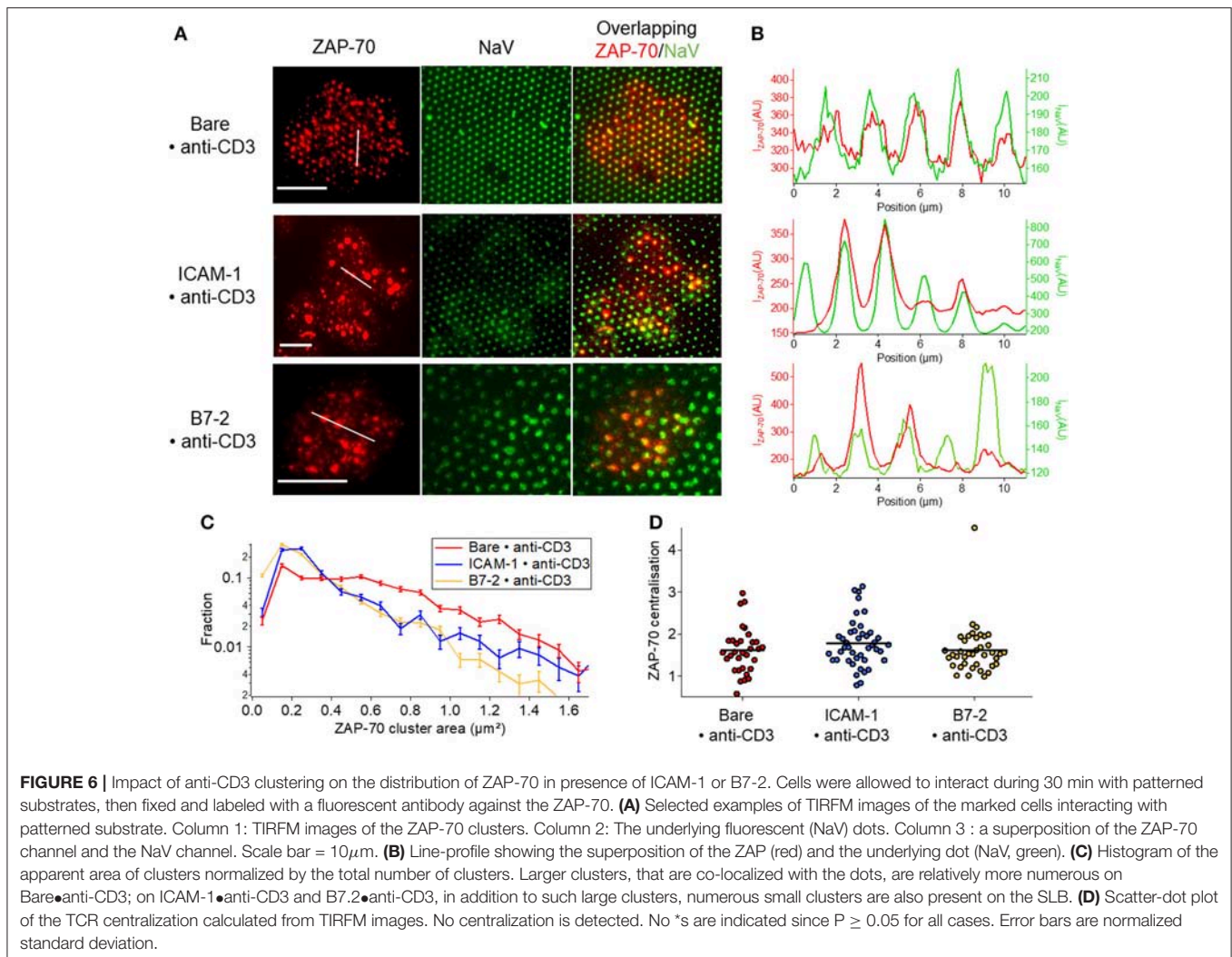


FIGURE 5 | Impact of anti-CD3 clustering on the distribution of T cell receptor (TCR) in absence or presence of ICAM-1 or B7-2. Cells were allowed to interact during 30 min with the substrate, and were fixed and labeled with a fluorescent antibody against TCR. **(A)** Selected examples of RICM and TIRFM images of the TCR-labeled cells interacting with homogeneous (top) or patterned (bottom) substrates. Scale bar = $10\mu\text{m}$. **(B)** Line-profile showing the superposition of TCR (red) and underlying dots (NaV, green), see A for the position of the line. **(C)** Histograms of the apparent area of clusters normalized by the total number of clusters. For each case, the data presented corresponds to 1 or 2 experiments, number of cells vary from 7 to 17, and the number of analyzed clusters from about 700 to 4,000. In each case, larger clusters are more numerous on the patterned substrates, compared to the homogeneous counterpart. **(D)** Scatter-dot plot of the TCR centralization calculated from TIRFM images. No centralization is detected. No *s are indicated since $P \geq 0.05$ for all cases. Error bars are normalized standard deviation.



reminiscent of their confinement on corrallated SLBs (20, 24). The centralization of TCR occurs when TCR molecules are coupled to the retrograde actin flow which draws them backwards toward the centre of the cell (20, 46). Here, unligated TCR molecules diffuse freely till they meet their immobilized ligands and then they themselves get immobilized according to the pattern of the underlying ligands. The lack of centralization here shows that unligated TCR does not couple to any retrograde actin flow that may exist.

The significance of micro-clusters have long been debated in literature, and the consensus is converging toward the view that they act as platforms from which long phosphatases are excluded, thus allowing phosphorylation of signaling molecules (47). Recently, elegant use of micro-nano patterning that presented or not a surface topography, and therefore presumably induced or not a corresponding texturing of the membrane, strengthened this view (48). In our experiments however, the connection between cell spreading, as a marker of activation, and membrane topography as detected by RISM, is not clear. Strikingly, here we show that cell spreading and actin organization is only weakly

impacted by TCR clustering alone, it is the presence of ICAM-1 (but not B7.2) that renders clustering important (Tables S1, S2, Figures S7A,B).

In all patterning studies, in addition to the activating molecules, the means of passivation out of the active zones becomes important (49), as emphasized by us previously (30). In Dillard et al. (30), the surface density of the passivating polymer (PLL-PEG) was varied and it was shown that this has a major impact on cell spreading but only a minor impact on local TCR or ZAP-70 clustering. In the current SLB system too this effect is evident—if the SLB is additionally doped with PEG-carrying lipids, the cells spread less and they do so more slowly. Interestingly, the local membrane topography and gathering of TCR are not impacted (Figure S8).

Here, by using biotinylated lipids and biotinylated BSA interchangeably, we could compare each pattern type with its equivalent homogeneous SLB substrate. In the range of density probed here, the additional presence of ICAM-1 in the homogeneous case did not affect cell spreading (compare bare+anti-CD3 and ICAM+anti-CD3). ICAM-1 however

dramatically increased the adhesion area in the patterned case. Cell spread more on ICAM•anti-CD3 both with respect to bare•anti-CD3 as well as ICAM+anti-CD3. This observation points to a possible crucial role for ICAM-1 in amplifying the effect of TCR-clustering. In fact, it has been reported that TCR micro-clusters form “mini-synapses” where the central core is surrounded by a ring of LFA-1 (50). One possible role of this ring is to compactify the clusters to render them denser, consistent with reports that only TCR-dense clusters are signaling-competent (51). Interestingly pre-labeling of the TCR with an antibody (anti-V β 8) in solution prior to spreading on bare•anti-CD3 leads to highly augmented spreading, perhaps because the antibody promotes cross-linking and cluster compaction (**Figure S9**). Experiments where the ICAM-1 is presented as dots and with anti-CD3 on the SLB shows that clustering of ICAM-1 has no impact (**Figure S5**).

However, CD28 co-stimulation of the T cell has a very different impact, as could be expected (52–54). Both on B7.2 + anti-CD3 and B7.2•anti-CD3, the cells spread less as compared to all other cases reported. This may be related to previous reports which showed that CD28 inhibits cell spreading by acting on LFA-1 related down-stream signaling (55). Here, a similar effect seems to be operational even though LFA-1 ligands are not present. Importantly, there was also no difference between B7.2 + anti-CD3 and B7.2•anti-CD3, showing that co-stimulation of CD28 has no effect on amplification of TCR-clustering induced activation. Very interestingly, even though cell spreading is limited, the actin forms a ring, contrary to expectations based on other studies where actin ring was associated with enhanced spreading (12).

Globally, considering all the substrates together (**Figure S7**), the enhancement in activation (as quantified by cell spreading and corroborated by actin ring formation) on ICAM1•anti-CD3 as compared to all the other substrates, cannot be explained by topographical differences and CD45 expulsion alone³. Soliciting LFA-1 but not CD28 triggers enhanced spreading even though the actin organization is similar in both cases and retrograde flow is present (data not shown). The friction model of spreading (5), links the actin retrograde flow and its coupling to ligand/receptor kinetics to cell spreading area. Presence of retrograde flow but diminished spreading would indicate a lack of transfer of

traction to the substrate in case of CD28, leading us to infer that the link between the TCR-complex (*via* CD3) and actin is enhanced by activation of LFA-1 and weakened by ligation of CD28. Thus while co-stimulation by CD28 and engagement of LFA-1 are used to activate/adhere T cells, the impact on spreading is complementary and the two together may regulate the engagement of TCR with actin, a step crucial for later centralization of the TCR into cSMAC.

5. CONCLUSION

Using our engineered substrates, we have evidenced the role of integrins in enhancing the impact of TCR clustering on cell spreading and actin organization, and shown that the co-receptor CD28 has no such role in amplification of the effect of TCR-clustering. The work presented here underlines the potential of nano-patterned substrates to decipher fundamental questions in T cell biology. Engineering the interface, combined with genetic engineering of the cell, can become a powerful and indispensable tool in immunobiology and be adapted for improved translational devices in immunotherapy.

AUTHOR CONTRIBUTIONS

EB did experiments and analysis. KS and LL conceived and directed the project and participated in data analysis. EB, JN, LL, and KS interpreted the results. KS and EB wrote the manuscript.

FUNDING

This work was partially funded by European Research Council *via* grant no. 307104 FP/2007-2013/ERC-Stg SYNINTER. JAN laboratory is supported by the Fondation pour la Recherche Médicale (Equipe FRM DEQ20180339209).

ACKNOWLEDGMENTS

We thank Rajat Varma for sharing analysis code for TCR analysis, Martine Biarnes and Laurence Borge for help with cell culture, and Igor Ozerov for help with sputtering. Nanofabrication was done at PLANETE clean-room facility of CINaM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02085/full#supplementary-material>

³The height difference between dots and SLB for Bare•anti-CD3 is about 27 nm dots being taller, for ICAM-1•anti-CD3 is about 15 nm, dots being shorter and for B7.2•anti-CD3 again 15 nm but with dots being taller.

REFERENCES

1. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. Immunological synapse: a molecular machine controlling T cell activation. *Science* (1999) 285:221–7.
2. Monks CRF, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) 395:82–6.
3. Cretel E, Touchard D, Bongrand P, Pierres A. A new method for rapid detection of T lymphocyte decision to proliferate after encountering activating surfaces. *J Immunol Methods* (2011) 364:33–9. doi: 10.1016/j.jim.2010.10.007
4. Kaizuka Y, Douglass AD, Varma R, Dustin ML, Vale RD. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc Natl Acad Sci USA*. (2007) 104:2029620–301. doi: 10.1073/pnas.0710258105

5. Dillard P, Varma R, Sengupta K, Limozin L. Ligand-mediated friction determines morphodynamics of spreading T cells. *Biophys J*. (2014) 107:2629–38. doi: 10.1016/j.bpj.2014.10.044
6. Crites TJ, Padhan K, Muller J, Krogsgaard M, Gudla PR, Lockett SJ, et al. TCR microclusters pre-exist and contain molecules necessary for TCR signal transduction. *J Immunol*. (2014) 193:56–67. doi: 10.4049/jimmunol.1400315
7. Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol*. (2005) 6:1253–62. doi: 10.1038/ni1272
8. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* (2006) 25:117–27. doi: 10.1016/j.immuni.2006.04.010
9. Davis DM. Intrigue at the immune synapse. *Sci Am*. (2006) 294:48–55. doi: 10.1038/scientificamerican0206-48
10. Carbone CB, Kern N, Fernandes RA, Hui E, Su X, Garcia KC, et al. *In vitro* reconstitution of T cell receptor-mediated segregation of the CD45 phosphatase. *Proc Natl Acad Sci USA*. (2017) 114:E9338–45. doi: 10.1073/pnas.1710358114
11. Chang VT, Fernandes RA, Ganzinger KA, Lee SF, Siebold C, McColl J, et al. Initiation of T cell signaling by CD45 segregation at close contacts. *Nat Immunol*. (2016) 17:574–82. doi: 10.1038/ni.3392
12. Hammer JA, Burkhardt JK. Controversy and consensus regarding myosin II function at the immunological synapse. *Curr Opin Immunol*. (2013) 25:300–6. doi: 10.1016/j.coi.2013.03.010
13. Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol*. (2002) 158:1263–75. doi: 10.1083/jcb.200203043
14. Hong J, Murugesan S, Betzig E, Hammer JA. Contractile actomyosin arcs promote the activation of primary mouse T cells in a ligand-dependent manner. *PLoS ONE* (2017) 12:e0183174. doi: 10.1371/journal.pone.0183174
15. Yi J, Wu XS, Crites T, Hammer JA. Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells. *Mol Biol Cell*. (2012) 25:834–52. doi: 10.1091/mbc.E11-08-0731
16. Murugesan S, Hong J, Yi J, Li D, Beach JR, Shao L, et al. Formin-generated actomyosin arcs propel T cell receptor microcluster movement at the immune synapse. *J Cell Biol*. (2016) 3:383–99. doi: 10.1083/jcb.201603080
17. Beemiller P, Krummel MF. Regulation of T-cell receptor signaling by the actin cytoskeleton and poroelastic cytoplasm. *Immunol Rev*. (2013) 256:148–59. doi: 10.1111/imr.12120
18. Swaminathan V, Kalappurakkal JM, Mehta SB, Nordenfelt P, Moore TI, Koga N, et al. Actin retrograde flow actively aligns and orients ligand-engaged integrins in focal adhesions. *Proc Natl Acad Sci USA*. (2017) 114:10648–53. doi: 10.1073/pnas.1701136114
19. Burkhardt JK, Carrizosa E, Shaffer MH. The actin cytoskeleton in T cell activation. *Annu Rev Immunol*. (2008) 26:233–59. doi: 10.1146/annurev.immunol.26.021607.090347
20. Hartman NC, Nyeb JA, Groves JT. Cluster size regulates protein sorting in the immunological synapse. *Proc Natl Acad Sci USA*. (2009) 106:12729–34. doi: 10.1073/pnas.0902621106
21. Choudhuri K, Wiseman D, Brown MH, Gould K, van der Merwe PA. T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature* (2005) 436:578–82. doi: 10.1038/nature03843
22. James JR, Vale RD. Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature* (2012) 487:64–9. doi: 10.1038/nature11220
23. Dustin ML, Groves JT. Receptor signaling clusters in the immune synapse. *Annu Rev Biophys*. (2012) 41:543–56. doi: 10.1146/annurev-biophys-042910-155238
24. Mossman KD, Campi G, Groves JT, Dustin ML. Altered TCR Signaling from geometrically repatterned immunological synapses. *Science* (2005) 310:1191–3. doi: 10.1126/science.1119238
25. Doh J, Irvine DJ. Immunological synapse arrays: patterned protein surfaces that modulate immunological synapse structure formation in T cells. *Proc Natl Acad Sci USA*. (2006) 103:5700–5. doi: 10.1073/pnas.0509404103
26. Schwarzenbacher M, Kaltenbrunner M, Brameshuber M, Hesch C, Paster W, Weghuber J, et al. Micropatterning for quantitative analysis of protein-protein interactions in living cells. *Nat Meth*. (2008) 5:1053–60. doi: 10.1038/nmeth.1268
27. Shen K, Milone M, Dustin ML, Kam LC. Nanoengineering of immune cell function. *Mater Res Soc Symp Proc*. (2009) 1209:1209-YY03-01. doi: 10.1557/PROC-1209-YY03-01
28. Hivroz C, Saitakis M. Biophysical aspects of T lymphocyte activation at the immune synapse. *Front Immunol*. (2016) 7:1–12. doi: 10.3389/fimmu.2016.00046
29. Tabdianov E, Gondarenko S, Kumari S, Liapis A, Dustin ML, Sheetz MP, et al. Micropatterning of TCR and LFA-1 ligands reveals complementary effects on cytoskeleton mechanics in T cells. *Integr Biol*. (2015) 7:1272–84. doi: 10.1039/c5ib00032g
30. Dillard P, Pi F, Lellouch AC, Limozin L, Sengupta K. Nano-clustering of ligands on surrogate antigen presenting cells modulates T cell membrane adhesion and organization. *Integr Biol*. (2016) 8:287–301. doi: 10.1039/C5IB00293A
31. Deeg J, Axmann M, Matic J, Liapis A, Depoil D, Afrose J, et al. T cell activation is determined by the number of presented antigens. *Nano Lett*. (2013) 13:5090–7. doi: 10.1021/nl403266t
32. Matic J, Deeg J, Scheffold A, Goldstein I, Spatz JP. Fine tuning and efficient T cell activation with stimulatory aCD3 nanoarrays. *Nano Lett*. (2013) 13:5090–7. doi: 10.1021/nl4022623
33. Lu X, Gibbs JS, Hickman HD, David A, Dolan BP, Jin Y, et al. Endogenous viral antigen processing generates peptide-specific MHC class I cell-surface clusters. *Proc Natl Acad Sci USA*. (2012) 109:15407–12. doi: 10.1073/pnas.1208696109
34. Ferez M, Castro M, Alarcon B, van Santen HM. Cognate peptide-MHC complexes are expressed as tightly apposed nanoclusters in virus-infected cells to allow TCR crosslinking. *J Immunol*. (2013) 192:52–8. doi: 10.4049/jimmunol.1301224
35. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annu Rev Immunol*. (2009) 27:591–619. doi: 10.1146/annurev.immunol.021908.132706
36. Bashour KT, Gondarenko A, Chen H, Shen K, Liu X, Huse M, et al. CD28 and CD3 have complementary roles in T-cell traction forces. *Proc Natl Acad Sci USA*. (2014) 111:2241–6. doi: 10.1073/pnas.1315606111
37. Pi F, Dillard P, Limozin L, Charrier A, Sengupta K. Nanometric protein-patch arrays on glass and polydimethylsiloxane for cell adhesion studies. *Nano Lett*. (2013) 13:3372–8. doi: 10.1021/nl401696m
38. Pi F, Dillard P, Alameddine R, Benard E, Ozerov I, Charrier A, et al. Functional organized organic nano-dots : a versatile platform for manipulating and imaging whole cells on surfaces. *Nano Lett*. (2015) 8:5178–84. doi: 10.1021/acs.nanolett.5b01400
39. Benard E, Pi F, Ozerov I, Charrier A, Sengupta K. Ligand nano-cluster arrays in a supported lipid bilayer. *J Vis Exp*. (2017) 122:e55060. doi: 10.3791/55060
40. Fenz SF, Merkel R, Sengupta K. Diffusion and intermembrane distance: case study of avidin and E-cadherin mediated adhesion. *Langmuir* (2008) 25:1074–85. doi: 10.1021/la803227s
41. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Meth*. (2012) 9:676–82. doi: 10.1038/nmeth.2019
42. Dietrich C, Merkel R, Tampe R. Diffusion measurement of fluorescence-labeled amphiphilic molecules with a standard fluorescence microscope. *Biophys J*. (1997) 72:676–82.
43. Sengupta K, Aranda-Espinoza H, Smith L, Janmey P, Hammer D. Spreading of neutrophils: from activation to migration. *Biophys J*. (2006) 91:4638–48. doi: 10.1529/biophysj.105.080382
44. Bunnell SC. Multiple microclusters: diverse compartments within the immune synapse, Vol. 340. Berlin/Heidelberg: Immunological synapse Springer (2010). Available online at: http://link.springer.com/10.1007/978-3-642-03858-7_7
45. Kumari S, Vardhana S, Cammer M, Curado S, Santos L, Sheetz MP, et al. T Lymphocyte Myosin IIA is Required for Maturation of the Immunological Synapse. *Front Immunol*. (2012) 3:230. doi: 10.3389/fimmu.2012.00230
46. Smoligovets AA, Smith AW, Wu HJ, Petit RS, Groves JT. Characterization of dynamic actin associations with T-cell receptor microclusters in primary T cells. *J Cell Sci*. (2012) 125:735–42. doi: 10.1242/jcs.092825

47. Su X, Ditlev JA, Hui E, Xing W, Banjade S, Okrut J, et al. Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* (2016) 352:595–9. doi: 10.1126/science.aad9964
48. Cai H, Muller J, Depoil D, Mayya V, Sheetz MP, Dustin ML, et al. Full control of ligand positioning reveals spatial thresholds for T cell receptor triggering. *Nat Nanotech.* 2018 13:610–7. doi: 10.1038/s41565-018-0113-3
49. Spatz JP. Nano- and micropatterning by organic-inorganic templating of hierarchical self-assembled structures. *Angew Chem Int Ed Engl.* (2002) 41:3359–62. doi: 10.1002/1521-3773(20020916)41:18<3359::AID-ANIE3359>3.0.CO;2-Y
50. Hashimoto-Tane A, Saito T. Dynamic regulation of TCR-microclusters and the microsynapse for T cell activation. *Front Immunol.* (2016) 7:255. doi: 10.3389/fimmu.2016.00255
51. Pigeon SV, Tabarin T, Yamamoto Y, Ma Y, Bridgeman JS, Cohnen A, et al. Functional role of T-cell receptor nanoclusters in signal initiation and antigen discrimination. *Proc Natl Acad Sci USA.* (2016) 113:E5454–63. doi: 10.1073/pnas.1607436113
52. Bachmann MF, McKall-Faienza K, Schmits R, Bouchard D, Beach J, Speiser DE, et al. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* (1997) 7:549–57.
53. Nunés JA, Truneh A, Olive D, Cantrell DA. Signal transduction by CD28 costimulatory receptor on T cells B7-1 and B7-2 regulation of tyrosine kinase adaptor molecules. *J Biol Chem.* (1996) 3:1591–9.
54. Yokosuka T, Saito T. Dynamic regulation of T-cell costimulation through TCR-CD28 microclusters. *Immunol Rev.* (2009) 229:27–40. doi: 10.1111/j.1600-065X.2009.00779.x
55. Strazza M, Azoulay-Alfaguter I, Dun B, Baquero-Buitrago J, Mor A. CD28 inhibits T cell adhesion by recruiting CAPRI to the plasma membrane. *J Immunol.* (2015) 194:2871–7. doi: 10.4049/jimmunol.1401492

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Membrane Ultrastructure and T Cell Activation

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OPEN ACCESS

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 13 June 2018

Accepted: 31 August 2018

Published: 25 September 2018

Citation:

Pettmann J, Santos AM, Dushek O
and Davis SJ (2018) Membrane
Ultrastructure and T Cell Activation.
Front. Immunol. 9:2152.
doi: 10.3389/fimmu.2018.02152

The immune system serves as a crucial line of defense from infection and cancer, while also contributing to tissue homeostasis. Communication between immune cells is mediated by small soluble factors called cytokines, and also by direct cellular interactions. Cell-cell interactions are particularly important for T cell activation. T cells direct the adaptive immune response and therefore need to distinguish between self and foreign antigens. Even though decades have passed since the discovery of T cells, exactly why and how they are able to recognize and discriminate between antigens is still not fully understood. Early imaging of T cells was very successful in capturing the early stages of conjugate formation of T cells with antigen-presenting cells upon recognition of peptide-loaded major histocompatibility complexes by the T cell receptor (TCR). These studies lead to the discovery of a “supramolecular activation cluster” now known as the immunological synapse, followed by the identification of microclusters of TCRs formed upon receptor triggering, that eventually coalesce at the center of the synapse. New developments in light microscopy have since allowed attention to turn to the very earliest stages of T cell activation, and to resting cells, at high resolution. This includes single-molecule localization microscopy, which has been applied to the question of whether TCRs are pre-clustered on resting T cells, and lattice light-sheet microscopy that has enabled imaging of whole cells interacting with antigen-presenting cells. The utilization of lattice light-sheet microscopy has yielded important insights into structures called microvilli, which are small membrane protrusions on T cells that seem likely to have a large impact on T cell recognition and activation. Here we consider how imaging has shaped our thinking about T cell activation. We summarize recent findings obtained by applying more advanced microscopy techniques and discuss some of the limitations of these methods.

Keywords: T cell signaling, microvilli, invadosome-like protrusions, membrane topology, microscopy, microclusters, immunological synapse

INTRODUCTION

T cells are the central players in adaptive immunity. They control and orchestrate the immune response but are also involved in direct cytotoxicity toward tumors or virus-infected cells. A unique and crucial feature of T cells is their ability to distinguish between self and foreign peptides presented by major histocompatibility complex (MHC) proteins with high sensitivity and specificity. Antigen-presenting cells (APCs)

process and present peptide-loaded MHC (pMHC) which is subsequently recognized by the antigen receptor expressed by T cells, i.e., the T cell receptor (TCR). Exactly how this binding event leads to receptor triggering, while self-peptides are ignored, is not well understood yet. Interestingly, T cell activation is known to be accompanied by profound changes in the spatial organization of TCRs and downstream signaling molecules.

T cells have been studied using numerous functional, genomic and imaging-based approaches. Microscopy has yielded especially valuable insights into the dynamics of T cell behavior and signaling *in vitro* and *in vivo*. Imaging membrane proteins on T cells during cellular activation led to the discovery of putative signaling assemblies, first in the form of the immune synapse, and later as precursor accumulations of TCRs, called microclusters (1–3). Recent technical developments in microscopy have allowed imaging of T cells in unprecedented temporal and spatial resolution. These developments have included single-molecule localization microscopy (SMLM), enabling super-resolution imaging, and lattice light-sheet (LLS) microscopy for 3D live, high-resolution cell imaging. Using these and other technologies, attention is now beginning to turn to the 3D topology of the cell membrane. Structures called microvilli or invadosome-like protrusions (ILPs) have been implicated in antigen probing and in the receptor triggering process (4–7). These structures are generally thought to be distinct from the accumulation of TCRs and other molecules in microclusters, observed on planar surfaces such as glass or supported lipid bilayers [SLBs; (1–3)].

In this review, we provide an overview of the common microscopy techniques used to image T cells (see **Box 1**) and discuss the types of membrane structures that have been observed in a variety of contexts. We consider the limitations in the imaging approaches used to characterize microclusters, ILPs and microvilli, and suggest that there could be substantial overlap in the cellular process resulting in the appearance of these structures in the course of imaging experiments.

THE IMMUNOLOGICAL SYNAPSE

During the late 1990s, enabled by new developments in confocal fluorescence imaging, Kupfer and Dustin and their colleagues described a large accumulation of TCRs at the center of contacts made by activated T cells with APCs or SLBs for the first time. The mature immunological synapse (IS), as it came to be called, consists of three subdomains: a central supramolecular activation cluster (cSMAC) containing TCRs, which is surrounded by a ring of ICAM-1 (Intercellular Adhesion Molecule 1) binding integrin LFA-1 (Lymphocyte function-associated antigen 1) molecules, called the peripheral SMAC (pSMAC), and a second outer ring called the distal SMAC (dSMAC), where the phosphatase CD45 accumulates. The exclusion of the large phosphatase CD45 from the TCR and its associated kinases has been proposed as a mechanism of T cell activation, referred to as the kinetic-segregation (KS) model (9). It might be expected that phosphatase exclusion to the dSMAC implicates the IS and KS-based signaling in T cell activation *per se*. However,

early markers of signaling such as calcium fluxes precede the formation of the IS by minutes and the cSMAC has very little phosphotyrosine or downstream signaling effectors associated with it (3, 10). Furthermore, a synapse is not always observed at T cell/APC interfaces, depending on the APC used and the strength of the antigenic stimulus (11). Rather than being the driver of signaling during the earliest stages of T cell activation, the IS is more likely involved in processes like TCR downregulation (10, 12), via endocytosis (13) and the secretion of TCR-containing exosomes (14). Moreover, the IS is now considered to have a major role in the delivery of effector functions (15, 16) and co-stimulation (17).

MICROCLUSTERS

Total internal reflection fluorescence (TIRF)-based imaging (see **Box 1**) of the earliest stages of T cell activation prior to full IS formation has revealed the formation of TCR ‘microclusters’ (see **Figure 1A**). These submicron-scale structures form seconds after the T cell contacts an antigen-presenting surface. They are enriched in signaling molecules such as Lck, ZAP70, LAT, and SLP-76, and their formation precedes calcium fluxing (1–3, 18). After a cell has spread on SLBs that contain pMHC + ICAM-1, microclusters are observed to start moving toward the center of the contact to form the cSMAC (2, 3). Microclusters also form when T cells contact glass coated with anti-CD3 antibodies (1), but only mobile ligands (e.g., on SLBs or expressed by APCs) allow the movement of microclusters and the formation of a cSMAC. Within the cSMAC, the TCRs are mostly dissociated from downstream signaling molecules (3, 10).

Rather than arising in the IS at the cSMAC, continuous signaling appears to correlate with the formation of new microclusters in the p- and dSMAC (10), prompting the suggestion that these structures sustain T cell activation or are even the primary signaling units (19). However, other evidence from imaging cells during the earliest stages of activation is inconsistent with this view (see also Membrane Protrusions and Signaling). Inhibition of pMHC binding by a competitive antibody was shown to block the formation of new microclusters in the periphery, and also calcium fluxes, whereas existing microclusters and the cSMAC are mostly resistant to the treatment (10). A similar result can be obtained by using the actin polymerization inhibitor Latrunculin A (10). Actin is important for the formation of microclusters and, recently, actin foci that colocalize with microclusters have been identified (20). These Wiskott-Aldrich Syndrome protein (WASP)-dependent actin foci are synthesized *de novo* upon receptor triggering and contribute to downstream signaling. Interestingly, close inspection of the data of Yokosuka et al. also reveals that phosphorylated (*i.e.* activated) ZAP70 assembles into microclusters in the dSMAC early during T cell activation, without the detectable co-accumulation of TCRs (3, 10). These observations suggest that microclusters, like the synapse, might be the product of signaling rather than the cause of it.

BOX 1 | Techniques used to study microclusters and membrane protrusions.

The properties of the structures observed on the surface of T cells are to some extent dependent on the methodology used to study them. The substrate stimulating the cells can also have a significant influence on what structures are observed. However, there are also technical and physical limitations to resolving the full complexity, in particular the topology of the membrane. Here we give an overview of commonly used microscopy techniques used to study those phenomena.

Confocal microscopy

Conventional confocal microscopy allows high-contrast imaging at a diffraction-limited resolution of about 250 nm in the xy-plane. This technique was used when the immunological synapse was initially discovered by the group of Kupfer et al. between T cell-APC conjugates (8). The major drawback for such an application is the axial resolution (z direction) of only about 700 nm. Consequently, high-resolution images can only be obtained when the cells form a horizontal interface, as they would on glass.

Total internal reflection fluorescence (TIRF) microscopy

This diffraction-limited technique provides very high sensitivity, even allowing the tracking of single fluorescent molecules and their movement. By illuminating the sample from an angle, causing reflection of the light, only a 100–200 nm section of the sample next to the glass is illuminated, making this method highly suitable for imaging cell/transparent substrate interfaces. The original studies describing TCR microclusters utilized this method in combination with glass-supported bilayers for high-resolution live cell imaging (2, 3). Many subsequent studies used the same combination.

Variable angle total internal reflection fluorescence (VA-TIRF) microscopy

VA-TIRF is an adaptation of TIRF microscopy in which the angle of illumination is changed, allowing the mapping of the height of structures close to the glass. Jung et al. use this technique in combination with SMLM (see below) to map TCRs and other molecules in relation to the tips of microvilli (5).

Single-molecule localization microscopy (SMLM)

This super resolution microscopy technique allows the localization of fluorescently tagged molecules with a precision of less than 50 nm. Common implementations are photoactivated localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM). A combination of these methods was used by Razvag et al. to investigate the segregation of CD45 from the TCR (7). Currently, the number of colors in routine applications is limited to two. Furthermore, the acquisition can take a significant amount of time, making it mostly unsuitable for live cell imaging.

Lattice light-sheet (LLS) microscopy

Lattice light-sheet microscopy, as utilized by Cai et al. in their study of microvilli dynamics on T cells (6), is a microscopy technique that combines light sheet microscopy with structured illumination microscopy (SIM), a form of super-resolution microscopy. By producing a “sheet” of light for illumination, rather than relying on exclusion of out-of-focus light as in a confocal microscope, this method allows fast and gentle imaging. Compared to confocal microscopy, particularly the z resolution is significantly better, which allows, in combination with the outstanding scanning rate, the analysis of 3D structures like microvilli and their dynamics on live cells.

MICROVILLI

Lymphocytes and other leukocytes have a complex surface topology that is dominated to a large extent by round, finger-like protrusions termed microvilli (21). Scanning and transmission electron microscopy (SEM and TEM, respectively) has revealed that microvilli are 70–150 nm in diameter and from 100 nm to several μm in length (median length 300–400 nm), on resting T cells (5, 22). Microvilli can also be found on myeloid cells, but the surfaces of monocytes, polymorphonuclear leukocytes (22), and dendritic cells (23) are dominated by “ruffles”. Ruffles vary in size significantly, whereas the size of microvilli is less variable. Majstoravich et al. observed no large differences in the size of microvilli on primary human lymphocytes, primary murine lymphocytes and a pre-B lymphoma cell line, even though the median diameters of these cells vary significantly, suggesting that the function(s) of microvilli might depend on their size, which is therefore tightly regulated (22).

Until recently the microvilli of T cells had not been characterized dynamically and their actual functions are still unclear. Microvilli contain actin filaments and are highly mobile (6, 24). Since lymphocytes and most other cells are covered by a dense glycocalyx (25–27), which creates a barrier that acts against receptor/ligand interactions (28, 29), cells need to exert force to form close contacts, allowing ligand/receptor binding to occur. For T cells, microvilli have been implicated in force-driven penetration of the glycocalyx (4). Consequently, it seems reasonable to expect that the tips of the microvilli are the sites of initial TCR triggering.

Studies of membrane protrusions were previously hindered by the inability to image dynamic 3D structures, such as microvilli, with high resolution on live cells. The techniques used to study the IS and microclusters are generally not very suitable for such experiments (see **Box 1**). TIRF microscopy benefits from, but is also inherently restricted to imaging very close to a coverslip (within <200 nm). Confocal microscopy is generally capable of 3D imaging but suffers from significantly decreased resolution in the z-direction. Furthermore, scanning is too slow to capture a whole cell in high spatial and temporal resolution. Recognizing these limitations, Krummel and Betzig and their colleagues used a high-speed imaging technique with good z-resolution called lattice light-sheet (LLS; see **Box 1**) microscopy for imaging the movement of dynamic structures such as microvilli on live T cells (either in the resting state or forming APC conjugates), while using an adaptation of TIRF microscopy to visualize contacts formed at the tips of the microvilli [termed surface contact mapping (SCM); see **Box 1** and **Figure 1B**; (6)] on SLBs. Microvilli observed using LLS imaging of live T cells interacting with DCs, or with SCM for T cells contacting SLBs, differed significantly in their height [see also Invadosome-like Protrusions]. Nevertheless, both approaches revealed microvilli moving rapidly over the entire interface in search of cognate antigen, surveying the majority of the opposing surface within a minute. Following encounter with cognate antigen, individual microvillar contacts were stabilized. Strikingly, microvillar search and stabilization were not decreased when ZAP70 was inhibited, implying that searching and stabilization are independent of downstream TCR

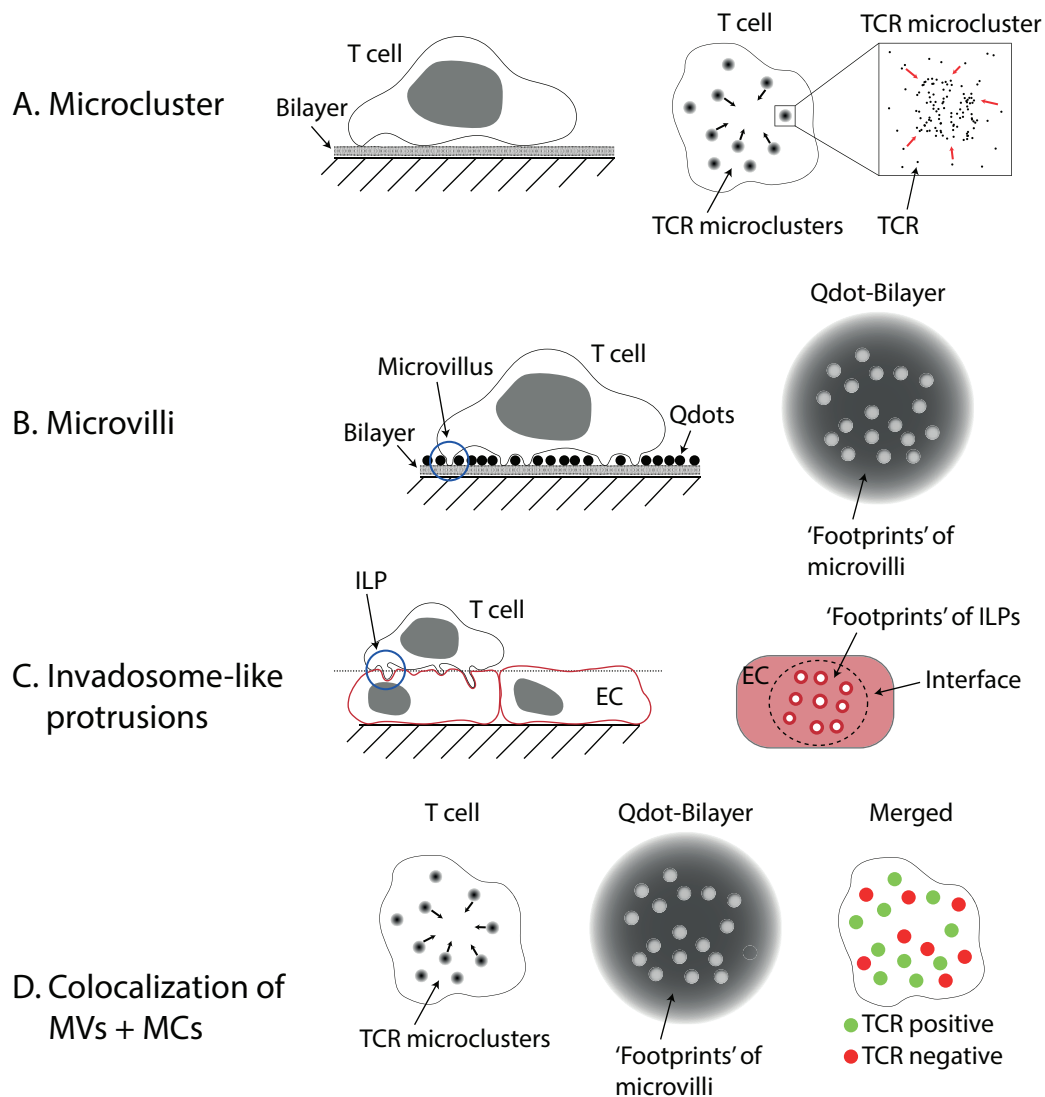


FIGURE 1 | Microvilli and Invadosome-like protrusions. **(A)** Microclusters are accumulations of TCRs found on activated T cells imaged on lipid bilayers or glass. On bilayers, they can move centripetally to form the central supramolecular activation cluster (cSMAC). **(B)** Microvilli have been imaged using different techniques. Here a method called “quantum dot enabled surface contact mapping” is shown. T cells are placed on a bilayer coated with small (<20 nm), fluorescent quantum dots (Qdots). Microvilli can be observed in TIRF mode (see **Box 1**) by imaging the displacement of Qdots (“footprints”). **(C)** Invadosome-like protrusions (ILPs) are observed on T cells placed on a monolayer of endothelial cells (EC). When the membranes of the endothelial cells are imaged, “footprints” of ILPs can be seen as circular areas with decreased membrane dye fluorescence. The dotted line indicates the image plane shown in the right drawing. **(D)** Using a combination of the techniques described in **(A,B)** microclusters (MCs) and microvilli (MVs) can be imaged at the same time. Cai et al. (6) found that microclusters and microvilli colocalized on activated T cells. Notably, not all microvilli showed colocalization, however.

signaling. When they imaged both the footprints of microvilli and the TCR using SCM, they observed strong colocalization (see **Figure 1D**). However, not every single microvillus was linked to the formation of a TCR microcluster (this might be susceptible to the threshold set to define TCR-positive structures), but microvilli lacking microclusters were selectively retained when cells were treated with Latrunculin A. Importantly, the authors found the microclusters that were localized on microvillar tips migrated centripetally, in the manner of “classical” microclusters (2, 3, 18). Together, these data suggest that structures previously observed in TIRF imaging experiments and referred to as

“microclusters” might comprise, at least in some cases, the activation-dependent local accumulation of TCRs at the tips of microvilli.

INVADOSOME-LIKE PROTRUSIONS

Invadosome-like protrusions (ILPs) are structures, initially identified using confocal microscopy, that form during the diapedesis of T cells (30). These membrane protrusions can penetrate deep into endothelial cells forming pores for

subsequent transcellular migration. As shown by Sage et al., they are also involved in probing for antigens. In their study, endothelial monolayers were imaged after addition of T cells, and ‘footprints’ of the T cells pushing into the endothelial cells were observed [see **Figure 1C**; (4)]. Similar to microclusters and microvilli, the ILPs of activated T cells are enriched in TCRs, downstream signaling molecules and phosphotyrosine-containing molecules (4). In a small number of cells, structures resembling the cSMAC were found. Transmission electron microscopy revealed that ILPs have mean diameters of 350 nm in the absence, and 280 nm in the presence of antigen, respectively. Intriguingly, ILP tips are 9-fold more likely to form close contacts, that is sites of less than 20 nm intermembrane spacing very likely to facilitate TCR engagement of pMHC, than other regions of the contact. The authors speculated that microcontacts, small contacts observed to form when cells land on glass, are mechanically “frustrated” ILPs. One of the main differences between glass and cellular systems is the presence of a thick glycocalyx, and it is worth also noting that cells are many orders of magnitude less stiff compared to glass or plastic (31, 32). The stiffness of the substrate used has been shown to modify the response of T cells (33), which would, in principle, be explained by a force-dependent component of T cell triggering (34).

How ILPs might relate to microvilli has not been directly investigated. ILPs have mostly been characterized using endothelial cells as APCs, but they have also been found in B-T cell and DC-T cell conjugates (4), whereas MVs have mostly been studied on SLBs and in DC-T cell conjugates. Microvilli and ILPs share numerous similarities, including the enrichment of downstream signaling molecules, dependence of their formation on actin, and stabilization in the presence of cognate antigen (4–6). Curiously, the antigen-dependent stabilization of ILPs observed by Sage et al. was much more pronounced than that observed by Cai et al. for microvilli, implying that there could be functional differences among these structures. The long-lasting stabilization of ILPs might be specifically required for diapedesis, the process they were initially associated with. Notably, L-selectin, an adhesion receptor important for the initial binding required for diapedesis (tethering), is enriched on the tips of microvilli (5, 35–37). This localization to the tips is likely important, since redistribution of L-selectin using chimeric proteins impairs lymphocyte attachment under flow (35, 37).

Whereas the lengths of ILPs measured using TEM (mean length 430 nm) corresponds well with what has also been reported for microvilli using both TEM and SEM (median 300–400 nm), the diameters of these structures measured by electron microscopy are significantly different [$\sim$ 350 nm for ILPs vs. 70–150 nm for microvilli; (4, 5, 22)]. Cai et al. (6) reported much larger diameters for microvilli (mean $\sim$ 540 nm) using both LLS and SCM microscopy, perhaps due to differences in cell activation status. When Cai et al. investigated the membrane topology of T cells interacting with SLBs, they observed only small variations in membrane/SLB separation across the contact ($\sim$ 50 nm), compared with the much longer microvilli seen in cell-cell conjugates or on resting cells (5, 22, 23). Similar topology was observed by Carbone et al. using scanning angle interference microscopy, in experiments in which giant unilamellar vesicles

formed contacts with SLBs created by model (i.e., FKBP/FRB-based) receptor/ligand pairs in the presence of CD45 (38). We speculate that, to some extent, microvillus length is determined by the depth of the glycocalyx, which can be as much as 500 nm deep in the case of endothelial cells (27). The diameters of these structures might, however, be related to their role in interface scanning and antigen recognition (see Membrane Protrusions and Signaling). A striking observation consistent with this idea is that whereas human embryonic kidney cells do not form microvillar contacts with protein-coated glass surfaces in the manner of lymphocytes, they seem compelled to do so following their expression of a glycocalyx comprised of the membrane-anchored extracellular domain of CD45 (39). Perhaps the first task of these types of structures, therefore, is to punch through the glycocalyx (on both sides of a contact), allowing proper, cognate interactions. It is important to note, however, that in the context of natural killer and cytotoxic T cell interactions with their targets, marked membrane invaginations observed at the contacts are transient and that, in the course of minutes, the interfaces flatten and exhibit wider undulations (40). This suggests that the complex topology of the contacts is only important, if at all, during the earliest stages of interaction.

Based on these studies collectively, we propose that microvilli and ILPs are highly related structures whose assignment to either category depends only on how they are used by different types of cells: for probing antigen presenting cells for the presence of TCR ligands or, more vigorously, to initiate diapedesis. It is possible that the differences observed originate largely from the cell type used (murine/human, CD4⁺/CD8⁺, naïve/effector/memory) and the methods used to observe ILPs (mostly indirectly as membrane invaginations) and MVs (directly using light and electron microscopy). Future comparisons of structures observed using the same methods and cells would yield valuable insight into the variety and functions of membrane protrusions on T cells. Hereafter we use the term membrane protrusions to refer to both microvilli and ILPs.

MEMBRANE PROTRUSIONS AND SIGNALING

A class of super-resolution techniques broadly named single-molecule localization microscopy, is based on the sequential excitation of small subsets of fluorophores, allowing the fluorescence point spread functions of diffraction-limited spots to be used to accurately determine the position of molecules with sub-diffraction resolution (see **Box 1**). Variable angle (VA)-TIRF microscopy (also see **Box 1**), on the other hand, allows measurement of the distance of fluorophores from a surface illuminated under TIRF conditions. Combining SMLM with VA-TIRF, Jung et al. characterized the distribution of the TCR and other molecules on the 3D surface of T cells (5). They observed that the TCR and L-selectin (a microvillus marker) were apparently enriched on resting T cells, i.e., pre-clustered on the tips of microvilli, and that CD44 formed rings around those sites. Latrunculin A completely blocked the formation of microvilli and clustering of the TCR (5).

Yi and Samelson (41) have suggested that membrane protrusions may create a structural scaffold for the formation of microclusters following T cell activation. In this way, they would serve as a physical barrier for the diffusion of molecules, enhancing signaling. The notion that membrane protrusions are dynamic, actin-containing foci also fits with the idea that force is an important contributor to T cell activation (34), and the response of the TCR/pMHC interaction to force seems in some cases to vary with the antigen (42). Lifetimes of agonist bonds are prolonged (due to formation of “catch” bonds) when forces are imposed on the interaction, whilst those of non-agonists are shortened (owing to “slip” bond formation); such effects were proposed to improve antigen discrimination (43, 44). Membrane protrusions might be ideally suited to divining such effects: first, the protrusion penetrates the glycocalyx, forming a close contact where interactions can occur, followed by a pulling force that elicits the catch/slip bond behavior of the interaction. It is unclear, however, why bond half-times would need to be extended in this way rather than through other, more straightforward thermodynamic processes, or how they especially would be selected for in the thymus. Also, it could be expected that adjacent adhesion molecules would have the effect of distributing and reducing local forces on the TCR, limiting such effects. Indeed, it is a strong argument against an important role for forces that adhesion molecules enhance TCR sensitivity rather than diminish it (45). It also needs to be emphasized that although forces have been detected using DNA-based nanoparticle tension sensors when T cells interact with immobilized anti-CD3 or pMHC (46), it is yet to be shown that this applies to T cell/APC contacts.

It was also proposed that membrane protrusions could add an important structural element to the kinetic-segregation model of phosphatase exclusion-based TCR signaling (41). The problem with this proposal is that although CD45 exclusion occurs upon ILP formation (4), the data for microvilli is somewhat equivocal. Chang et al. (39) observed spontaneous segregation of CD45 at microvillar-sized contacts formed by T cells interacting with artificial surfaces in a TIRF-based study and noted that this sufficed to initiate T cell activation. A similar study utilizing super-resolution imaging of T cells responding to glass-immobilized anti-CD3 antibodies reported a rather more complex reorganization of signaling proteins with a CD45-depletion zone ~250 nm in diameter, but without directly implicating membrane protrusions *per se* (7). Direct analyses of microvillar contacts analyzed using VA-TIRF and resting cells or SCM and activated cells, however, revealed only limited, if any, exclusion of CD45 (5, 6). One possible explanation for these discrepancies is that only ILPs, and the “frustrated” versions of these structures that may form on resilient artificial surfaces, may create compressive forces large enough to readily observe phosphatase exclusion. A smaller, less easily observed level of segregation, albeit one sufficient to initiate signaling, might only be achieved by more-subtle, microvillar-based cell-cell contacts. It is also possible that phosphatase exclusion occurs on length scales smaller than the resolution limit of TIRF microscopy. Further studies are needed to determine under what conditions, if at all, CD45 exclusion occurs at the tips of membrane protrusions

during early cell-cell contact. This is presently very challenging, although the advent of single-molecule light-sheet imaging (47), or three-dimensional super-resolution imaging (48), offer ways to tackle this problem.

BACK TO THE BEGINNING: THE RESTING T CELL SURFACE

The remarkable, imaging-led progress in understanding the ultra-structural changes accompanying T cell activation has brought the field full circle to the problem of the resting, or “ground” state of the T cell, so that the drivers of signaling-dependent changes can be properly understood. The earliest electron microscopy-based data suggested that the TCR is pre-clustered on resting cells (49, 50). Subsequent single-molecule fluorescence-based studies of TCR stoichiometry and mobility implied instead, however, that the mobile TCRs expressed by T cells are largely if not wholly monovalent (51, 52), and that all TCRs are apparently mobile (53). The new proposal, i.e., that TCRs are freely diffusing and monovalent, was in turn quickly overtaken by new data obtained using SMLM, which supported the idea that the TCR was indeed pre-clustered in resting cells. Using high-speed photoactivated localization microscopy-based imaging, Lillemeier and colleagues proposed that the TCR is organized into “protein-islands” <70–140 nm in diameter (54). It was furthermore suggested that TCRs, LAT, CD4 and Lck were present in separate clusters on resting T cells on immobilized poly L-lysine, which then concatenate upon activation, yielding microclusters (54, 55).

But the notion that the TCR and other signaling proteins are pre-organized on resting cells has once again been challenged. Baumgart et al. (56) demonstrated that PALM and direct stochastic optical reconstruction microscopy (dSTORM) are generally prone to reporting artefactual protein clustering due to inhomogeneous stochastic fluorophore blinking, i.e., the erroneous detection of clusters due to overcounting. Whereas it was reported that the kinase Lck is clustered in domains with diameters of 50 nm (57), by titrating the levels of label, Baumgart showed that Lck is more likely homogeneously distributed in both resting and activated T cells. When Schütz and colleagues applied this approach to the TCR, they did not observe overt receptor clustering in non-activated CD4⁺ T cells in dSTORM and PALM experiments (58). An additional source of uncertainty is that by virtue of super-resolution experiments being TIRF-based, imaging has to be done on transparent, i.e., glass substrates that may or may not preserve the resting status of the imaged cell. Making matters worse, in many instances the cationic homopolymer poly L-lysine (PLL), widely thought to be inert, has been used to coat the glass surfaces used in the imaging experiments, presumably to enhance cell adherence. Santos et al. recently demonstrated, however, that PLL is not inert and that it produces levels of calcium signaling comparable to that measurable with the most potent combinations of activating antibodies in present use. Compared to cells in suspension, e.g., in hydrogels, Santos et al. showed also with super-resolution imaging that the organization of

the TCR is profoundly altered following T cell contact with PLL-coated glass (47, 59). Most recently, using a variety of complementary non-invasive imaging/spectroscopy approaches, Huppa and colleagues showed that the TCRs that engage antigen are monomeric (60).

CONCLUSION

Fluorescence-based light microscopy techniques, old and new, have already yielded paradigm-shifting insights into the ultrastructure and behavior of the T cell surface. Inevitably, controversies will arise as we gain experience with pioneering technologies and start to understand and accommodate their limitations. Because so much of what results in effective immunity occurs at the T cell surface, the stakes will always be high. For us, the two outstanding technological challenges are: (1) how do we study the resting T cell surface without perturbing it, and (2) how do we “get at” cell-cell contacts within seconds of the initiation of signaling, with the necessary time and spatial resolution. On the biology side, we would like to know: (1) what is the typical resting organization of a receptor expressed by a T cell, and what do exceptions to this behavior imply; or is the distribution of receptors and signaling proteins on the resting T cell surface best described as random? (2) What sub diffraction-limited ultrastructural changes accompany and, perhaps, drive early signaling, if any? (3) How and why

do microclusters form, and how do they relate to microvilli, if at all? (4) Are membrane protrusions essentially all the same structures? (5) Why do T cells interrogate their targets using membrane protrusions, in any case? We can expect more surprises.

AUTHOR CONTRIBUTIONS

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

FUNDING

OD and SD are each supported by Wellcome Trust Senior Research Fellowships in Basic Biomedical Science (207537/Z/17/Z and 207547/Z/17/Z). SD is additionally funded by the Medical Research Council (MC_UU_12010). JP is funded by the Wellcome Trust PhD Studentship in Science (203737/Z/16/Z).

ACKNOWLEDGMENTS

We would like to thank Athena Cavounidis for carefully reading and correcting the review and Chris Tang for reviewing the manuscript. We also thank Anton van der Merwe for providing helpful feedback and insight on this review.

REFERENCES

- Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol.* (2002) 158:1263–75. doi: 10.1083/jcb.200203043
- Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med.* (2005) 202:1031–6. doi: 10.1084/jem.20051182
- Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol.* (2005) 6:1253–62. doi: 10.1038/ni1272
- Sage PT, Varghese LM, Martinelli R, Sciuto TE, Kamei M, Dvorak AM, et al. Antigen recognition is facilitated by invadosome-like protrusions formed by memory/effector T cells. *J Immunol.* (2012) 188:3686–99. doi: 10.4049/jimmunol.1102594
- Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) 113:E5916–24. doi: 10.1073/pnas.1605399113
- Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) 356:eaa13118. doi: 10.1126/science.aa13118
- Razvag Y, Neve-Oz Y, Sajman J, Reches M, Sherman E. Nanoscale kinetic segregation of TCR and CD45 in engaged microvilli facilitates early T cell activation. *Nat Commun.* (2018) 9:732. doi: 10.1038/s41467-018-03127-w
- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) 395:82–6. doi: 10.1038/25764
- Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol.* (2006) 7:803–9. doi: 10.1038/ni1369
- Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* (2006) 25:117–27. doi: 10.1016/j.immuni.2006.04.010
- Thauland TJ, Parker DC. Diversity in immunological synapse structure. *Immunology* (2010) 131:466–72. doi: 10.1111/j.1365-2567.2010.03366.x
- Vardhana S, Choudhuri K, Varma R, Dustin ML. Essential role of ubiquitin and TSG101 protein in formation and function of the central supramolecular activation cluster. *Immunity* (2010) 32:531–40. doi: 10.1016/j.immuni.2010.04.005
- Singleton K, Parvaze N, Dama KR, Chen KS, Jennings P, Purdie B, et al. A large T Cell invagination with CD2 enrichment resets receptor engagement in the immunological synapse. *J Immunol.* (2006) 177:4402–13. doi: 10.4049/jimmunol.177.7.4402
- Choudhuri K, Llodra J, Roth EW, Tsai J, Gordo S, Wucherpfennig KW, et al. Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature* (2014) 507:118–23. doi: 10.1038/nature12951
- Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol.* (2006) 7:247–55. doi: 10.1038/ni1304
- Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* (2006) 443:462–5. doi: 10.1038/nature05071
- Yokosuka T, Kobayashi W, Sakata-Sogawa K, Takamatsu M, Hashimoto-Tane A, Dustin ML, et al. Spatiotemporal regulation of T cell costimulation by TCR-CD28 microclusters and protein kinase C theta translocation. *Immunity* (2008) 29:589–601. doi: 10.1016/j.immuni.2008.08.011
- Krummel ME, Sjaastad MD, Wulfig C, Davis MM. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science* (2000) 289:1349–52. doi: 10.1126/science.289.5483.1349

19. Dustin ML, Groves JT. Receptor signaling clusters in the immune synapse. *Annu Rev Biophys.* (2012) 41:543–56. doi: 10.1146/annurev-biophys-042910-155238
20. Kumari S, Depoil D, Martinelli R, Judokusumo E, Carmona G, Gertler FB, et al. Actin foci facilitate activation of the phospholipase C-gamma in primary T lymphocytes via the WASP pathway. *Elife* (2015) 4:e04953 doi: 10.7554/eLife.04953
21. Polliack A. The contribution of scanning electron microscopy in haematology: its role in defining leucocyte and erythrocyte disorders. *J Microsc.* (1981) 123(Pt. 2):177–87. doi: 10.1111/j.1365-2818.1981.tb01293.x
22. Majstoravich S, Zhang J, Nicholson-Dykstra S, Linder S, Friedrich W, Siminovich KA, et al. Lymphocyte microvilli are dynamic, actin-dependent structures that do not require Wiskott-Aldrich syndrome protein (WASP) for their morphology. *Blood* (2004) 104:1396–403. doi: 10.1182/blood-2004-02-0437
23. Fisher PJ, Bulur PA, Vuk-Pavlovic S, Prendergast FG, Dietz AB. Dendritic cell microvilli: a novel membrane structure associated with the multifocal synapse and T-cell clustering. *Blood* (2008) 112:5037–45. doi: 10.1182/blood-2008-04-149526
24. Greicius G, Westerberg L, Davey EJ, Buentke E, Scheynius A, Thyberg J, et al. Microvilli structures on B lymphocytes: inducible functional domains? *Int Immunol.* (2004) 16:353–64. doi: 10.1093/intimm/dxh031
25. Bell GI, Dembo M, Bongrand P. Cell adhesion. Competition between nonspecific repulsion and specific bonding. *Biophys J.* (1984) 45:1051–64. doi: 10.1016/S0006-3495(84)84252-6
26. Springer TA. Adhesion receptors of the immune system. *Nature* (1990) 346:425–34. doi: 10.1038/346425a0
27. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. *Annu Rev Biomed Eng.* (2007) 9:121–67. doi: 10.1146/annurev.bioeng.9.060906.151959
28. Ardman B, Sikorski MA, Staunton DE. CD43 interferes with T-lymphocyte adhesion. *Proc Natl Acad Sci USA.* (1992) 89:5001–5. doi: 10.1073/pnas.89.11.5001
29. Paszek MJ, DuFort CC, Rossier O, Bainer R, Mouw JK, Godula K, et al. The cancer glycocalyx mechanically primes integrin-mediated growth and survival. *Nature* (2014) 511:319–25. doi: 10.1038/nature13535
30. Carman CV, Sage PT, Sciuto TE, de la Fuente MA, Geha RS, Ochs HD, et al. Transcellular diapedesis is initiated by invasive podosomes. *Immunity* (2007) 26:784–97. doi: 10.1016/j.immuni.2007.04.015
31. Bui N, Saitakis M, Dogniaux S, Buschinger O, Bohineust A, Richert A, et al. Human primary immune cells exhibit distinct mechanical properties that are modified by inflammation. *Biophys J.* (2015) 108:2181–90. doi: 10.1016/j.bpj.2015.03.047
32. Saitakis M, Dogniaux S, Goudot C, Bui N, Asnacios S, Maurin M, et al. Different TCR-induced T lymphocyte responses are potentiated by stiffness with variable sensitivity. *Elife* (2017) 6:e23190. doi: 10.7554/eLife.23190
33. Hivroz C, Saitakis M. Biophysical aspects of T lymphocyte activation at the immune synapse. *Front Immunol.* (2016) 7:46. doi: 10.3389/fimmu.2016.00046
34. Huse M. Mechanical forces in the immune system. *Nat Rev Immunol.* (2017) 17:679–90. doi: 10.1038/nri.2017.74
35. von Andrian UH, Hasslen SR, Nelson RD, Erlandsen SL, Butcher EC. A central role for microvillous receptor presentation in leukocyte adhesion under flow. *Cell* (1995) 82:989–99. doi: 10.1016/0092-8674(95)90278-3
36. Bruehl RE, Springer TA, Bainton DF. Quantitation of L-selectin distribution on human leukocyte microvilli by immunogold labeling and electron microscopy. *J Histochem Cytochem.* (1996) 44:835–44. doi: 10.1177/44.8.8756756
37. Buscher K, Riese SB, Shakibaei M, Reich C, Dervede J, Tauber R, et al. The transmembrane domains of L-selectin and CD44 regulate receptor cell surface positioning and leukocyte adhesion under flow. *J Biol Chem.* (2010) 285:13490–7. doi: 10.1074/jbc.M110.102640
38. Carbone CB, Kern N, Fernandes RA, Hui E, Su X, Garcia KC, et al. *In vitro* reconstitution of T cell receptor-mediated segregation of the CD45 phosphatase. *Proc Natl Acad Sci USA.* (2017) 114:E9338–45. doi: 10.1073/pnas.1710358114
39. Chang VT, Fernandes RA, Ganzinger KA, Lee SF, Siebold C, McColl J, et al. Initiation of T cell signaling by CD45 segregation at 'close contacts'. *Nat Immunol.* (2016) 17:574–82. doi: 10.1038/ni.3392
40. Stinchcombe JC, Salio M, Cerundolo V, Pende D, Arico M, Griffiths GM. Centriole polarisation to the immunological synapse directs secretion from cytolytic cells of both the innate and adaptive immune systems. *BMC Biol.* (2011) 9:45. doi: 10.1186/1741-7007-9-45
41. Yi JC, Samelson LE. Microvilli set the stage for T-cell activation. *Proc Natl Acad Sci USA.* (2016) 113:11061–2. doi: 10.1073/pnas.1613832113
42. Robert P, Aleksic M, Dushek O, Cerundolo V, Bongrand P, van der Merwe PA. Kinetics and mechanics of two-dimensional interactions between T cell receptors and different activating ligands. *Biophys J.* (2012) 102:248–57. doi: 10.1016/j.bpj.2011.11.4018
43. Liu B, Chen W, Evavold BD, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* (2014) 157:357–68. doi: 10.1016/j.cell.2014.02.053
44. Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell* (2018) 174:672–87 e627. doi: 10.1016/j.cell.2018.06.017
45. Bachmann MF, Barner M, Kopf M. Cd2 sets quantitative thresholds in T cell activation. *J Exp Med.* (1999) 190:1383–92. doi: 10.1084/jem.190.10.1383
46. Liu Y, Blanchfield L, Ma VP, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci USA.* (2016) 113:5610–5. doi: 10.1073/pnas.1600163113
47. Ponjavic A, McColl J, Carr AR, Santos AM, Kulenkampff K, Lippert A, et al. Single-molecule light-sheet imaging of suspended T cells. *Biophys J.* (2018) 114:2200–11. doi: 10.1016/j.bpj.2018.02.044
48. Carr AR, Ponjavic A, Basu S, McColl J, Santos AM, Davis S, et al. Three-dimensional super-resolution in eukaryotic cells using the double-helix point spread function. *Biophys J.* (2017) 112:1444–54. doi: 10.1016/j.bpj.2017.02.023
49. Schamel WW, Arechaga I, Risueno RM, van Santen HM, Cabezas P, Risco C, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J Exp Med.* (2005) 202:493–503. doi: 10.1084/jem.20042155
50. Kumar R, Ferez M, Swamy M, Arechaga I, Rejas MT, Valpuesta JM, et al. Increased sensitivity of antigen-experienced T cells through the enrichment of oligomeric T cell receptor complexes. *Immunity* (2011) 35:375–87. doi: 10.1016/j.immuni.2011.08.010
51. James JR, White SS, Clarke RW, Johansen AM, Dunne PD, Sleep DL, et al. Single-molecule level analysis of the subunit composition of the T cell receptor on live T cells. *Proc Nat Acad Sci USA.* (2007) 104:17662–7. doi: 10.1073/pnas.0700411104
52. Dunne PD, Fernandes RA, McColl J, Yoon JW, James JR, Davis SJ, et al. DySCO: quantitating associations of membrane proteins using two-color single-molecule tracking. *Biophys J.* (2009) 97:L5–7. doi: 10.1016/j.bpj.2009.05.046
53. James JR, McColl J, Oliveira MI, Dunne PD, Huang E, Jansson A, et al. The T cell receptor triggering apparatus is composed of monovalent or monomeric proteins. *J Biol Chem.* (2011) 286:31993–2001. doi: 10.1074/jbc.M111.219212
54. Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol.* (2010) 11:90–6. doi: 10.1038/ni.1832
55. Roh KH, Lillemeier BF, Wang F, Davis MM. The coreceptor CD4 is expressed in distinct nanoclusters and does not colocalize with T-cell receptor and active protein tyrosine kinase p56lck. *Proc Natl Acad Sci USA.* (2015) 112:E1604–1613. doi: 10.1073/pnas.1503532112
56. Baumgart F, Arnold AM, Leskova K, Staszek K, Folser M, Weghuber J, et al. Varying label density allows artifact-free analysis of membrane-protein nanoclusters. *Nat Methods* (2016) 13:661–4. doi: 10.1038/nmeth.3897
57. Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat Immunol.* (2013) 14:82–9. doi: 10.1038/ni.2488

58. Rossboth B, Arnold AM, Ta H, Platzer R, Kellner F, Huppa JB, et al. TCRs are randomly distributed on the plasma membrane of resting antigen-experienced T cells. *Nat Immunol.* (2018) 19:821–7. doi: 10.1038/s41590-018-0162-7
59. Santos AM, Ponjavic A, Fritzsche M, Fernandes RA, de la Serna JB, Wilcock MJ, et al. Capturing resting T cells: the perils of PLL. *Nat Immunol.* (2018) 19:203–5. doi: 10.1038/s41590-018-0048-8
60. Brameshuber M, Kellner F, Rossboth BK, Ta H, Alge K, Sevcsik E, et al. Monomeric TCRs drive T cell antigen recognition. *Nat Immunol.* (2018) 19:487–6. doi: 10.1038/s41590-018-0092-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mapping Determinants of Cytokine Signaling via Protein Engineering

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

Mark Walter,
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Ghent University, Belgium

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Specialty section:

This article was submitted to
Cytokines and Soluble Mediators in
Immunity,
a section of the journal
Frontiers in Immunology

Received: 30 May 2018

Accepted: 30 August 2018

Published: 27 September 2018

Citation:

Gorby C, Martinez-Fabregas J,
Wilmes S and Moraga I (2018)
Mapping Determinants of Cytokine
Signaling via Protein Engineering.
Front. Immunol. 9:2143.
doi: 10.3389/fimmu.2018.02143

Cytokines comprise a large family of secreted ligands that are critical for the regulation of immune homeostasis. Cytokines initiate signaling via dimerization or oligomerization of the cognate receptor subunits, triggering the activation of the Janus Kinases (JAKs)/signal transducer and activator of transcription (STATs) pathway and the induction of specific gene expression programs and bioactivities. Deregulation of cytokines or their downstream signaling pathways are at the root of many human disorders including autoimmunity and cancer. Identifying and understanding the mechanistic principles that govern cytokine signaling will, therefore, be highly important in order to harness the therapeutic potential of cytokines. In this review, we will analyze how biophysical (ligand-receptor binding geometry and affinity) and cellular (receptor trafficking and intracellular abundance of signaling molecules) parameters shape the cytokine signalosome and cytokine functional pleiotropy; from the initial cytokine binding to its receptor to the degradation of the cytokine receptor complex in the proteasome and/or lysosome. We will also discuss how combining advanced protein engineering with detailed signaling and functional studies has opened promising avenues to tackle complex questions in the cytokine signaling field.

Keywords: cytokine signaling, protein engineering, JAK/STAT signaling pathway, endosomal trafficking, endosomes signaling

INTRODUCTION

Cytokines comprise a large family of soluble factors, which control virtually every aspect of mammalian physiology (1–5). Deregulation of cytokines or cytokine-related pathways can result in human diseases such as asthma, severe combined immunodeficiency (SCID) and certain cancers (6–13), making this family highly relevant to human health. A poor mechanistic understanding of how cytokine signaling is initiated and regulated in space and time, however, has hindered the translation of these ligands to the clinic.

The cytokine signaling paradigm encompasses the binding of a cytokine to its surface receptors, followed by the activation of receptor associated tyrosine kinases of the Janus Kinases family (JAKs) (1, 14). JAKs in turn phosphorylate tyrosines in the cytokine receptor intracellular domains (ICD), generating docking sites for the signal transducer and activator of transcription (STAT) factors (2, 3, 15, 16). Upon receptor binding, STATs are phosphorylated by JAKs, forming homo- and hetero-dimers, which translocate to the nucleus, bind specific promoter sequences and induce defined gene expression programs and bioactivities (17–19). In recent years however, a series of biophysical and protein engineering studies have provided new evidence which highlights the large complexity of signaling triggered by a cytokine-cytokine receptor complex. This complexity allows cytokines to produce a wide range of biological responses despite using a very minimal set of surface

receptors and effector signaling molecules. In this review, we will focus on cytokines that engage the JAK/STAT signaling pathway and on how the engineering of agonistic surrogate cytokines has expanded our understanding of cytokine signaling and biology; in addition we will discuss future directions in the context of cytokine-based therapies.

STOICHIOMETRY OF THE CYTOKINE-CYTOKINE RECEPTOR COMPLEX IN THE PLASMA MEMBRANE

One of the most debated questions in the cytokine field concerns the stoichiometry of the cytokine receptor complex in the absence of ligand (20, 21). At first glance, this question seems to be unimportant, given that all models agree that ligand binding is the initial step for activating cytokine receptors. However, how cytokine receptors are activated by cytokine binding has clear functional implications—in particular for targeted engineering of desired cytokine properties. Two opposing models have emerged in the past years. The first model postulates that cytokine receptors exist as preformed inactive dimers in the plasma membrane that become active upon cytokine binding through a conformational/structural rearrangement. Evidence supporting this model is found primarily in homo-dimeric systems such as erythropoietin (Epo) (22–26), thrombopoietin (Tpo) (27, 28), and Growth Hormone (GH) (29–31), although some reports in hetero-dimeric systems have also been reported (32–35). The erythropoietin receptor (EpoR) was found to exist as a dimer in crystals that did not include Epo (23), and at the cell surface by immunofluorescence (26). Similar observations were made for the GH receptor (GH-R) via co-immunoprecipitation of differentially tagged receptors or fluorescence and bioluminescence resonance energy transfer techniques (29, 30).

A second model postulates that cytokine receptors diffuse freely in the plasma membrane as monomers and only upon cytokine binding are recruited into a complex to trigger signaling. According to this model, cytokine receptor assembly is driven by affinities, interaction rate constants and the respective concentrations of all involved reactants. This leads to dynamic equilibria between monomeric and assembled receptors subunits, which can be tuned by affinities and receptor concentrations according to the law of mass action. There are also several lines of evidence supporting this model: (a) this model predicts a step-wise formation of cytokine receptor complexes. Indeed, all cytokines described to date bind one of the receptor chains with significantly higher affinity than the other one and step-wise complex formation has been shown for several cytokines both in *in vitro* and *in vivo* studies, including Epo and IFN systems (36–38). (b) cytokine receptor chimeras where the extracellular domains and/or transmembrane domains are swapped by those of any other receptor still trigger signaling in a ligand dependent manner (39–45). (c) surrogate cytokine ligands, e.g., antibodies (46–49) can trigger signaling, arguing against precise conformational changes required for signal activation. (d) Single particle fluorescence imaging studies in several cytokine systems has shown that receptor subunits exist as monomers on

the surface of live cells at physiologically relevant cell surface densities, and only form dimers upon ligand binding (48, 50–55). Additionally, cytokines with mutations in the low-affinity chain binding site (“site 2”) fail to induce receptor dimers in agreement with the classical two-step binding mode.

A point to consider from all these studies is that in many instances modified/tagged receptor constructs that are ectopically expressed are used. Thus, the possibility that these modifications inhibit or induce receptor assembly on their own cannot be formally excluded, making it difficult to decide which model is true for a given cytokine receptor system. Nonetheless, due to the strong evidences supporting either model, it is plausible that both models are correct to some extent and that their relative contribution to cytokine signaling could vary depending on cellular context, i.e., receptor and signaling molecules abundance, as has been reported for the Epo system.

CYTOKINE-CYTOKINE RECEPTOR COMPLEX STABILITY VS. ACTIVITY

A key factor contributing to signaling and bioactivity potency and specificity by cytokines is the stability of the cytokine-cytokine receptor interactions. Type I Interferons (IFNs) have been used as a model system to study how receptor complex stability influences signaling. The type I IFN family comprises more than 15 different subtypes, all binding to the same receptor complex formed by IFNAR1 and IFNAR2 subunits and activating to the same extent the same JAK/STAT pathway (36, 56–58). Yet, different IFN subtypes induce anti-viral and anti-cancer responses with very different potencies (59–64). While all IFNs exhibit a comparable antiviral activity, only IFN β has an exceptional antiproliferative activity, which is linked to its anti-cancer potential. A series of biophysical, structural and engineering studies has started to address this apparent lack of correlation between signaling and activity output in this family. Early studies elegantly showed that complex stability critically contributed to the differential activities exhibited by IFNs (59, 65–69). Indeed, an IFN α 2 variant, engineered to mimic the properties of IFN β by enhancing IFNAR1 binding affinity acquired potent antiproliferative activity (59, 66, 67). More recently, structural and engineering studies have shown that the topologies of the IFN receptor complexes formed by the different IFNs are very similar and that their differential activities likely result from different receptor binding kinetics and signal activation (64, 70). Indeed, this differential kinetics of STAT activation by type I IFNs result in the induction of two sets of genes: robust genes that drive the antiviral response and only require short pulses of IFN at low concentrations, and tuneable genes that require sustained activation with higher doses of IFNs and are linked to the anti-cancer responses. Induction of robust genes is not very sensitive to changes in complex stability, while the induction of tuneable genes is (71–73).

The ability of cytokine receptors to translate binding stability into biological output potency is not restricted to type I IFNs and can be found in other cytokine systems. IL-4 and IL-13 are two important immunomodulatory cytokines that bind the same receptor complex comprised of IL-4R α and IL-13R α 1 and

activate STAT6 (8, 74). Yet, their activities are not completely overlapping, with each cytokine exhibiting pockets of specificity (75–77). Biophysical and structural studies have shown that the kinetics of complex formation by these two cytokines is at the root of their differences (5, 78, 79). While IL-4 binds first IL-4R α with high affinity and then recruits IL-13R α 1, IL-13 first binds IL-13R α 1 and then recruits IL-4R α . Importantly, in most immune cells IL-4R α appears to be the limiting factor (78, 80–83). As a consequence, IL-4 by recruiting IL-4R α with higher efficiency can activate signaling more efficiently than IL-13 and overall elicit more potent biological responses (77). Despite this, IL-13 can elicit specific biological responses not induced by IL-4, for which we still lack a mechanistic understanding.

Viruses have taken advantage of the functional plasticity exhibited by the cytokine system. Viruses often encode open-reading frames that share sequence identity with known human cytokines and mimic their biological properties (56, 84–86). A classic example of this is viral IL-10 (vIL-10) (87). IL-10 is a key immune-modulatory cytokine that controls the extent and potency of the immune response by engaging a surface receptor formed by IL-10R α and IL-10R β receptor subunits to activate STAT3 (11, 56, 88, 89). Interestingly two viruses, cytomegalovirus (CMV) and Epstein-Barr (EBV), encode in their genomes homologs of this cytokine (87). Of particular interest is the ebvIL-10, which it is better known as vIL-10. Despite sharing a high degree of sequence and structural homology with human IL-10 (hIL-10), vIL-10 only engages the anti-inflammatory responses elicited by hIL-10 (90–92). vIL-10 inhibits the expression of MHC class II in monocytes and macrophages and the proliferation of T cells (93), but fails to promote other hIL-10 activities such as induction of thymocytes and mast cell proliferation or upregulation of MHC class II by B cells (94–96). This differential effect could be traced to the different complex stabilities elicited by the two ligands, with vIL-10 binding more weakly to IL-10R α than hIL-10 (91). Overall, these studies describe an intricate relationship between ligand-receptor complex stability and signaling and biological outcomes by cytokines, which could act as a source of functional heterogeneity and potentially be exploited for therapeutic purposes.

ADDITIONAL FACTORS CONTRIBUTING TO CYTOKINE-CYTOKINE RECEPTOR COMPLEX STABILITY

Signal activation by cytokines is a very efficient process where cytokines exhibiting a wide range of binding affinities activate signaling to a similar extent. This suggests that other factors beyond the sole affinity of the ligand for its receptor contribute to form and stabilize the cytokine receptor complex (**Figure 1**). Here we will highlight three cellular determinants that have been the focus of attention in recent studies:

The role of the endosomal compartment in cytokine signaling initiation and diversification has been proposed but not formally proven (97–102). Early work in the EGF system showed that EGF mutants with impaired EGFR binding affinity paradoxically elicited more potent signaling responses (103). Through a series

of studies the authors showed that receptor complexes formed by these mutants did not survive the endosomal acidic pH leading to dissociation and recycling of the ligands and receptor to the membrane, contributing to more sustained signal activation by these mutants (103). More recently, studies utilizing TIRF microscopy have revealed that the endosomal compartment contributes to the formation and stabilization of the cytokine-cytokine receptor complex, thus ensuring signaling fitness at a wide range of environmental conditions (50, 104). Whether endosomes serve as signaling platforms where cytokine receptors encounter alternative signaling molecules to fine-tune their activities, however, still remains an open question. Some evidence of this can be found in a study which showed that phosphorylated JAK1 and Tyk2 could be found in EEA1 positive endosomes upon IFN stimulation (105). Additionally, mutations in the G-CSF receptor that altered its intracellular traffic differentially affected the signaling output and bioactivities engaged by this receptor (106, 107). However, to date no direct evidence demonstrating that endosomes function as signaling hubs for cytokine receptors has been described. This dearth of knowledge originates from the technical challenge that following and blocking cytokine receptor complexes to intracellular compartments represents. Future studies combining biochemistry and imaging methodologies will be required to address this long-standing question.

Another factor contributing to cytokine-cytokine receptor complex formation and stabilization is the actin cytoskeleton. Two recent studies in the IFN and IL-4 systems have shown that cytokine receptors are confined to cytoskeletal microcompartments at the plasma membrane, which allows quick reassembly of the cytokine-cytokine receptor complex after dissociation (52, 55). Manipulation of these actin compartments with small molecule inhibitors altered signaling downstream of the IFN or IL-4 complexes (52, 55).

Yet another factor contributing to cytokine-cytokine receptor complex stability are the JAK kinases associated to the receptors intracellular domains. Early studies with type I IFN showed that mutations in JAKs which did not affect receptor surface expression decreased the number of high affinity IFN binding sites in cells, suggesting an inside-out communication between JAKs and the IFN receptor (108). More recent studies have confirmed this initial observation and provided mechanistic insight into this JAK-receptor communication. A first study showed that two JAK2 molecules could interact *in trans* via their kinase/pseudokinase domains when bound to the GH-R homodimer, contributing to signaling initiation and propagation (31, 109). A follow up study showed that a productive JAK1-Tyk2 interaction was required to obtain maximal dimerization efficiency in the IFN system. Indeed, lack of JAK1 resulted in a reduction of the number of complexes formed by IFN α 2, which could not be assigned to lower levels of IFNAR1 or IFNAR2 on the surface (51).

Overall these studies suggest that the cytokine system has developed a series of check points to ensure that the cytokine-cytokine receptor complex is formed and activates signaling. The next topic we will address is whether we can exploit these different factors contributing to cytokine receptor complex formation to fine-tune cytokine signaling and responses.

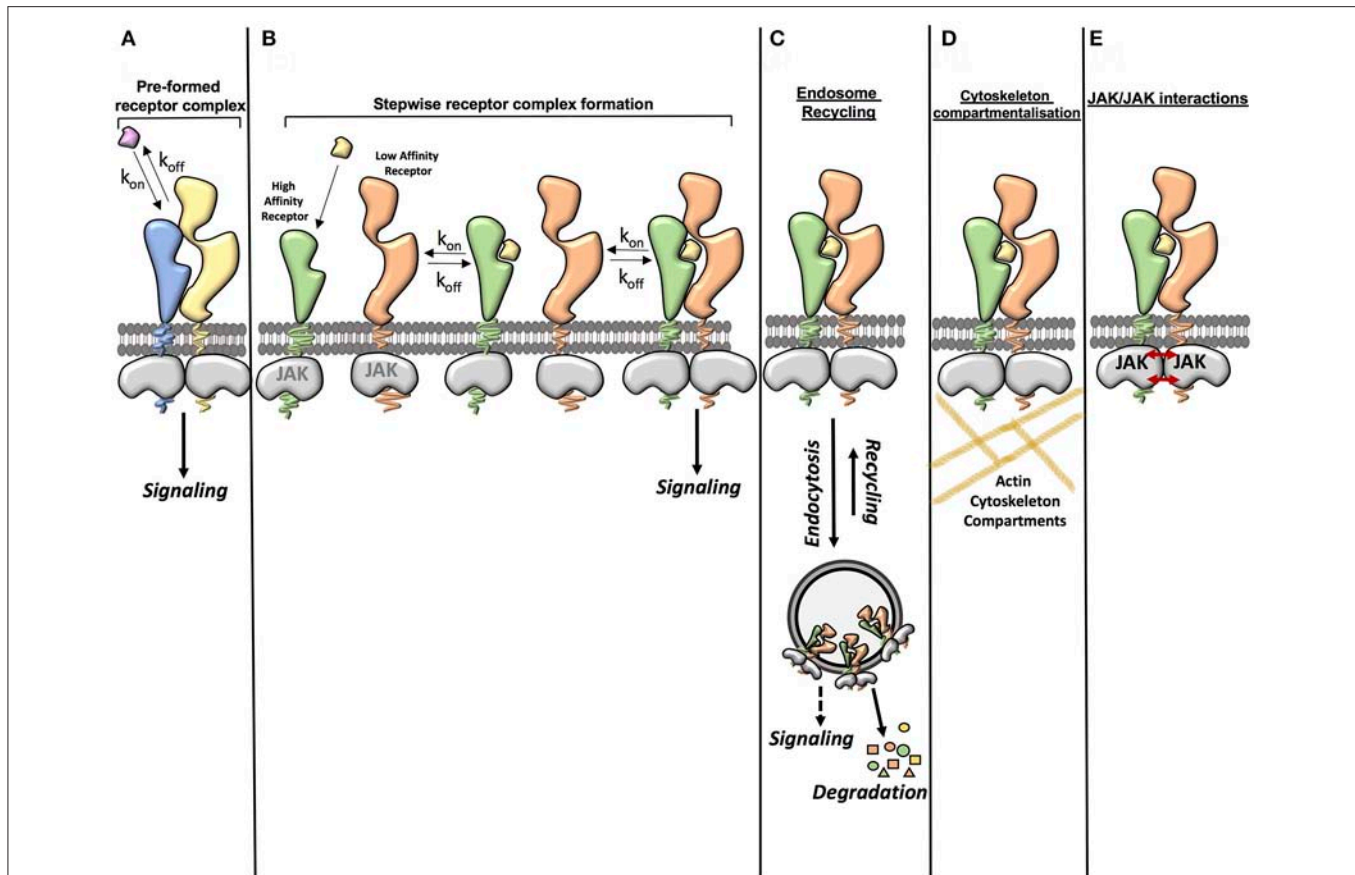


FIGURE 1 | Factors contributing to cytokine-cytokine receptor complex stability. **(A)** Ligand affinity for the receptor (pre-formed receptor complex). The affinity of the ligand for the pre-formed receptor can enhance the stability of the ligand-receptor complex. **(B)** Ligand affinity for the receptor (stepwise receptor complex formation). The ligand first binds to the high affinity receptor chain. This then allows recruitment of the second receptor chain. The affinity of the ligand for the high affinity receptor chain and the affinity of the ligand-high affinity receptor complex for the second receptor chain can influence the stability of the complete complex. **(C)** Endosome recycling. Endocytosis of the ligand-receptor complex from the cell surface to intracellular vesicles depletes ligands and receptors from the plasma membrane, negatively regulating signaling. However some studies have suggested that there is a possibility that some ligand-receptor complexes may also be stabilized in the endosome, leading to a stable complex being formed. Under certain circumstances the recycling of receptor components and ligands from the endosome back to the plasma membrane also contributes to the formation of a stable complex. **(D)** Cytoskeleton compartmentalization. Cytoskeleton components such as actin can contribute to the stability of a receptor complex by confining the movement of the receptor and thus increasing the opportunity for rebinding of the ligand. **(E)** JAK/JAK interactions. Some studies have shown that interactions between JAKs can aid dimerization of the receptor complex and that JAKs can act in trans in certain receptor systems.

EXPLOITING CYTOKINE ENGINEERING TO DISCOVER NEW CYTOKINE BIOLOGY

Manipulation of cytokine binding properties via protein engineering is a valuable tool with which we can better understand cytokine biology and to fine-tune cytokine responses. Above we have already introduced some examples focused on the IFN system that help to better understand IFN biology. Next, we will describe additional examples in other cytokine systems which highlight the potential of cytokine engineering to address complex biological problems.

IL-2 plays a critical role in regulating T cell responses, making it an attractive target to treat autoimmune diseases and cancer (5, 110–113). However, its use in the clinic is limited due to severe toxicity resulting from its functional pleiotropy

(114–117). IL-2 can engage two types of receptor complexes on the surface of responsive cells: the high affinity receptor complex comprised of IL-2R α , IL-2R β , and γ_c receptor subunits and the intermediate affinity complex formed by IL-2R β and γ_c subunits (5). Thus, T cells control their sensitivity to IL-2 by modulating their levels of the alpha receptor (113). Many attempts to improve the clinical efficacy of IL-2 by fine tuning its receptor binding properties have been carried out over the years. One of the first studies was performed by Shanafelt and colleagues, who proposed that IL-2-derived toxicity resulted from engagement of the intermediate receptor present on NK cells, which are believed to be the major source of the cytokines and inflammatory mediators causing most of the toxicity associated with high-dose IL-2 therapy (118). In order to specifically target IL-2 to T cells and thus decrease its toxicity they used site

directed mutagenesis to reduce the binding affinity of IL-2 to IL-2R β (119). This IL-2 mutant could not engage the intermediate affinity receptor, but still could activate signaling in the context of the high affinity receptor, leading to a more than 3,000-fold specificity for T cells over NK cells (119). In an experimental lung metastasis model, sensitive to IL-2 therapy, this IL-2 mutant showed similar levels of tumor inhibition to IL-2 but elicited lower levels of morbidity as scored by general health examination (119). However, in a later phase I trial this mutant did not show advantage over wt IL-2 in anti-tumor responses or toxicity, highlighting the complexity of this cytokine in an *in vivo* setting (120). Another example of IL-2 engineering is found in studies by the Wittrup lab. Using yeast surface display, the authors engineered an IL-2 variant with high affinity for IL-2R α . This variant induced T cell proliferation more potently than wt IL-2, suggesting that it could be a better alternative than wt for cancer immunotherapy since lower doses of the variant would be required to show efficacy which could result in lower toxicity (121–123).

More recently, studies by Garcia and collaborators have provided a series of IL-2 variants that have furthered our understanding of IL-2 biology. Using yeast surface display, Levin and colleagues engineered an IL-2 variant (Super-2) binding 200-fold tighter to IL-2R β than wt (124). Super-2 can signal through the intermediate affinity receptor as potently as through the high affinity receptor, thus negating the regulatory role of the α subunit. This in turn resulted in a stronger anti-tumor response by Super-2 with a significantly lower toxicity when compared to wt IL-2 (124). In a second study, Suman and colleagues used Super-2 as a backbone to engineer a series of Super-2-based cytokines where binding to γ c chain was reduced (125). Strikingly, the authors observed that rather than a complete loss in response, these new variants activated signaling with different amplitudes ranging from 100% activity to 50 and 10% in accordance with their binding affinity (125). Interestingly, the IL-2 mutant activating 50% activity could induce proliferation of activated T cells, but not of naïve T cells, suggesting different signaling thresholds required for proliferation in different T cell differentiation stages (125).

In addition, a recent study has shown that IL-2 receptor binding specificity can also be altered in a mutation-independent manner by introducing PEG molecules in the IL-2 region interacting with IL-2R α . This new IL-2 variant, named NKTR-214, has shown promising anti-tumor responses and decreased toxicity and it is now finding its way to the clinic (126).

The IL-4/IL-13 system has also been the subject of protein engineering studies. As described above, IL-4 binds two surface receptor complexes: The type I receptor, consisting of the IL-4R α and γ c subunits, which is found exclusively on hematopoietic cells; and the type II receptor, composed of the IL-4R α and IL-13R α 1 chains, which is also shared by IL-13 (5, 78). A recent work by Junttila and collaborators shed some light onto the differential activities elicited by the two IL-4 complexes. Using yeast surface display, the authors engineered two IL-4 variants exhibiting high specificity for either the type I or the type II IL-4 receptors (127). Detailed functional characterization of these variants revealed that while T cell responses were exclusively

dependent on the type I IL-4 complex, in agreement with the specific expression of this receptor in T cells, dendritic cell maturation was dependent on the IL-4 type II complex (127). These results agreed with previously published observations and revealed functional dichotomy between the Type I and Type II IL-4 receptors (128, 129).

The impact of complex formation kinetics and stability on signaling and activities by the IL-4/IL-13 complex was further explored in a recent work (50). In this study, we engineered a range of IL-13 variants exhibiting different binding affinities for the IL-13R α 1 receptor subunit. When we functionally characterized these variants, we observed that large decreases in binding affinity were required to marginally alter signaling efficiency. Further increases in binding affinities, however, did not improve signaling by IL-13. Through a series of modeling simulations and experiments we concluded that transition of the cytokine-cytokine receptor complex to the endosomal compartment was the limiting rate factor for signaling potency in the IL-13 system. Cytokine-cytokine receptor complexes capable of undergoing endocytosis would be stabilized due to the high local cytokine receptor concentration achieved in the limited area of endosomes. Further stabilization of the complex beyond that required to transit to the endosomes will have minimal influence on signaling (50). Indeed, our data agreed with a recent study highlighting the role of the endosomal compartment in the formation of the IL-4/IL-13 complexes (104, 130). Interestingly, this disconnect between binding affinity and signaling output was not found when more complex biological responses, e.g., TF-1 cell proliferation and dendritic cell differentiation, were analyzed, which in turn directly correlated with the stability of the IL-13 complex (50). A possible explanation for this apparent lack of correlation between signaling and activity could be found in the different times used to study these processes. While signaling is measured during the first few hours of cytokine stimulation, biological responses often take days to be observed. Surface receptors, signaling molecules and ligand concentrations could be altered with time, leading to functional diversification from an apparently similar starting point. An example of this can be found in the type I IFN system, where IFN stimulation leads to the upregulation of negative regulators that preferentially inhibit short-lived IFN complexes (51, 131, 132).

CONCLUSIONS AND REMARKS

In this mini-review, we have summarized recent studies that have underlined the intricate interplay of cytokine-receptor complex stability and signaling and biological responses. Additionally we have discussed recent findings that support a scaffolding role for the JAK kinases in complex formation, as well as interesting observations regarding the contribution of the actin cytoskeleton and the endosomal compartment to signaling robustness by cytokine-receptor complexes. However, key standing questions remains in the field such as how binding of a cytokine to its receptor triggers signaling, how signaling specificity is generated, are endosomes contributing to fine tune

cytokine signaling and biology and how cytokine functional pleiotropy is generated. In order to answer these questions, which would allow us to rationally manipulate cytokine responses and harness their full therapeutic potential, future studies will need to take advantage of recent advances in cryo-EM and membrane protein structural biology to fully understand the complex interconnectivity of the cytokine/cytokine receptor/JAK/STAT complex. Additionally, advance microscopy studies combined with proximity labeling methodologies such as bioID could provide us with new insights into the role that the endosomal compartment plays in shaping cytokine signaling and responses.

REFERENCES

- Haan C, Kreis S, Margue C, Behrmann I. Jaks and cytokine receptors—an intimate relationship. *Biochem Pharmacol.* (2006) 72:1538–46. doi: 10.1016/j.bcp.2006.04.013
- Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol.* (2007) 178:2623–9. doi: 10.4049/jimmunol.178.5.2623
- Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. *J Biol Chem.* (2007) 282:20059–63. doi: 10.1074/jbc.R700016200
- Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol.* (2009) 9:480–90. doi: 10.1038/nri2580
- Wang X, Lupardus P, Laporte SL, Garcia KC. Structural biology of shared cytokine receptors. *Annu Rev Immunol.* (2009) 27:29–60. doi: 10.1146/annurev.immunol.24.021605.090616
- Foster PS, Martinez-Moczygemba M, Huston DP, Corry DB. Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease. *Pharmacol Ther.* (2002) 94:253–64. doi: 10.1016/S0163-7258(02)00220-6
- Kovanen PE, Leonard WJ. Cytokines and immunodeficiency diseases: critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways. *Immunol Rev.* (2004) 202:67–83. doi: 10.1111/j.0105-2896.2004.00203.x
- Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Curr Allergy Asthma Rep.* (2004) 4:123–31. doi: 10.1007/s11882-004-0057-6
- Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol.* (2006) 6:595–601. doi: 10.1038/nri1901
- Boyman O, Purton JF, Surh CD, Sprent J. Cytokines and T-cell homeostasis. *Curr Opin Immunol.* (2007) 19:320–6. doi: 10.1016/j.coi.2007.04.015
- Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev.* (2008) 226:205–18. doi: 10.1111/j.1600-065X.2008.00706.x
- Romo de Vivar Chavez A, de Vera ME, Liang X, Lotze MT. The biology of interleukin-2 efficacy in the treatment of patients with renal cell carcinoma. *Med Oncol.* (2009) 26(Suppl. 1):3–12. doi: 10.1007/s12032-008-9162-z
- Steel JC, Waldmann TA, Morris JC. Interleukin-15 biology and its therapeutic implications in cancer. *Trends Pharmacol Sci.* (2012) 33:35–41. doi: 10.1016/j.tips.2011.09.004
- Yamaoka K, Saharinen P, Pesu M, Holt VE III, Silvennoinen O, O'Shea JJ. The Janus kinases (Jaks). *Genome Biol.* (2004) 5:253. doi: 10.1186/gb-2004-5-12-253
- Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. *Annu Rev Immunol.* (1998) 16:293–322. doi: 10.1146/annurev.immunol.16.1.293
- Heim MH. The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *J Recept Signal Transduct Res.* (1999) 19:75–120. doi: 10.3109/10799899909036638
- Ramana CV, Chatterjee-Kishore M, Nguyen H, Stark GR. Complex roles of Stat1 in regulating gene expression. *Oncogene* (2000) 19:2619–27. doi: 10.1038/sj.onc.1203525
- Ihle JN. The Stat family in cytokine signaling. *Curr Opin Cell Biol.* (2001) 13:211–7. doi: 10.1016/S0955-0674(00)00199-X

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by Wellcome Trust (grant # 202323/Z/16/Z to IM), European Research Council (grant # 714680 to IM, JM-F); SW was funded by the EMBO (ALTF 454-2017); CG was funded by Wellcome Trust 4 year Ph.D. (203752/Z/16/Z).

- Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol.* (2002) 3:651–62. doi: 10.1038/nrm909
- Ballinger MD, Wells JA. Will any dimer do? *Nat Struct Biol.* (1998) 5:938–40. doi: 10.1038/29111
- Atanasova M, Whitty A. Understanding cytokine and growth factor receptor activation mechanisms. *Crit Rev Biochem Mol Biol.* (2012) 47:502–30. doi: 10.3109/10409238.2012.729561
- Constantinescu SN, Ghaffari S, Lodish HF. The erythropoietin receptor: structure, activation and intracellular signal transduction. *Trends Endocrinol Metab.* (1999) 10:18–23. doi: 10.1016/S1043-2760(98)00101-5
- Livnah O, Stura EA, Middleton SA, Johnson DL, Jolliffe LK, Wilson IA. Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science* (1999) 283:987–90. doi: 10.1126/science.283.5404.987
- Remy I, Wilson IA, Michnick SW. Erythropoietin receptor activation by a ligand-induced conformation change. *Science* (1999) 283:990–3. doi: 10.1126/science.283.5404.990
- Constantinescu SN, Huang LJ, Nam H, Lodish HF. The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. *Mol Cell* (2001) 7:377–85. doi: 10.1016/S1097-2765(01)00185-X
- Constantinescu SN, Keren T, Socolovsky M, Nam H, Henis YI, Lodish HF. Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. *Proc Natl Acad Sci USA.* (2001) 98:4379–84. doi: 10.1073/pnas.081069198
- Staerk J, Defour JP, Pecquet C, Leroy E, Antoine-Poirel H, Brett I, et al. Orientation-specific signalling by thrombopoietin receptor dimers. *EMBO J.* (2011) 30 4398–413. doi: 10.1038/emboj.2011.315
- Matthews EE, Thevenin D, Rogers JM, Gotow L, Lira PD, Reiter LA, et al. Thrombopoietin receptor activation: transmembrane helix dimerization, rotation, and allosteric modulation. *FASEB J.* (2011) 25:2234–44. doi: 10.1096/fj.10-178673
- Gent J, van Kerkhof P, Roza M, Bu G, Strous GJ. Ligand-independent growth hormone receptor dimerization occurs in the endoplasmic reticulum and is required for ubiquitin system-dependent endocytosis. *Proc Natl Acad Sci USA.* (2002) 99:9858–63. doi: 10.1073/pnas.152294299
- Brown RJ, Adams JJ, Pelekanos RA, Wan Y, McKinsty WJ, Palethorpe K, et al. Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat Struct Mol Biol.* (2005) 12:814–21. doi: 10.1038/nsmb977
- Brooks AJ, Dai W, O'Mara ML, Abankwa D, Chhabra Y, Pelekanos RA, et al. Mechanism of activation of protein kinase JAK2 by the growth hormone receptor. *Science* (2014) 344:1249783. doi: 10.1126/science.1249783
- Pillet AH, Laverne V, Pasquier V, Gesbert F, Theze J, Rose T. IL-2 induces conformational changes in its preassembled receptor core, which then migrates in lipid raft and binds to the cytoskeleton meshwork. *J Mol Biol.* (2010) 403:671–92. doi: 10.1016/j.jmb.2010.08.056
- Tenhuberg S, Schuster B, Zhu L, Kovaleva M, Scheller J, Kallen KJ. gp130 dimerization in the absence of ligand: preformed cytokine

- receptor complexes. *Biochem Biophys Res Commun.* (2006) 346:649–57. doi: 10.1016/j.bbrc.2006.05.173
34. Malka Y, Hornakova T, Royer Y, Knoops L, Renauld JC, Constantinescu SN, et al. Ligand-independent homomeric and heteromeric complexes between interleukin-2 or -9 receptor subunits and the gamma chain. *J Biol Chem.* (2008) 283:33569–77. doi: 10.1074/jbc.M803125200
 35. Krause CD, Digioia G, Izotova LS, Xie J, Kim Y, Schwartz BJ, et al. Ligand-independent interaction of the type I interferon receptor complex is necessary to observe its biological activity. *Cytokine* (2013) 64:286–97. doi: 10.1016/j.cyt.2013.06.309
 36. Piehler J, Thomas C, Garcia KC, Schreiber G. Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation. *Immunol Rev.* (2012) 250:317–34. doi: 10.1111/imr.12001
 37. Moraga I, Spangler J, Mendoza JL, Garcia KC. Multifarious determinants of cytokine receptor signaling specificity. *Adv Immunol.* (2014) 121:1–39. doi: 10.1016/B978-0-12-800100-4.00001-5
 38. Spangler JB, Moraga I, Mendoza JL, Garcia KC. Insights into cytokine-receptor interactions from cytokine engineering. *Annu Rev Immunol.* (2015) 33:139–67. doi: 10.1146/annurev-immunol-032713-120211
 39. Ohashi H, Maruyama K, Liu YC, Yoshimura A. Ligand-induced activation of chimeric receptors between the erythropoietin receptor and receptor tyrosine kinases. *Proc Natl Acad Sci USA.* (1994) 91:158–62. doi: 10.1073/pnas.91.1.158
 40. Fujiwara H, Hanissian SH, Tsytsykova A, Geha RS. Homodimerization of the human interleukin 4 receptor alpha chain induces Cepsilon germline transcripts in B cells in the absence of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci USA.* (1997) 94:5866–71. doi: 10.1073/pnas.94.11.5866
 41. Pattyn E, Van Ostade X, Schauvliege L, Verhee A, Kalai M, Vandekerckhove J, et al. Dimerization of the interferon type I receptor IFNAR2–2 is sufficient for induction of interferon effector genes but not for full antiviral activity. *J Biol Chem.* (1999) 274:34838–45. doi: 10.1074/jbc.274.49.34838
 42. Schaeffer M, Schneiderbauer M, Weidler S, Tavares R, Warmuth M, de Vos G, et al. Signaling through a novel domain of gp130 mediates cell proliferation and activation of Hck and Erk kinases. *Mol Cell Biol.* (2001) 21:8068–81. doi: 10.1128/MCB.21.23.8068-8081.2001
 43. Kawahara M, Ogo Y, Tsumoto K, Kumagai I, Ueda H, Nagamune T. Growth control of hybridoma cells with an artificially induced EpoR-gp130 heterodimer. *Cytotechnology* (2006) 52:171–9. doi: 10.1007/s10616-006-9035-2
 44. Heller NM, Qi X, Gesbert F, Keegan AD. The extracellular and transmembrane domains of the gammaC and interleukin (IL)-13 receptor alpha1 chains, not their cytoplasmic domains, dictate the nature of signaling responses to IL-4 and IL-13. *J Biol Chem.* (2012) 287:31948–61. doi: 10.1074/jbc.M112.348896
 45. Moraga I, Spangler JB, Mendoza JL, Gakovic M, Wehrman TS, Krutzik P, et al. Synthetins are surrogate cytokine and growth factor agonists that compel signaling through non-natural receptor dimers. *Elife* (2017). 6:e22882. doi: 10.7554/eLife.22882
 46. Cwirla SE, Balasubramanian P, Duffin DJ, Wagstrom CR, Gates CM, Singer SC, et al. Peptide agonist of the thrombopoietin receptor as potent as the natural cytokine. *Science* (1997) 276:1696–9. doi: 10.1126/science.276.5319.1696
 47. Elliott S, Lorenzini T, Yanagihara D, Chang D, Elliott G. Activation of the erythropoietin (EPO) receptor by bivalent anti-EPO receptor antibodies. *J Biol Chem.* (1996) 271:24691–7. doi: 10.1074/jbc.271.40.24691
 48. Moraga I, Wernig G, Wilmes S, Gryshkova V, Richter CP, Hong WJ, et al. Tuning cytokine receptor signaling by re-orienting dimer geometry with surrogate ligands. *Cell* (2015) 160:1196–208. doi: 10.1016/j.cell.2015.02.011
 49. Rui H, Lebrun JJ, Kirken RA, Kelly PA, Farrar WL. JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerization. *Endocrinology* (1994) 135:1299–306. doi: 10.1210/endo.135.4.7925093
 50. Moraga I, Richter D, Wilmes S, Winkelmann H, Jude K, Thomas C, et al. Instructive roles for cytokine-receptor binding parameters in determining signaling and functional potency. *Sci Signal.* (2015) 8:ra114. doi: 10.1126/scisignal.aab2677
 51. Wilmes S, Beutel O, Li Z, Francois-Newton V, Richter CP, Janning D, et al. Receptor dimerization dynamics as a regulatory valve for plasticity of type I interferon signaling. *J Cell Biol.* (2015) 209:579–93. doi: 10.1083/jcb.201412049
 52. You C, Marquez-Lago TT, Richter CP, Wilmes S, Moraga I, Garcia KC, et al. Receptor dimer stabilization by hierarchical plasma membrane microcompartments regulates cytokine signaling. *Sci Adv.* (2016) 2:e1600452. doi: 10.1126/sciadv.1600452
 53. Ho CCM, Chhabra A, Starkl P, Schnorr PJ, Wilmes S, Moraga I, et al. Decoupling the functional pleiotropy of stem cell factor by tuning c-kit signaling. *Cell* (2017) 168:1041–1052 e18. doi: 10.1016/j.cell.2017.02.011
 54. Kim AR, Ulirsch JC, Wilmes S, Unal E, Moraga I, Karakukcu M, et al. Functional Selectivity in Cytokine Signaling Revealed Through a Pathogenic EPO Mutation. *Cell* (2017) 168:1053–1064 e15. doi: 10.1016/j.cell.2017.02.026
 55. Richter D, Moraga I, Winkelmann H, Birkholz O, Wilmes S, Schulte M, et al. Ligand-induced type II interleukin-4 receptor dimers are sustained by rapid re-association within plasma membrane microcompartments. *Nat Commun.* (2017) 8:15976. doi: 10.1038/ncomms15976
 56. Walter MR. Structural analysis of IL-10 and Type I interferon family members and their complexes with receptor. *Adv Protein Chem.* (2004) 68:171–223. doi: 10.1016/S0065-3233(04)68006-5
 57. de Weerd NA, Samarajiwa SA, Hertzog PJ. Type I interferon receptors: biochemistry and biological functions. *J Biol Chem.* (2007) 282:20053–7. doi: 10.1074/jbc.R70006200
 58. Pestka S. The interferons: 50 years after their discovery, there is much more to learn. *J Biol Chem.* (2007) 282:20047–51. doi: 10.1074/jbc.R70004200
 59. Jaitin DA, Roisman LC, Jaks E, Gavutis M, Piehler J, Van der Heyden J, et al. Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. *Mol Cell Biol.* (2006) 26:1888–97. doi: 10.1128/MCB.26.5.1888-1897.2006
 60. Jaks E, Gavutis M, Uze G, Martal J, Piehler J. Differential receptor subunit affinities of type I interferons govern differential signal activation. *J Mol Biol.* (2007) 366:525–39. doi: 10.1016/j.jmb.2006.11.053
 61. Marijanovic Z, Ragimbeau J, van der Heyden J, Uze G, Pellegrini S. Comparable potency of IFNalpha2 and IFNbeta on immediate JAK/STAT activation but differential down-regulation of IFNAR2. *Biochem J.* (2007) 407:141–51. doi: 10.1042/BJ20070605
 62. Moraga I, Harari D, Schreiber G, Uze G, Pellegrini S. Receptor density is key to the alpha2/beta interferon differential activities. *Mol Cell Biol.* (2009) 29:4778–87. doi: 10.1128/MCB.01808-08
 63. Subramaniam PS, Khan SA, Pontzer CH, Johnson HM. Differential recognition of the type I interferon receptor by interferons tau and alpha is responsible for their disparate cytotoxicities. *Proc Natl Acad Sci USA.* (1995) 92:12270–4. doi: 10.1073/pnas.92.26.12270
 64. Thomas C, Moraga I, Levin D, Krutzik PO, Podoplelova Y, Trejo A, et al. Structural linkage between ligand discrimination and receptor activation by type I interferons. *Cell* 146:621–32. doi: 10.1016/j.cell.2011.06.048
 65. Chill JH, Quadt SR, Levy R, Schreiber G, Anglister J. The human type I interferon receptor: NMR structure reveals the molecular basis of ligand binding. *Structure* (2003) 11:791–802. doi: 10.1016/S0969-2126(03)00120-5
 66. Kalie E, Jaitin DA, Abramovich R, Schreiber G. An interferon alpha2 mutant optimized by phage display for IFNAR1 binding confers specifically enhanced antitumor activities. *J Biol Chem.* (2007) 282:11602–11. doi: 10.1074/jbc.M610115200
 67. Kalie E, Jaitin DA, Podoplelova Y, Piehler J, Schreiber G. The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities. *J Biol Chem.* (2008) 283:32925–36. doi: 10.1074/jbc.M806019200
 68. Roisman LC, Jaitin DA, Baker DP, Schreiber G. Mutational analysis of the IFNAR1 binding site on IFNalpha2 reveals the architecture of a weak ligand-receptor binding-site. *J Mol Biol.* (2005) 353:271–81. doi: 10.1016/j.jmb.2005.08.042
 69. Li H, Sharma N, General IJ, Schreiber G, Bahar I. Dynamic modulation of binding affinity as a mechanism for regulating interferon signaling. *J Mol Biol.* (2017) 429:2571–89. doi: 10.1016/j.jmb.2017.06.011
 70. Mendoza JL, Schneider WM, Hoffmann HH, Vercauteren K, Jude KM, Xiong A, et al. The IFN-lambda-IFN-lambdaR1-IL-10Rbeta complex reveals

- structural features underlying type III IFN functional plasticity. *Immunity* (2017) 46:379–92. doi: 10.1016/j.immuni.2017.02.017
71. Schreiber G. The molecular basis for differential type I interferon signaling. *J Biol Chem.* (2017) 292:7285–94. doi: 10.1074/jbc.R116.774562
 72. Urin V, Levin D, Sharma N, Harari D, Schreiber G. Fine Tuning of a type I interferon antagonist. *PLoS ONE* (2015) 10:e0130797. doi: 10.1371/journal.pone.0130797
 73. Levin D, Schneider WM, Hoffmann HH, Yarden G, Busetto AG, Manor O, et al. Multifaceted activities of type I interferon are revealed by a receptor antagonist. *Sci Signal.* (2014) 7:ra50. doi: 10.1126/scisignal.2004998
 74. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol.* (2010) 28:445–89. doi: 10.1146/annurev-immunol-030409-101212
 75. Chomarat P, Banchereau J. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int Rev Immunol.* (1998) 17:1–52. doi: 10.3109/08830189809084486
 76. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol.* (2012) 13:58–66. doi: 10.1038/ni.2182
 77. Junttila IS, Mizukami K, Dickensheets H, Meier-Schellersheim M, Yamane H, Donnelly RP, et al. Tuning sensitivity to IL-4 and IL-13: differential expression of IL-4Ralpha, IL-13Ralpha1, and gamma c regulates relative cytokine sensitivity. *J Exp Med.* (2008) 205:2595–608. doi: 10.1084/jem.20080452
 78. LaPorte SL, Juo ZS, Vacklavikova J, Colf LA, Qi X, Heller NM, et al. Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell* (2008) 132:259–72. doi: 10.1016/j.cell.2007.12.030
 79. Kelly-Welch AE, Hanson EM, Boothby MR, Keegan AD. Interleukin-4 and interleukin-13 signaling connections maps. *Science* (2003) 300:1527–8. doi: 10.1126/science.1085458
 80. Zhang JL, Buehner M, Sebald W. Functional epitope of common gamma chain for interleukin-4 binding. *Eur J Biochem.* (2002) 269:1490–9. doi: 10.1046/j.1432-1033.2002.02796.x
 81. Zhang JL, Simeonowa I, Wang Y, Sebald W. The high-affinity interaction of human IL-4 and the receptor alpha chain is constituted by two independent binding clusters. *J Mol Biol.* (2002) 315:399–407. doi: 10.1006/jmbi.2001.5243
 82. Andrews AL, Holloway JW, Puddicombe SM, Holgate ST, Davies DE. Kinetic analysis of the interleukin-13 receptor complex. *J Biol Chem.* (2002) 277:46073–8. doi: 10.1074/jbc.M209560200
 83. Andrews AL, Holloway JW, Holgate ST, Davies DE. IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets. *J Immunol.* (2006) 176:7456–61. doi: 10.4049/jimmunol.176.12.7456
 84. Boulanger MJ, Chow D, Brevnova E, Martick M, Sandford G, Nicholas J, et al. Molecular mechanisms for viral mimicry of a human cytokine: activation of gp130 by HHV-8 interleukin-6. *J Mol Biol.* (2004) 335:641–54. doi: 10.1016/j.jmb.2003.10.070
 85. Alami A. Viral mimicry of cytokines, chemokines and their receptors. *Nat Rev Immunol.* (2003) 3:36–50. doi: 10.1038/nri980
 86. Chatterjee M, Osborne J, Bestetti G, Chang Y, Moore PS. Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* (2002) 298:1432–5. doi: 10.1126/science.1074883
 87. Redpath S, Ghazal P, Gascoigne NR. Hijacking and exploitation of IL-10 by intracellular pathogens. *Trends Microbiol.* (2001) 9:86–92. doi: 10.1016/S0966-842X(00)01919-3
 88. Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol.* (2004) 22:929–79. doi: 10.1146/annurev.immunol.22.012703.104622
 89. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol.* (2001) 19:683–765. doi: 10.1146/annurev.immunol.19.1.683
 90. Schreiber G, Walter MR. Cytokine-receptor interactions as drug targets. *Curr Opin Chem Biol.* (2010) 14:511–9. doi: 10.1016/j.cbpa.2010.06.165
 91. Yoon SI, Jones BC, Logsdon NJ, Walter MR. Same structure, different function crystal structure of the Epstein-Barr virus IL-10 bound to the soluble IL-10R1 chain. *Structure* (2005) 13:551–64. doi: 10.1016/j.str.2005.01.016
 92. Jones BC, Logsdon NJ, Josephson K, Cook J, Barry PA, Walter MR. Crystal structure of human cytomegalovirus IL-10 bound to soluble human IL-10R1. *Proc Natl Acad Sci USA.* (2002) 99:9404–9. doi: 10.1073/pnas.152147499
 93. Liu Y, de Waal Malefyt R, Briere F, Parham C, Bridon JM, Banchereau J, et al. The EBV IL-10 homologue is a selective agonist with impaired binding to the IL-10 receptor. *J Immunol.* (1997) 158:604–13.
 94. Vieira P, de Waal-Malefyt R, Dang MN, Johnson KE, Kastelein R, Fiorentino DF, et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1. *Proc Natl Acad Sci USA.* (1991) 88:1172–6. doi: 10.1073/pnas.88.4.1172
 95. MacNeil IA, Suda T, Moore KW, Mosmann TR, Zlotnik A. IL-10, a novel growth cofactor for mature and immature T cells. *J Immunol.* (1990) 145:4167–73.
 96. Go NF, Castle BE, Barrett R, Kastelein R, Dang W, Mosmann TR, et al. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med.* (1990) 172:1625–31. doi: 10.1084/jem.172.6.1625
 97. Becker V, Schilling M, Bachmann J, Baumann U, Raue A, Maiwald T, et al. Covering a broad dynamic range: information processing at the erythropoietin receptor. *Science* (2010) 328:1404–8. doi: 10.1126/science.1184913
 98. Bulut GB, Sulahian R, Ma Y, Chi NW, Huang LJ. Ubiquitination regulates the internalization, endolysosomal sorting, and signaling of the erythropoietin receptor. *J Biol Chem.* 286 6449–57. doi: 10.1074/jbc.M110.186890
 99. Keeler C, Jablonski EM, Albert YB, Taylor BD, Myszkowski DG, Clevenger CV, et al. The kinetics of binding human prolactin, but not growth hormone, to the prolactin receptor vary over a physiologic pH range. *Biochemistry* (2007) 46:2398–410. doi: 10.1021/bi061958v
 100. Claudinon J, Monier MN, Lamaze C. Interfering with interferon receptor sorting and trafficking: impact on signaling. *Biochimie* (2007) 89:735–43. doi: 10.1016/j.biochi.2007.03.014
 101. Shah M, Patel K, Mukhopadhyay S, Xu F, Guo G, Sehgal PB. Membrane-associated STAT3 and PY-STAT3 in the cytoplasm. *J Biol Chem.* (2006) 281:7302–8. doi: 10.1074/jbc.M508527200
 102. German CL, Sauer BM, Howe CL. The STAT3 beacon: IL-6 recurrently activates STAT 3 from endosomal structures. *Exp Cell Res.* (2010) 317:1955–69. doi: 10.1016/j.yexcr.2011.05.009
 103. Reddy CC, Niyogi SK, Wells A, Wiley HS, Lauffenburger DA. Engineering epidermal growth factor for enhanced mitogenic potency. *Nat Biotechnol.* (1996) 14:1696–9. doi: 10.1038/nbt1296-1696
 104. Gandhi H, Worch R, Kurgonaite K, Hintersteiner M, Schwille P, Bokel C, et al. Dynamics and interaction of interleukin-4 receptor subunits in living cells. *Biophys J.* (2014) 107:2515–27. doi: 10.1016/j.bpj.2014.07.077
 105. Payelle-Brogard B, Pellegrini S. Biochemical monitoring of the early endocytic traffic of the type I interferon receptor. *J Interferon Cytokine Res.* (2010) 30:89–98. doi: 10.1089/jir.2009.0044
 106. Avalos BR, Gasson JC, Hedvat C, Quan SG, Baldwin GC, Weisbart RH, et al. Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. *Blood* (1990) 75:851–7.
 107. Irandoust MI, Aarts LH, Roovers O, Gits J, Erkeland SJ, Touw IP. Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor. *EMBO J.* (2007) 26:1782–93. doi: 10.1038/sj.emboj.7601640
 108. Gauzzi MC, Barbieri G, Richter MF, Uze G, Ling L, Fellous M, et al. The amino-terminal region of Tyk2 sustains the level of interferon alpha receptor 1, a component of the interferon alpha/beta receptor. *Proc Natl Acad Sci USA.* (1997) 94:11839–44. doi: 10.1073/pnas.94.22.11839
 109. Waters MJ, Brooks AJ, Chhabra Y. A new mechanism for growth hormone receptor activation of JAK2, and implications for related cytokine receptors. *JAKSTAT* (2014) 3:e29569. doi: 10.4161/jkst.29569
 110. Boyman O, Cho JH, Sprent J. The role of interleukin-2 in memory CD8 cell differentiation. *Adv Exp Med Biol.* (2010) 684:28–41. doi: 10.1007/978-1-4419-6451-9_3
 111. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol.* (2011) 12:551–9. doi: 10.1038/ni.2030

112. Boyman O, Krieg C, Homann D, Sprent J. Homeostatic maintenance of T cells and natural killer cells. *Cell Mol Life Sci.* (2012) 69:1597–608. doi: 10.1007/s00018-012-0968-7
113. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes. *Annu Rev Immunol.* (2018) 36:411–433. doi: 10.1146/annurev-immunol-042617-053352
114. Pellegrini M, Mak TW, Ohashi PS. Fighting cancers from within: augmenting tumor immunity with cytokine therapy. *Trends Pharmacol Sci.* (2010) 31:356–63. doi: 10.1016/j.tips.2010.05.003
115. Smith FO, Downey SG, Klapper JA, Yang JC, Sherry RM, Royal RE, et al. Treatment of metastatic melanoma using interleukin-2 alone or in conjunction with vaccines. *Clin Cancer Res.* (2008) 14:5610–8. doi: 10.1158/1078-0432.CCR-08-0116
116. Klapper JA, Downey SG, Smith FO, Yang JC, Hughes MS, Kammula US, et al. High-dose interleukin-2 for the treatment of metastatic renal cell carcinoma: a retrospective analysis of response and survival in patients treated in the surgery branch at the National Cancer Institute between 1986 and 2006. *Cancer* (2008) 113:293–301. doi: 10.1002/cncr.23552
117. Boyman O, Surh CD, Sprent J. Potential use of IL-2/anti-IL-2 antibody immune complexes for the treatment of cancer and autoimmune disease. *Expert Opin Biol Ther.* (2006) 6:1323–31. doi: 10.1517/14712598.6.12.1323
118. Smith KA. Interleukin-2: inception, impact, and implications. *Science* (1988) 240:1169–76. doi: 10.1126/science.3131876
119. Shanafelt AB, Lin Y, Shanafelt MC, Forte CP, Dubois-Stringfellow N, Carter C, et al. A T-cell-selective interleukin 2 mutein exhibits potent antitumor activity and is well tolerated in vivo. *Nat Biotechnol.* (2000) 18:1197–202. doi: 10.1038/81199
120. Margolin K, Atkins MB, Dutcher JP, Ernstoff MS, Smith JW, 2nd, Clark JI, et al. Phase I trial of BAY 50–4798, an interleukin-2-specific agonist in advanced melanoma and renal cancer. *Clin Cancer Res.* (2007) 13:3312–9. doi: 10.1158/1078-0432.CCR-06-1341
121. Rao BM, Driver I, Lauffenburger DA, Wittrup KD. High-affinity CD25-binding IL-2 mutants potently stimulate persistent T cell growth. *Biochemistry* (2005) 44:10696–701. doi: 10.1021/bi050436x
122. Rao BM, Driver I, Lauffenburger DA, Wittrup KD. Interleukin 2 (IL-2) variants engineered for increased IL-2 receptor alpha-subunit affinity exhibit increased potency arising from a cell surface ligand reservoir effect. *Mol Pharmacol.* (2004) 66:864–9. doi: 10.1124/mol.66.4
123. Rao BM, Girvin AT, Ciardelli T, Lauffenburger DA, Wittrup KD. Interleukin-2 mutants with enhanced alpha-receptor subunit binding affinity. *Protein Eng.* (2003) 16:1081–7. doi: 10.1093/protein/gzg111
124. Levin AM, Bates DL, Ring AM, Krieg C, Lin JT, Su L, et al. Exploiting a natural conformational switch to engineer an interleukin-2 ‘superkine’. *Nature* (2012) 484:529–33. doi: 10.1038/nature10975
125. Mitra S, Ring AM, Amarnath S, Spangler JB, Li P, Ju W, et al. Interleukin-2 activity can be fine tuned with engineered receptor signaling clamps. *Immunity* (2015) 42:826–38. doi: 10.1016/j.immuni.2015.04.018
126. Charych DH, Hoch U, Langowski JL, Lee SR, Addepalli MK, Kirk PB, et al. NKTR-214, an Engineered cytokine with biased IL2 receptor binding, increased tumor exposure, and marked efficacy in mouse tumor models. *Clin Cancer Res.* (2016) 22:680–90. doi: 10.1158/1078-0432.CCR-15-1631
127. Junttila IS, Creusot RJ, Moraga I, Bates DL, Wong MT, Alonso MN, et al. Redirecting cell-type specific cytokine responses with engineered interleukin-4 superkines. *Nat Chem Biol.* (2012) 8:990–8. doi: 10.1038/nchembio.1096
128. Lutz MB, Schnare M, Menges M, Rossner S, Rollinghoff M, Schuler G, et al. Differential functions of IL-4 receptor types I and II for dendritic cell maturation and IL-12 production and their dependency on GM-CSF. *J Immunol.* (2002) 169:3574–80. doi: 10.4049/jimmunol.169.7.3574
129. Shanafelt AB, Forte CP, Kasper JJ, Sanchez-Pescador L, Wetzel M, Gundel R, et al. An immune cell-selective interleukin 4 agonist. *Proc Natl Acad Sci USA.* (1998) 95:9454–8. doi: 10.1073/pnas.95.16.9454
130. Kurgonait K, Gandhi H, Kurth T, Pautot S, Schwiller P, Weidemann T, et al. Essential role of endocytosis for interleukin-4-receptor-mediated JAK/STAT signalling. *J Cell Sci.* (2015) 128:3781–95. doi: 10.1242/jcs.170969
131. Francois-Newton V, Magno de Freitas Almeida G, Payelle-Brogard B, Monneron D, Pichard-Garcia L, Piehler J, et al. USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon alpha response. *PLoS ONE* (2011) 6:e22200. doi: 10.1371/journal.pone.0022200
132. Porritt RA, Hertzog PJ. Dynamic control of type I IFN signalling by an integrated network of negative regulators. *Trends Immunol.* (2015) 36:150–60. doi: 10.1016/j.it.2015.02.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dimensions and Interactions of Large T-Cell Surface Proteins

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OPEN ACCESS

Edited by:

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United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 01 July 2018

Accepted: 06 September 2018

Published: 27 September 2018

Citation:

Junghans V, Santos AM, Lui Y,
Davis SJ and Jönsson P (2018)
Dimensions and Interactions of Large
T-Cell Surface Proteins.
Front. Immunol. 9:2215.
doi: 10.3389/fimmu.2018.02215

The first step of the adaptive immune response involves the interaction of T cells that express T-cell receptors (TCRs) with peptide-loaded major histocompatibility complexes expressed by antigen-presenting cells (APCs). Exactly how this leads to activation of the TCR and to downstream signaling is uncertain, however. Recent findings suggest that one of the key events is the exclusion of the large receptor-type tyrosine phosphatase CD45, from close contacts formed at sites of T-cell/APC interaction. If this is true, a full understanding of how close contact formation leads to signaling would require insights into the structures of, and interactions between, large membrane proteins like CD45 and other proteins forming the glycocalyx, such as CD43. Structural insights into the overall dimensions of these proteins using crystallographic methods are hard to obtain, and their conformations on the cell surface are also unknown. Several imaging-based optical microscopy techniques have however been developed for analyzing protein dimensions and orientation on model cell surfaces with nanometer precision. Here we review some of these methods with a focus on the use of hydrodynamic trapping, which relies on liquid flow from a micropipette to move and trap membrane-associated fluorescently labeled molecules. Important insights that have been obtained include (i) how protein flexibility and coverage might affect the effective heights of these molecules, (ii) the height of proteins on the membrane as a key parameter determining how they will distribute in cell-cell contacts, and (iii) how repulsive interactions between the extracellular parts of the proteins influences protein aggregation and distribution.

Keywords: CD45, hydrodynamic trapping, glycoproteins, kinetic-segregation model, protein dimensions, protein interactions

INTRODUCTION

The high specificity of the adaptive immune system is ensured by the interaction of molecular complexes on the surface of T lymphocytes called T-cell receptors (TCRs) with peptide-loaded major histocompatibility complexes (pMHCs) on antigen presenting cells (APCs). This interaction leads to activation of the TCR and further downstream signaling. However, how the TCR is triggered and initiates signaling is highly debated (1). Different mechanisms, including mechanotransduction (2–4) and receptor clustering (5, 6) to mention two examples, have been argued to play an important part in this. In addition, it has been observed that close contact zones form during the initial contact of T cells with model cell surfaces (7–9) and that the cell-surface bound phosphatase CD45 is partly excluded from these contacts, whereas shorter

molecules such as the TCR and the protein kinase Lck are less affected (**Figure 1A**). This is in contrast to the formation of the supramolecular activation clusters that form minutes after T-cell activation (12, 13), whose organization is dependent on active transport by cytoskeletal motor proteins (14). The kinetic-segregation model has proposed that early, spontaneous molecular reorganization at close contact zones would lead to a local shift in the balance between phosphatases and kinases that results in the phosphorylation of the TCR complex and further downstream signaling if the TCR stays for a sufficiently long period in the CD45-depleted contact zone (15).

The possibility of sterically-based molecular segregation at cellular contacts poses the questions of how large proteins are organized and interact in general as well as the physicochemical mechanism driving segregation in these contacts. Various studies have been undertaken to understand this better including experiments using contacting cells (7, 16–18), immune cells interacting with functionalized surfaces (8, 19–21), and studies of fully artificial model systems (22, 23), to mention a few examples. It has long been argued that the key parameter that would drive segregation is the length of the protein compared to the cell-cell gap (15). However, whereas most of the cell-surface molecules involved in starting an immune response have been structurally characterized (24–26), the structures of the larger isoforms of CD45, as well as other bulky glycoproteins such as CD43, are less defined. Here we review several of the imaging-based optical microscopy techniques that have been developed for analyzing protein dimensions and orientation on model cell surfaces. We focus especially on the use of hydrodynamic trapping, which relies on liquid flow from a micropipette to manipulate membrane-associated molecules. Whereas values for the height and interactions between T-cell proteins would be needed to better understand the principles of the kinetic-segregation model, they will also be important to understand how proteins distribute on cell surfaces and in cell-cell contacts in general.

STRUCTURAL STUDIES OF CD43 AND CD45

The T-cell glycocalyx consists of mainly CD43 and the receptor-type protein tyrosine phosphatase (RPTP) CD45, which are two heavily glycosylated glycoproteins. CD43 is comprised of a mucin-like extracellular region only, whereas CD45 has a folded region as well as variable N-terminal stretches of mucin-like sequence, with mucins being unfolded and extended, serine-, threonine-, and proline-rich polypeptide segments bearing large numbers of O-linked oligosaccharides (27, 28). The particular isoforms of CD45 that are expressed depend on cell type, developmental stage and cell activation state (29). These isoforms arise due to alternative splicing, which alters the length of the mucin-like region, with the smallest (CD45R0) and longest (CD45RABC) having mucin-like regions of 41 and 202 residues, respectively.

The crystal structure of the folded part of CD45 has been determined and comprises a beads-on-a-string arrangement of four fibronectin-like (FN3) domains (8). The organization and

dimensions of the FN3 domains of CD45 are comparable to those in RPTP μ (30), another large type II RPTP, but the domain interfaces are larger, suggesting that flexibility in the extracellular region of CD45 is unhelpful for the function of CD45. Also, the topologies of the two N-terminal-most FN3 domains of CD45 are degenerate, i.e., some of the β -strands are absent or severely truncated, implying that the detail of the structure is unimportant. For this class of proteins, wherein levels of conservation can be as high as >90%, the sequence conservation of CD45 is remarkably low ($\sim$ 18% among mammalian species). The little conservation that there is, is mostly concentrated at the domain interfaces where it likely serves to influence the shape and rigidity of the extracellular domain of CD45, although additional conservation at the top of the folded part might impact on the positioning of the mucin-like region of the protein. The extracellular region of CD45 is also notable for being rich in cysteines that likely stabilize the protein *via* the formation of disulfide bonds; other large type II RPTPs either completely lack, or contain many fewer cysteines. Together, these observations point to the mechanical properties of the folded part of the extracellular domain of CD45 being integral to its function, as would be expected if it acts as a “lever” that excludes the attached cytoplasmic phosphatase from cell-cell contacts.

Obtaining the overall dimensions of glycoproteins using crystallographic methods is often difficult due to the presence of large amounts of sugar-based side chains which tends to work against the formation of crystals. The height of the intact ectodomains of CD43 and CD45 have instead been estimated using electron microscopy. Whereas the single mucin-like extracellular region of CD43 has a length of 45 nm (31), the $\sim$ 15 nm folded region of CD45 combines with the mucin-like segments to give extracellular regions spanning 22 nm (CD45R0) to 40 nm (CD45RABC) (8, 32, 33). Comparisons with TCR-ligand complexes ($\sim$ 15 nm) (10, 11) suggested that even the smallest CD45 isoform, CD45R0, would be sterically excluded from sites of TCR-ligand engagement if CD45 has an “upright” posture at the cell surface (**Figure 1B**).

THE PHYSICAL PROPERTIES OF CELL-SURFACE PROTEINS MEASURED *IN SITU*

Experiments have indicated that rigid membrane-anchored molecules can have markedly different surface heights due to their orientation, covering the range from being upright to lying flat on the surface depending on the conditions and their molecular properties (34–36). Rotation around the membrane-anchoring point can furthermore result in a significant change in effective height (34, 37). Several imaging techniques have been used to measure the effective height of membrane-anchored proteins and the gap size between contacting membranes. We will in this review focus on different optical methods. An alternative to these methods, not discussed here, is electron microscopy coupled with immunogold labeling, as implemented by Davis and colleagues, for example (38).

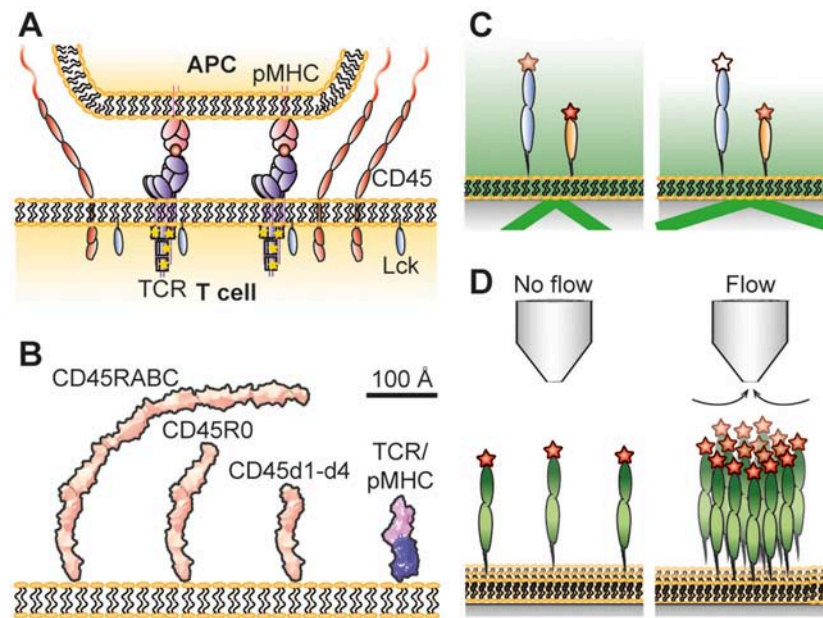


FIGURE 1 | (A) Schematic illustrations of the kinetic-segregation model. CD45 keeps TCR unphosphorylated but gets excluded when TCR binds pMHC. This leads to TCR phosphorylation by Lck and T-cell signaling if the TCR stays long enough in the contact. **(B)** Dimensions of CD45 and the TCR/pMHC complex. The structures of CD45d1-d4 (8), and TCR/pMHC complexes (10, 11) have been determined by crystallography, and that of CD45R0 by negative-staining electron microscopy (8). CD45RABC is modeled on the CD45R0 structure, using the assumption that the mucin-like segment of RABC has the same length/mass ratio as in R0. The R0 and RABC forms of CD45 are each larger than the complex formed by the TCR and pMHC. **(C)** Principle of VA-TIRFM. Fluorophores at various heights will experience a different electromagnetic field when varying the incidence angle of the light, making it possible to determine their vertical position. **(D)** Hydrodynamic trapping uses the liquid flow from a micropipette to locally accumulate molecules. Measuring how much the molecules accumulate at different trap strengths makes it possible to determine the molecules' orientations and the intermolecular interactions between the molecules. Not drawn to scale.

Reflection interference contrast microscopy (RICM) measures the gap size between contacting membranes by utilizing the interference pattern arising from light rays being reflected at different sample interfaces (39). From this information it has been possible to measure the gap size in artificial cell-cell contacts with nanometer precision (23, 40). The gap size in these studies was defined by the dimensions of binding receptor/ligand pairs bridging the two contacting surfaces. Another way to measure the length of receptor/ligand pairs is the microbead-based fluorescence imaging assay developed by Biswas and Groves (41). However, to measure the height of individual membrane proteins requires other methods. Fluorescence interference contrast microscopy (FLIC) (34, 42), scanning angle interference microscopy (SAIM) (43, 44) and variable angle total internal reflection fluorescence microscopy (VA-TIRFM) (8, 45) have all been used for this purpose. FLIC and SAIM are fluorescence-based interference methods, where the interference pattern arises due to different optical path lengths between the fluorophore and a reflective surface. For FLIC this is typically achieved by having oxide terraces of varying thickness between a reflective silicon wafer and the studied sample (42). The interference pattern will be different for each terrace which can be used to fit the effective height of the fluorophore above the surface. This has, for example, been used to measure the effective height of membrane-anchored glycopolymers (34) and reconstituted membrane proteins (36).

SAIM creates the altering interference pattern by varying the incidence angle of the light. This makes it possible to make measurements on more heterogeneous samples compared to FLIC (46). Carbone et al. used this property of SAIM to measure the difference in height inside and outside artificial cell-cell contacts formed *via* a receptor/ligand pair, where the latter were enriched with CD45R0 (22). From this it could be concluded that membrane-anchored CD45R0 stands upright with an effective height similar to the full length of the protein. VA-TIRFM is a non-interferometric technique in which the sample is illuminated with light at an angle that is above that needed for total internal reflection (Figure 1C). This creates an evanescent electromagnetic field that decays exponentially with distance from the surface/sample interface (47). The decay length of the light depends on the incidence angle of the incoming light, and from the resulting change in emitted intensity from a fluorophore it is possible to estimate the vertical distance between the fluorophore and the surface (8). The relative heights of different CD45 isoforms anchored to a supported lipid bilayer have been studied using this technique (8). Both CD45R0 and CD45RABC had a significantly larger height than the folded, mucin-free region (i.e., FN3 domains 1–4 only). Unexpectedly, however, the apparent difference in height between CD45R0 and CD45RABC was only moderate. From these results it appears that the difference in effective height of membrane-anchored CD45RABC and CD45R0 could be much smaller than expected

from the lengths of the proteins. A possible explanation for this, supported by hydrodynamic trapping studies, is that the larger protein can rotate around its anchoring point, leading to a lower effective height.

There are also other fluorescence-based methods that can be used to measure molecular height and orientation. Förster resonance energy transfer has been used extensively to map <10 nm distances and conformational changes in macromolecules (48). A related technique that extends the distance range to 100 nm, but retains the nm resolution, is metal-induced energy transfer (MIET) (49, 50). MIET measures the lifetime of fluorescent molecules above a metallized surface, which changes with the distance to the surface, and has a 3 nm resolution in living cells (50). The orientation of the membrane and membrane-associated molecules can also be estimated using polarization microscopy (51–53). This is based on the fact that the excitation and emission of fluorescent probes depends on the polarization of the incident light relative to the orientation of the fluorescent molecule. The average orientation of a labeled macromolecule can then be estimated if it is known how the fluorescent moiety orients relative to the labeled molecule (51, 52). In addition, there has been a rapid development of various superresolution fluorescence imaging techniques over the last decade with nm resolution along the optical axis (54). This includes, but is not limited to, biplane imaging (55), stochastic optical reconstruction microscopy with optical astigmatism (56), interferometric photoactivated localization microscopy (55), and 4Pi nanoscopy (57, 58). Protein heights can also be measured using two-color fluorescence imaging by relating the position of a dye on the end of the studied protein and another dye in the membrane or on the cytoplasmic part of the protein (18).

HYDRODYNAMIC TRAPPING

Measuring the Height of Membrane-Anchored Molecules

Hydrodynamic trapping uses the focused liquid flow from a micropipette to move and accumulate membrane-anchored molecules (59). It is the drag force on the molecules due to the flow that causes them to move in the direction of flow; larger molecules experience a higher drag force than smaller molecules (60). A micropipette is first positioned above a supported lipid bilayer (SLB) anchoring the studied proteins. After applying negative pressure over the micropipette, the proteins start to accumulate until a steady state concentration profile is reached (Figure 1D). During this process, the surface coverage can be increased by several orders of magnitude (59, 61). The steady state accumulation depends on the dimensions of the protein, with a protein standing upright accumulating more than a protein positioned at an angle (60). The accumulation will also be affected by intermolecular interactions among the proteins. By relating the amount of accumulation to the trap strength at different positions it is possible to determine both the orientation as well as the intermolecular interactions from a single measurement (62).

Using hydrodynamic trapping we found that the largest CD45 isoform, membrane-anchored CD45RABC, interacts with other CD45RABC molecules as if the protein was a 40 nm long rod that is free to rotate around its anchoring point (62). This means that the average height of the protein on the surface would be ~25 nm at low protein densities (Figure 2A). This is similar to the height of membrane-anchored CD45R0 measured by Carbone et al. (22), and can explain why Chang et al. (8) only found a modest difference in height between membrane-anchored CD45R0 and CD45RABC. However, it should be noted that the concentration of both CD43 and CD45 in the cell's glycocalyx is high, ~1,000 molecules/ μm^2 each (8, 63), and so interactions with neighboring proteins will cause CD45 to adopt a more upright position on the cell surface (Figure 2A). Indeed, using Monte Carlo simulations, similar to those described in Junghans et al. (62), we found that this effect would increase the effective height of CD45 by ~5 nm. Thus, the effective height depends not only on the total length of the protein, but also on other properties including flexibility and protein density.

It should be mentioned that only the interaction between the extracellular region of the proteins was studied and that the transmembrane domain of the proteins was replaced with a histidine tag that binds to nickel-chelating lipids in the SLB (62). Whereas this affects the mobility of the proteins compared to their native state on the cell surface, it should only moderately influence the steady state distribution in the hydrodynamic trap. However, it remains to be investigated whether the orientation and interaction of the proteins are affected by the mode of membrane attachment. This could, for example, be studied by comparing height measurements of the proteins on cells, or by using different model systems incorporating the full protein in a cushioned supported lipid bilayer (64).

Measuring Intermolecular Interactions

How the effective height of surface proteins affects protein distribution in cell-cell contacts will be discussed in the next section. However, protein exclusion from cell-cell contacts is also influenced by crowding effects, and it has been observed that proteins smaller than the gap size can also be excluded (16, 18, 23, 65, 66). This can be due to interactions with stationary adhesion molecules in the contact but also with mobile proteins on the contacting surface. This topic has recently been covered in detail (23), however, it should be noted that the more repulsive the interaction between the proteins, the more pronounced the effect of crowding will be. Measuring these interactions can be done using hydrodynamic trapping (62), which will also indicate under what conditions the membrane-anchored molecules will cluster.

Protein rotation and glycosylation can both significantly increase the repulsion between membrane-anchored proteins. In fact, the latter can dominate the interaction between the proteins even if the total molecular weight of the added sugars is only a fraction of the total weight (62). Protein rotation around the anchoring point will also increase repulsion due to more frequent collisions with other proteins (62). Another effect of protein rotation and glycosylation is that a larger attractive energy is required to bring the proteins together into clusters.

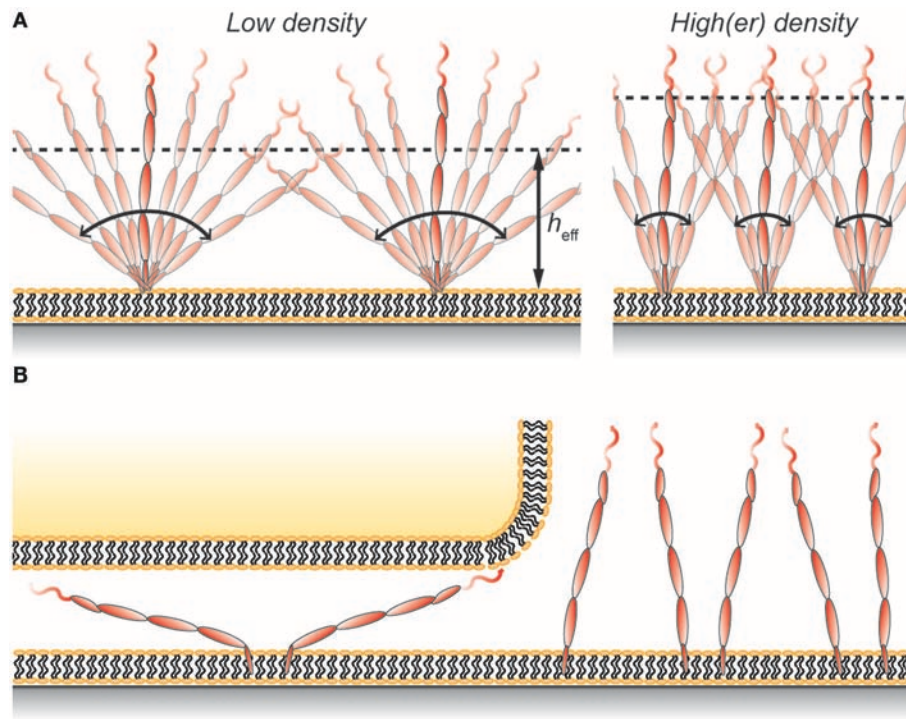


FIGURE 2 | (A) Protein rotation gives rise to an effective height, h_{eff} , that is lower than the full length of the protein. The value of h_{eff} depends, in addition to the properties of the protein, also on the protein density on the membrane. **(B)** Rotating membrane-anchored proteins can fit in gaps smaller than the length of the protein even without deforming the membrane. However, this restricts the possible angles the protein can have and comes at a cost in entropy, which leads to protein exclusion at the contact.

For example, no clustering or aggregation of the extracellular part of CD45RABC was observed when accumulating the protein at a trapping strength of $9 k_B T$ from a surface coverage of 1,000 molecules/ μm^2 (62). Most of this repulsive energy came from free rotation of the protein around the anchoring point. If the rotation can be restricted by other means, such as binding to a receptor on a contacting cell, the repulsive energy would be reduced, and the likelihood of aggregation increase. An example of this is the cadherin adhesion proteins where lateral clustering of the proteins in junctions is amplified by *trans* interactions across the contacting cells (67, 68).

PROTEIN DISTRIBUTION AT CELL-CELL CONTACTS AND ITS DEPENDENCE ON HEIGHT

Proteins larger than the cell-cell gap generally get excluded, but the extent of exclusion can vary significantly depending on the system and the number of adhesion molecules creating the contact (16, 23). In understanding this it must be considered that the cell membrane is not rigid and can therefore bend to encompass molecules larger than the average cell-cell gap. This leads to an increase in energy of the system which is balanced by the cost of excluding a protein from the cell-cell contact (16). The number of adhesion molecules bridging the gap also influences

the exclusion. For a high density of adhesion molecules (10,000 molecules/ μm^2) Schmid et al. found a relatively sharp transition between partially and fully excluded proteins, and that proteins 2 nm larger than the 10 nm gap were essentially completely excluded (23). Alakoskela et al. used a lower density of adhesion molecules (500 molecules/ μm^2) bridging two cell surfaces and forming a 15 nm cell-cell gap (16) and in this case the transition from no exclusion to exclusion was more gradual and even 25 nm diameter spherical particles were only excluded by 40–50%. This value is similar to the 60% exclusion of CD45R0 measured by Chang et al. in a 15 nm gap between a T cell and a supported lipid bilayer with a similar number of adhesion molecules (8). A slightly higher value for the exclusion in the study by Chang et al. agrees with the possibility that only the cell and not the supported lipid bilayer is able to deform and encompass the proteins. Thus, the similar effective height for the rod-like protein (8) and the spherical particle (16) gives comparable exclusion values, despite the different shapes. Having a mixture of adhesion molecules of different length can also influence exclusion. For example, only 10% exclusion of CD45R0 was observed by Cai et al. in a contact between a T cell and a functionalized supported lipid bilayer containing a mixture of 13 and 40 nm adhesion pairs (21).

Using a wholly non-cell based approach, Carbone et al. compared the exclusion of CD45R0 and CD45RABC in a 15 nm gap between a giant unilamellar vesicle and a supported lipid bilayer (22). In these experiments the exclusion of CD45R0

was ~20% whereas CD45RABC was excluded by 40–50% under the same conditions. The lower values compared to those stated by Chang et al. (8) could be due to the vesicle being more flexible than the T-cell membrane, or that the T-cell cytoskeleton exerts additional excluding forces on CD45. But if the effective heights of the two CD45 isoforms are similar, as implied by VA-TIRFM measurements (8), why was the CD45RABC isoform significantly more excluded in the experiments of Carbone et al.? A possible explanation is that the larger protein can rotate around its anchoring point resulting in a smaller effective height. One consequence of this is that the protein can fit into the gap even without deforming the membranes (**Figure 2B**). However, this comes with an entropic penalty since not all surface-to-protein angles will fit the gap. For example, excluding angles that makes the protein height larger than 25 nm (angles $>39^\circ$), which is the approximate height of CD45R0 (22), gives an estimated CD45RABC exclusion that is $(39/90^\circ)^{-1} = 2.3$ times larger than that of CD45R0. A more accurate analysis would have to take molecular interactions as well as additional membrane deformation into account, but this approximation still captures the essential behavior observed by Carbone et al. (22).

CONCLUDING REMARKS

We have discussed that it is not only important to know the molecular dimensions of membrane-anchored proteins to understand how they behave and distribute at cell-cell contacts but also how these proteins orient and interact on the membrane. Fluorescence-based techniques have been developed that allow the effective height of a specific membrane-anchored protein

to be obtained, however, in most of the studies done to date the protein is anchored to a model membrane. These model systems have nevertheless shown that the actual height *in vivo* could be different, especially if the membrane protein composition and density vary, as discussed above. Hence, general methods to measure the height of membrane-anchored molecules on the surface of cells are needed. Super-resolution fluorescence imaging is one technique that has shown promising developments in recent years to achieve this. However, knowing both the effective height of the protein and its crystal structure are not sufficient to predict how the protein will distribute at cell-cell contacts. The interactions between proteins and whether they can rotate also affects this. Hydrodynamic trapping can be used to measure these interactions and combined with knowledge of the protein structure reveal the conditions required for the proteins to partition into domains or aggregate on the membrane. Taking all of this into consideration, complementary techniques are needed to understand the complex behavior of these molecules and how they distribute at cell-cell contacts.

AUTHOR CONTRIBUTIONS

VJ, AS, SD, YL, and PJ wrote the paper. VJ, PJ, and YL made the figures.

FUNDING

This work was supported by grants from the Swedish Research Council (number: 621-2014-3907) and the Wellcome Trust. (number: 207547/Z/17/Z).

REFERENCES

- Chakraborty AK, Weiss A. Insights into the initiation of TCR signaling. *Nat Immunol.* (2014) 15:798–807. doi: 10.1038/ni.2940
- Lee MS, Glassman CR, Deshpande NR, Badgandi HB, Parrish HL, Uttamapinant C, et al. A mechanical switch couples T cell receptor triggering to the cytoplasmic juxtamembrane regions of CD3 ζ . *Immunity* (2015) 43:227–39. doi: 10.1016/j.immuni.2015.06.018
- Gil D, Schrum AG, Alarcón B, Palmer E. T cell receptor engagement by peptide-MHC ligands induces a conformational change in the CD3 complex of thymocytes. *J Exp Med.* (2005) 201:517–22. doi: 10.1084/jem.20042036
- Depoil D, Dustin ML. Force and affinity in ligand discrimination by the TCR. *Trends Immunol.* (2014) 35:597–603. doi: 10.1016/j.it.2014.10.007
- Taylor MJ, Husain K, Gartner ZJ, Mayor S, Vale RD. A DNA-based T cell receptor reveals a role for receptor clustering in ligand discrimination. *Cell* (2017) 169:108–19. doi: 10.1016/j.cell.2017.03.006
- Dushek O, Van der Merwe PA. An induced rebinding model of antigen discrimination. *Trends Immunol.* (2014) 35:153–8. doi: 10.1016/j.it.2014.02.002
- Choudhuri K, Wiseman D, Brown MH, Gould K, van der Merwe PA. T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature* (2005) 436:578–82. doi: 10.1038/nature03843
- Chang VT, Fernandes RA, Ganzinger KA, Lee SF, Siebold C, McColl J, et al. Initiation of T cell signaling by CD45 segregation at “close contacts.” *Nat Immunol.* (2016) 17:574–82. doi: 10.1038/ni.3392
- Santos AM, Ponjavic A, Fritzsche M, Fernandes RA, De La Serna JB, Wilcock MJ, et al. Capturing resting T cells: The perils of PLL correspondence. *Nat Immunol.* (2018) 19:203–5. doi: 10.1038/s41590-018-0048-8
- Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* (1996) 384:134–41. doi: 10.1038/384134a0
- Garcia KC, Degano M, Stanfield RL, Brunmark A, Jackson MR, Peterson PA, et al. An alphabeta T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. *Science* (1996) 274:209–19. doi: 10.1126/science.274.5285.209
- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) 395:82–6. doi: 10.1038/25764
- Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* (1999) 285:221–7. doi: 10.1126/science.285.5425.221
- Ritter AT, Angus KL, Griffiths GM. The role of the cytoskeleton at the immunological synapse. *Immunol Rev.* (2013) 256:107–17. doi: 10.1111/imr.12117
- Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol.* (2006) 7:803–9. doi: 10.1038/ni1369
- Alakoskela J-M, Koner AL, Rudnicka D, Köhler K, Howarth M, Davis DM. Mechanisms for size-dependent protein segregation at immune synapses assessed with molecular rulers. *Biophys J.* (2011) 100:2865–74. doi: 10.1016/j.bpj.2011.05.013
- Köhler K, Xiong S, Brzostek J, Mehrabi M, Eissmann P, Harrison A, et al. Matched sizes of activating and inhibitory receptor/ligand pairs are required

- for optimal signal integration by human natural killer cells. *PLoS ONE* (2010) 5:e15374. doi: 10.1371/journal.pone.0015374
18. James JR, Vale RD. Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature* (2012) 487:64–9. doi: 10.1038/nature11220
 19. Cordoba SP, Choudhuri K, Zhang H, Bridge M, Basat AB, Dustin ML, et al. The large ectodomains of CD45 and CD148 regulate their segregation from and inhibition of ligated T-cell receptor. *Blood* (2013) 121:4295–302. doi: 10.1182/blood-2012-07-442251
 20. Razvag Y, Neve-Oz Y, Sajman J, Reches M, Sherman E. Nanoscale kinetic segregation of TCR and CD45 in engaged microvilli facilitates early T cell activation. *Nat Commun.* (2018) 9:732. doi: 10.1038/s41467-018-03127-w
 21. Cai H, Muller J, Depoil D, Mayya V, Sheetz MP, Dustin ML, et al. Full control of ligand positioning reveals spatial thresholds for T cell receptor triggering. *Nat Nanotechnol.* (2018) 1–8. doi: 10.1038/s41565-018-0113-3
 22. Carbone CB, Kern N, Fernandes RA, Hui E, Su X, Garcia KC, Vale RD. *In vitro* reconstitution of T cell receptor-mediated segregation of the CD45 phosphatase. *Proc Natl Acad Sci USA.* (2017) 114:E9338–45. doi: 10.1073/pnas.1710358114
 23. Schmid EM, Bakalar MH, Choudhuri K, Weichsel J, Ann HS, Geissler PL, et al. Size-dependent protein segregation at membrane interfaces. *Nat Phys.* (2016) 12:704–11. doi: 10.1038/nphys3678
 24. Jones EY, Davis SJ, Williams AF, Harlos K, Stuart DI. Crystal structure at 2.8 Å resolution of a soluble form of the cell adhesion molecule CD2. *Nature* (1992) 360:232–239. doi: 10.1038/360232a0
 25. Yin Y, Wang XX, Mariuzza RA. Crystal structure of a complete ternary complex of T-cell receptor, peptide-MHC, and CD4. *Proc Natl Acad Sci USA.* (2012) 109:5405–10. doi: 10.1073/pnas.1118801109
 26. Yang Y, Jun CD, Liu JH, Zhang R, Joachimiak A, Springer TA, et al. Structural basis for dimerization of ICAM-1 on the cell surface. *Mol Cell* (2004) 14:269–76. doi: 10.1016/S1097-2765(04)00204-7
 27. Cyster JG, Fowell D, Neil Barclay A. Antigenic determinants encoded by alternatively spliced exons of CD45 are determined by the polypeptide but influenced by glycosylation. *Int Immunol.* (1994) 6:1875–81. doi: 10.1093/intimm/6.12.1875
 28. Hanisch F-G. O-Glycosylation of the Mucin Type. *Biol Chem.* (2001) 382:143–9. doi: 10.1515/BC.2001.022
 29. Hermiston ML, Xu Z, Weiss A. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol.* (2003) 21:107–37. doi: 10.1146/annurev.immunol.21.120601.140946
 30. Aricescu AR, Siebold C, Choudhuri K, Chang VT, Lu W, Davis SJ, et al. Structure of a tyrosine phosphatase adhesive interaction reveals a spacer-clamp mechanism. *Science* (2007) 317:1217–20. doi: 10.1126/science.1144646
 31. Cyster JG, Shotton DM, Williams AF. The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear protein epitopes that can be modified by glycosylation. *EMBO J.* (1991) 10:893–902. doi: 10.2307/2667032
 32. McCall MN, Shotton DM, Barclay AN. Expression of soluble isoforms of rat CD45. Analysis by electron microscopy and use in epitope mapping of anti-CD45R monoclonal antibodies. *Immunology* (1992) 76:310–7.
 33. Woollett GR, Williams AF, Shotton DM. Visualisation by low-angle shadowing of the leucocyte-common antigen. A major cell surface glycoprotein of lymphocytes. *EMBO J.* (1985) 4:2827–30.
 34. Godula K, Umbel ML, Rabuka D, Botyanski Z, Bertozzi CR, Parthasarathy R. Control of the molecular orientation of membrane-anchored biomimetic glycopolymers. *J Am Chem Soc.* (2009) 131:10263–8. doi: 10.1021/ja903114g
 35. Parthasarathy R, Rabuka D, Bertozzi CR, Groves JT. Molecular orientation of membrane-anchored mucin glycoprotein mimics. *J Phys Chem B* (2007) 111:12133–5. doi: 10.1021/jp072136q
 36. Kiessling V, Tamm LK. Measuring distances in supported bilayers by fluorescence interference-contrast microscopy: polymer supports and SNARE proteins. *Biophys J.* (2003) 84:408–18. doi: 10.1016/S0006-3495(03)74861-9
 37. Polley A, Orłowski A, Danne R, Gurtovenko AA, Bernardino de la Serna J, Eggeling C, et al. Glycosylation and lipids working in concert direct CD2 ectodomain orientation and presentation. *J Phys Chem Lett.* (2017) 8:1060–6. doi: 10.1021/acs.jpclett.6b02824
 38. McCann FE, Vanherberghen B, Eleme K, Carlin LM, Newsam RJ, Goulding D, et al. The size of the synaptic cleft and distinct distributions of filamentous Actin, Ezrin, CD43, and CD45 at activating and inhibitory human NK cell immune synapses. *J Immunol.* (2003) 170:2862–70. doi: 10.4049/jimmunol.170.6.2862
 39. Limozin L, Sengupta K. Quantitative reflection interference contrast microscopy (RICM) in soft matter and cell adhesion. *ChemPhysChem* (2009) 10:2752–68. doi: 10.1002/cphc.200900601
 40. Fenz SF, Merkel R, Sengupta K. Diffusion and intermembrane distance: case study of avidin and E-cadherin mediated adhesion. *Langmuir* (2009) 25:1074–85. doi: 10.1021/la803227s
 41. Biswas KH, Groves JT. A microbead supported membrane-based fluorescence imaging assay reveals intermembrane receptor–ligand complex dimension with nanometer precision. *Langmuir* (2016) 32:6775–80. doi: 10.1021/acs.langmuir.6b01377
 42. Groves JT, Parthasarathy R, Forstner MB. Fluorescence imaging of membrane dynamics. *Annu Rev Biomed Eng.* (2008) 10:311–38. doi: 10.1146/annurev.bioeng.10.061807.160431
 43. Carbone CB, Vale RD, Stuurman N. An acquisition and analysis pipeline for scanning angle interference microscopy. *Nat Methods* (2016) 13:897–8. doi: 10.1038/nmeth.4030
 44. Paszek MJ, Dufort CC, Rubashkin MG, Davidson MW, Thorn KS, Liphardt JT, et al. Scanning angle interference microscopy reveals cell dynamics at the nanoscale. *Nat Methods* (2012) 9:825–7. doi: 10.1038/nmeth.2077
 45. Ölveczky BP, Periasamy N, Verkman AS. Mapping fluorophore distributions in three dimensions by quantitative multiple angle-total internal reflection fluorescence microscopy. *Biophys J.* (1997) 73:2836–47. doi: 10.1016/S0006-3495(97)78312-7
 46. Ajo-Franklin CM, Ganesan PV, Boxer SG. Variable incidence angle fluorescence interference contrast microscopy for Z-imaging single objects. *Biophys J.* (2005) 89:2759–69. doi: 10.1529/biophysj.105.066738
 47. Stock K, Sailer R, Strauss WSL, Lyttke M, Steiner R, Schneckeburger H. Variable-angle total internal reflection fluorescence microscopy (VA-TIRFM): Realization and application of a compact illumination device. *J Microsc.* (2003) 211:19–29. doi: 10.1046/j.1365-2818.2003.01200.x
 48. Lerner E, Cordes T, Ingargiola A, Alhadid Y, Chung S, Michalet X, et al. Toward dynamic structural biology: Two decades of single-molecule Förster resonance energy transfer. *Science* (2018) 359:eaan1133. doi: 10.1126/science.aan1133
 49. Chizhik AM, Wollnik C, Ruhlandt D, Karedla N, Chizhik AI, Hauke L, et al. Dual-color metal-induced and Förster resonance energy transfer for cell nanoscopy. *Mol Biol Cell* (2018) 29:846–51. doi: 10.1091/mbc.E17-05-0314
 50. Chizhik AI, Rother J, Gregor I, Janshoff A, Enderlein J. Metal-induced energy transfer for live cell nanoscopy. *Nat Photonics* (2014) 8:124–7. doi: 10.1038/nphoton.2013.345
 51. Forkey JN, Quinlan ME, Goldman YE. Measurement of single macromolecule orientation by total internal reflection fluorescence polarization microscopy. *Biophys J.* (2005) 89:1261–71. doi: 10.1529/biophysj.104.053470
 52. Zhanghao K, Chen L, Yang X-S, Wang M-Y, Jing Z-L, Han H-B, et al. Super-resolution dipole orientation mapping via polarization demodulation. *Light Sci Appl.* (2016) 5:e16166. doi: 10.1038/lsa.2016.166
 53. Kabbani AM, Kelly C V. The detection of nanoscale membrane bending with polarized localization microscopy. *Biophys J.* (2017) 113:1782–94. doi: 10.1016/j.bpj.2017.07.034
 54. Sahl SJ, Hell SW, Jakobs S. Fluorescence nanoscopy in cell biology. *Nat Rev Mol Cell Biol.* (2017) 18:685–701. doi: 10.1038/nrm.2017.71
 55. Shtengel G, Galbraith JA, Galbraith CG, Lippincott-Schwartz J, Gillette JM, Manley S, et al. Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure. *Proc Natl Acad Sci USA.* (2009) 106:3125–30. doi: 10.1073/pnas.0813131106
 56. Huang B, Wang W, Bates M, Zhuang X. Three-dimensional super-resolution reconstruction microscopy. *Science* (2008) 319:810–3. doi: 10.1126/science.1153529
 57. Huang F, Sirinakis G, Allgeyer ES, Schroeder LK, Duim WC, Kromann EB, et al. Ultra-high resolution 3D imaging of whole cells. *Cell* (2016) 166:1028–40. doi: 10.1016/j.cell.2016.06.016
 58. Böhm U, Hell SW, Schmidt R. 4Pi-RESOLFT nanoscopy. *Nat Commun.* (2016) 7:1–8. doi: 10.1038/ncomms10504
 59. Jönsson P, McColl J, Clarke RW, Ostanin VP, Jönsson B, Klenerman D, et al. Hydrodynamic trapping of molecules in lipid bilayers. *Proc Natl Acad Sci USA.* (2012) 109:10328–33. doi: 10.1073/pnas.1202858109

60. Jönsson P, Jönsson B. Hydrodynamic forces on macromolecules protruding from lipid bilayers due to external liquid flows. *Langmuir* (2015) 31:12708–18. doi: 10.1021/acs.langmuir.5b03421
61. Johansson B, Höök F, Klennerman D, Jönsson P. Label-free measurements of the diffusivity of molecules in lipid membranes. *ChemPhysChem* (2014) 15:486–91. doi: 10.1002/cphc.201301136
62. Junghans V, Hladilkova J, Santos AM, Lund M, Davis SJ, Jönsson P. Hydrodynamic trapping measures the interaction between membrane-associated molecules. *Sci Rep.* (2018) 8:12479. doi: 10.1038/s41598-018-30285-0
63. Hui E, Vale RD. *In vitro* membrane reconstitution of the T-cell receptor proximal signaling network. *Nat Struct Mol Biol.* (2014) 21:133–42. doi: 10.1038/nsmb.2762
64. Castellana ET, Cremer PS. Solid supported lipid bilayers: from biophysical studies to sensor design. *Surf Sci Rep.* (2006) 61:429–44. doi: 10.1016/j.surfrep.2006.06.001
65. Zhu D-M, Dustin ML, Cairo CW, Golan DE. Analysis of two-dimensional dissociation constant of laterally mobile cell adhesion molecules. *Biophys J.* (2007) 92:1022–34. doi: 10.1529/biophysj.106.089649
66. Jönsson P, Southcombe JH, Santos AM, Huo J, Fernandes RA, McColl J, et al. Remarkably low affinity of CD4/peptide-major histocompatibility complex class II protein interactions. *Proc Natl Acad Sci USA.* (2016) 113:5682–7. doi: 10.1073/pnas.1513918113
67. Wu Y, Vendome J, Shapiro L, Ben-Shaul A, Honig B. Transforming binding affinities from three dimensions to two with application to cadherin clustering. *Nature* (2011) 475:510–3. doi: 10.1038/nature10183
68. Wu Y, Jin X, Harrison O, Shapiro L, Honig BH, Ben-Shaul A. Cooperativity between trans and cis interactions in cadherin-mediated junction formation. *Proc Natl Acad Sci USA.* (2010) 107:17592–7. doi: 10.1073/pnas.1011247107

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OPEN ACCESS

Biophysical Characterization of CD6—TCR/CD3 Interplay in T Cells

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Specialty section:

This article was submitted to
T cell Biology,
a section of the journal
Frontiers in Immunology

Received: 30 May 2018

Accepted: 19 September 2018

Published: 09 October 2018

Citation:

Meddens MBM, Mennens SFB,
Celikkol FB, te Riet J, Kanger JS,
Joosten B, Witsenburg JJ, Brock R,
Figdor CG and Cambi A (2018)
Biophysical Characterization of
CD6—TCR/CD3 Interplay in T Cells.
Front. Immunol. 9:2333.
doi: 10.3389/fimmu.2018.02333

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Activation of the T cell receptor (TCR) on the T cell through ligation with antigen-MHC complex of an antigen-presenting cell (APC) is an essential process in the activation of T cells and induction of the subsequent adaptive immune response. Upon activation, the TCR, together with its associated co-receptor CD3 complex, assembles in signaling microclusters that are transported to the center of the organizational structure at the T cell-APC interface termed the immunological synapse (IS). During IS formation, local cell surface receptors and associated intracellular molecules are reorganized, ultimately creating the typical bull's eye-shaped pattern of the IS. CD6 is a surface glycoprotein receptor, which has been previously shown to associate with CD3 and co-localize to the center of the IS in static conditions or stable T cell-APC contacts. In this study, we report the use of different experimental set-ups analyzed with microscopy techniques to study the dynamics and stability of CD6-TCR/CD3 interaction dynamics and stability during IS formation in more detail. We exploited antibody spots, created with microcontact printing, and antibody-coated beads, and could demonstrate that CD6 and the TCR/CD3 complex co-localize and are recruited into a stimulatory cluster on the cell surface of T cells. Furthermore, we demonstrate, for the first time, that CD6 forms microclusters co-localizing with TCR/CD3 microclusters during IS formation on supported lipid bilayers. These co-localizing CD6 and TCR/CD3 microclusters are both radially transported toward the center of the IS formed in T cells, in an actin polymerization-dependent manner. Overall, our findings further substantiate the role of CD6 during IS formation and provide novel insight into the dynamic properties of this CD6-TCR/CD3 complex interplay. From a methodological point of view, the biophysical approaches used to characterize these receptors are complementary and amenable for investigation of the dynamic interactions of other membrane receptors.

Keywords: T cell, immunological synapse, T-cell receptor (TCR), CD3, CD6, membrane receptor, receptor dynamics

INTRODUCTION

T cells play an important role in the execution of the adaptive immune response by regulating the activity of innate and other adaptive immune cells or directly executing effector functions, such as killing by cytotoxic T cells. In general, for T cells to execute their function, they need to become activated by antigens through interaction with an antigen-presenting cell (APC). Crucial to this activation is the interaction between the T cell receptor (TCR) on the T cell surface and the peptide-Major Histocompatibility Complex (pMHC) on the APC surface. Immediately after recognition of the pMHC, the TCR, associated with the CD3 receptor complex, combines with co-stimulatory receptors CD4/CD8 and CD28 on the T cell membrane in small so-called TCR microclusters where signaling is initiated (1, 2). During the T cell-APC contact, TCR microclusters are laterally transported during local cell surface receptor rearrangement creating a typical bull's eye-shaped pattern at the T cell-APC interface, termed “the immunological synapse” (IS) (3, 4). This lateral TCR microcluster transport results in TCR accumulation in the center of the IS, forming the central supramolecular activation cluster or “cSMAC,” together with co-stimulatory molecules such as CD2, CD4/CD8 and CD28 (4–6). Surrounding the central cSMAC is the peripheral supramolecular activation cluster (pSMAC), that exists of adhesion receptor LFA-1 and phosphatase CD45, both kept from the cSMAC due to size-dependent exclusion (7). This spatial organization of the receptors, together with the transport of TCR microclusters toward the cSMAC is dependent on the actomyosin cytoskeleton, which is excluded from the cSMAC region (8–11). Antigen binding on the extracellular side leads intracellularly to recruitment of tyrosine kinase Lck to the TCR/CD3 complex, where it phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tail of CD3 chains (12). Subsequently, tyrosine kinase ZAP70 can bind to the phosphorylated ITAM-motifs and recruit the transmembrane protein LAT (12, 13). LAT forms a signaling hub, the so-called LAT signalosome, which through various signaling molecules such as SLP-76 and GRB2, initiates downstream events, such as calcium fluxing, actin reorganization, integrin inside-out signaling and gene expression, leading to T cell activation and effector functions (12, 14).

CD6 is one of the cell surface co-receptors on the T cell membrane involved in T cell activation. CD6 is a transmembrane glycoprotein, part of the scavenger receptor cysteine-rich superfamily (SRCR-SF), that is expressed on thymocytes, mature T cells, a subset of B cells and NK cells, and brain parenchymal cells (15–18). On the T cell membrane, CD6 associates with its closely related family member CD5 (17, 19). Extracellularly, ligands for CD6 are Activated Leukocyte Cell Adhesion Molecule (ALCAM), which is present on antigen presenting cells and thymic epithelial cells, and the recently identified CD318, a glycoprotein expressed on epithelial cells, some hematopoietic cells and mesenchymal stem cells (16, 20–22).

Already early on, it was clear that CD6 was involved in T cell activation in mature T cells, since monoclonal antibodies targeting CD6 were able to induce T cell activation, subsequent

T cell proliferation and IL-2 receptor expression (23, 24). Since then, multiple studies have further substantiated a co-stimulatory role of CD6 in T cell activation (25–29). However, more recently this view was challenged by data from Oliveira and colleagues, who describe a role for CD6 as attenuator of early and late T cell responses in a ligand-independent manner (30). The exact role for CD6 in T cell signaling is therefore still under debate and most likely depends on a balance between stimulatory and inhibitory signals, provided among others by binding of its ligand (30, 31).

Multiple data hint at an interaction, either direct or indirect, between CD6 and the TCR. Co-precipitation studies have indicated that rat CD6 associates with protein kinases Lck, Fyn, ZAP-70, and Itk: protein kinases that also interact with and co-precipitate with the TCR or are part of the LAT signalosome (14, 32). This interaction is important for CD6 signaling, as inhibition of protein kinases abolishes CD6-induced T cell proliferation (26). Furthermore, CD6 physically associates with adaptor protein SLP-76 (33), which is involved in TCR microcluster signaling. Also, direct cross-linking of CD3 induces phosphorylation of CD6, which suggests cross-talk between TCR/CD3 complex and CD6 (34). More importantly, using co-precipitation Gimferrer and colleagues showed that CD6 and the TCR/CD3 complex interact (independently of CD5) (35). Also, co-localization of CD6 and TCR/CD3 in the cSMAC of the mature IS has been described through co-capping, FRET and DC-T cell co-cultures (29, 35). CD6 is important for mature IS formation as treatment with soluble recombinant CD6 leads to inhibition of IS maturation and resulted in inhibition of T cell proliferation (35).

Importantly, CD6 has recently reclaimed attention as a focus of research: the *CD6* gene, together with the gene for its ligand ALCAM, was identified as a susceptibility locus and a potential target for treatment of multiple sclerosis (36, 37). Furthermore, antibodies targeting CD6 are tested for treatment of various autoimmune diseases, such as psoriasis and rheumatoid arthritis (38–41). This renewed interest in CD6 underlines the importance of understanding CD6 signaling and interaction at the molecular level. For instance, although static co-localization of CD6 and TCR/CD3 complexes has been reported at the fully mature IS and signaling cross-talk between CD6 and CD3 has been identified, thorough characterization of (early) dynamics during IS formation and stability of CD6-TCR/CD3 interplay at the mature IS are still lacking.

Imaging techniques with high spatiotemporal resolution, such as Total Internal Reflection Fluorescence (TIRF) Microscopy, combined with biochemical or immunological assays, such as supported lipid bilayers (42), have been fundamental in unraveling the dynamics of multiple protein-protein interactions during IS formation (1, 11, 13). Here, we exploited different biophysical approaches including microcontact printing, fluorescence microscopy techniques, antibody-coated beads and magnetic tweezers to study the dynamics and stability of CD6-TCR/CD3 interplay in more detail. Overall, our findings provide novel insight into the dynamic properties of CD6—TCR/CD3 complex interplay during IS formation.

MATERIALS AND METHODS

Cell Lines and Transfection

Jurkat E6.1 lymphoma T cells were maintained in 1640 RPMI (PAA) supplemented with 10% Fetal Calf Serum (Greiner Bio-one), 1 mM Ultra-glutamine (U-glut, PAA) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, PAA). Jurkat cell lines stably expressing CD6-RFP, CD6-GFP, or LifeAct-GFP were obtained by electroporation using the Neon Transfection System for Electroporation (Invitrogen) according to the manufacturer's guidelines. Shortly, 5×10^5 Jurkat cells were transfected at 1325 Volt (10 ms, 3 pulses) with 3 µg of DNA in 100 µl Resuspension buffer. After transfection cells were seeded in 2 ml of 1640 RPMI with 10% FCS and 1% U-glut. Antibiotics were added after 3 h. Stable cell lines were sorted on RFP or GFP expression on a FACSaria cell sorter (BD Biosciences), and cells were maintained in complete RPMI medium as described above, additionally supplemented with 500 ng/ml geneticin (G418, Gibco).

Antibodies, Reagents and Expression Constructs

The following primary antibodies were used: Mouse IgG2A-anti-human CD3 antibodies clone T3B and clone OKT-3 (both referred to in the text as α CD3), and Mouse IgG1 anti-human LFA-1 antibody TS2/4 were obtained from in-house hybridoma production. Mouse IgG1 anti-human phosphotyrosine (P-Tyr-100), both unconjugated and conjugated to Alexa488, was obtained from Cell Signaling Technology; Mouse IgG1 anti-human CD6 (M-T605; referred to in the text as α CD6) was obtained from BD Biosciences. The following secondary antibodies were used: Goat anti-Rabbit-IgG(H+L)-Alexa647 and Goat-anti-Mouse-IgG1-Alexa488 (both from Invitrogen). Neutravidin-TexasRed was obtained from Thermo Fisher Scientific. For use in immunofluorescence staining, anti-CD3 antibody clone OKT-3 was biotinylated (Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific) at RT for 1.5 h, with a molecular ratio of IgG:Biotin at 1:15. Following the same procedure, for use in supported lipid bilayers, anti-human CD3 antibody OKT-3 was simultaneously biotinylated and conjugated to ATTO647 Carboxylic Acid, Succinimidyl ester (ATTO-TEC) at a molecular ratio of IgG:Biotin:dye at 1:15:15. In both cases, purification was performed with Zeba Desalting columns (Thermo Fisher Scientific). For preparation of supported lipid bilayers, the lipids POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and Biotin Capped PE (1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[Cap Biotinyl]), both from Avanti Polar Lipids Inc, were used, together with the fluorescent lipophilic tracer DiI (1,1'-Diocadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate; Invitrogen). For inhibition of actin polymerization Cytochalasin D (CytoD) was used (2.5 µg/ml, Sigma-Aldrich). The CD6-GFP plasmid was generated by cloning CD6 from the CD6-RFP construct into pGFP-N1 (Clontech) (29). LifeAct-GFP (43) was a kind gift of Michael Sixt (Institute of Science and Technology, Vienna, Austria).

Micro Contact Printing of Antibody Spots

PDMS stamps containing a regular pattern of 5 µm circular spots were prepared as described earlier (44). PDMS stamps were incubated for 1 h at RT with a protein solution containing 15 µg/ml Goat-anti-Rabbit IgG(H+L)-Alexa647 antibody to visualize the spots, and anti-human CD6 or anti-human CD3 clone T3B, the latter including (if indicated) mouse IgG2A isotype control antibody, to create spots containing 1 or 10% α CD3. Total concentration of primary antibody in the protein solution amounted to 100 µg/ml. After incubation, the stamps were thoroughly washed with distilled H₂O and dried under a N₂ stream. A glass microscope slide was cleaned by rinsing consecutively with distilled H₂O, 70% ethanol and 100% acetone, and was dried under a N₂ stream. The stamp was then manually pushed on the cleaned glass microscope slide for 20 s and removed, after which the stamped area was back-filled with 20 µg/ml fibronectin (from human plasma; Roche) in PBS for 1 h at RT. The microscope slide was washed in PBS and incubated with 1% BSA for 30 min to block all uncoated glass surface. The slide was subsequently washed with PBS and dried under a N₂ stream before cell seeding.

Preparation of Supported Lipid Bilayers (SLBs)

Coverslips were cleaned in 2% v/v Hellmanex III (Hellma-Analytix) solution and sonified for 15 min at RT after which they were rinsed with ultra clean water and ethanol and dried under a N₂ stream. SLBs were prepared by spin coating (45). To form SLBs, a lipid chloroform mixture containing 1 mM POPC, 0.01 mM Biotin Capped PE, supplemented with DiI, was dropped on a spinning coverslip. SLBs were hydrated with Hank's Balanced Salt Solution (HBSS; Gibco) throughout the preparation. After deposition, nonspecific binding was blocked by incubation with 10 mg/ml BSA in HBSS. Subsequently, SLBs were incubated with 0.5 µg/ml streptavidin (Thermo Fisher scientific). Finally, SLBs were incubated with 0.5 µg/ml biotinylated anti-human CD3 (OKT3)-ATTO647 for 15 min at RT, after which they were used directly for cell seeding.

Immunofluorescence

Immunofluorescent staining of CD3 was performed on wildtype Jurkat T cells on microprinted antibody spots. Immunofluorescent staining of phospho-tyrosine was performed on wildtype Jurkat T cells on microprinted antibody spots and on wildtype Jurkat T cells on SLBs. LFA-1 staining was performed on wildtype Jurkat T cells on SLBs. Cells were seeded on spots or SLBs for 15–30 min at 37°C. Samples were washed with PBS and subsequently fixed with 4% PFA in PBS for 15 min at RT. After fixation, samples were blocked for 1 h with blocking solution (PBS/3% BSA/10 mM glycine/1% human serum) at RT. For CD3 and phospho-tyrosine staining on antibody spots and for phospho-tyrosine staining on SLBs, permeabilization was performed simultaneously with blocking by adding 0.1% saponin to the blocking solution. 0.1% saponin was added to all subsequent antibody staining solutions. For LFA-1 staining, after blocking, cells on SLBs were incubated with primary Mouse

IgG1 anti-human LFA-1 antibody TS2/4 and subsequently Goat-anti-Mouse-IgG1-Alexa488. For phospho-tyrosine staining, after blocking/permeabilization, cells on antibody spots were incubated with Mouse IgG1 p-Tyr-100-Alexa488; cells on SLBs were incubated with primary Mouse IgG1 p-Tyr-100 and subsequently Goat-anti-Mouse-IgG1-Alexa488. For CD3 staining, after blocking/permeabilization, cells on antibody spots were incubated with OKT3-biotin and subsequently NeutrAvidin-Texas-Red. After immunofluorescence staining, samples on microprinted antibody spots were washed with phosphate buffer and embedded in Mowiol (Sigma-Aldrich). Immunofluorescence samples on SLBs were not embedded but imaged in PBS directly after preparation. To study the effect of inhibition of actin polymerization on IS formation, CD6-GFP Jurkat cells were taken from culture and incubated in HBSS with or without 0.5 μ M Cytochalasin D for 15 min at 37°C at a concentration of 800,000 cells per ml. Next, cell suspensions were added onto α CD3-containing SLBs, reaching a final cell concentration of 400,000 cells per ml. Samples were incubated for 30 min at 37°C. After incubation, samples were fixed with 4% PFA in PBS for 15 min at RT. Finally samples were washed once and imaged in PBS directly after preparation. CD6-GFP, α CD3-ATTO647, DiI, and brightfield signals were acquired. Samples of cells seeded on microprinted antibody spots were imaged on an Olympus FV1000 confocal laser scanning microscope with a 60 $\times$ 1.35 NA oil immersion objective. Samples of cytochalasin D treated cells on SLBs were imaged using a Leica DMI6000 widefield microscope equipped with a HC PL APO 63 $\times$ 1.40 NA oil immersion objective. Samples of LFA-1 and phospho-tyrosine staining in cells on SLBs were imaged with TIRF microscopy at an Olympus IX-71 wide field fluorescence microscope equipped with a 3-line TIRF system and a Hamamatsu ImagEM EM-CCD camera equipped with a PL APO 60 $\times$ /1.4 NA oil immersion TIRF objective.

Live Cell Imaging on SLBs

Live cell imaging in cells on SLBs was performed at 37°C with TIRF microscopy at the Olympus TIRF microscope setup described above. Prior to live cell imaging, Jurkat cells (LifeAct-GFP or CD6-GFP) were washed with PBS and resuspended in HBSS. Cells were added to the SLBs at the microscope, in a final concentration of 400,000 cells per ml HBSS. Directly after adding the cells, both cells and α CD3-ATTO647 coupled to the SLBs were imaged. Images were acquired at a frame rate of 300 ms/frame or 1 s/frame with an exposure time of 10–100 ms.

Cell-Bead Contact Experiments

Dynal CD3 beads coated with mouse monoclonal anti-CD3 antibody (Invitrogen) or fibronectin-coated beads, all with a diameter of 4.5 μ m, were used for bead experiments. Jurkat CD6-GFP cells were seeded on fibronectin-coated coverslips in imaging medium (RPMI 1640, 25 mM HEPES, 0.5% BSA). Subsequently, beads were added to the cells in a concentration of 5 μ M. Imaging of the CD6-GFP signal and the brightfield channel of cells with beads was performed on a Zeiss LSM510 meta confocal laser scanning microscope

equipped with a PL APO 63 $\times$ /1.4 NA oil immersion objective. Cells were imaged at RT to slow down internalization of the beads.

FRAP Measurements

All FRAP measurements were performed on a Zeiss LSM510 meta confocal laser scanning microscope equipped with a PL APO 63 $\times$ /1.4 NA oil immersion objective. For FRAP on antibody spots, Jurkat CD6-GFP cells were resuspended in phenol red-free medium, seeded onto microprinted surfaces and imaged at 37°C. FRAP was performed using a 2.1 μ m diameter circular region of interest (ROI). Photobleaching was performed at 100% laser power by scanning the bleached ROI for two iterations, yielding a total bleach time of 0.10 s and an average fluorescence loss of $\sim$ 50%. Recoveries were collected with time intervals of 200 ms using 488 nm excitation. Fluorescence intensity data for the bleached ROI and a control ROI were calculated using LSM software (Zeiss). After background correction and normalization to t_0 using a method that is known as double normalization (46), the single post-bleach curves were fitted using Origin (OriginLab) with the following model:

$$I(t) = A * e^{-t/\tau} \quad (1)$$

where $I(t)$ is the intensity in the bleached ROI at time t , A is the mobile fraction, and τ is the characteristic recovery time. The half-time recovery $t_{0.5}$ was calculated with:

$$t_{0.5} = \ln 2 * \tau \quad (2)$$

For FRAP measurements on cells in contact with beads, Jurkat CD6-GFP cells were resuspended in phenol red-free medium, incubated with α CD3-coated beads and seeded on fibronectin-coated surfaces. FRAP was performed using a 2 $\times$ 1 μ m rectangular ROI. Photobleaching was performed at 100% laser power by scanning the bleached ROI for 20 iterations, yielding a total bleach time of 1 s and average fluorescence loss of $\sim$ 50%. Recoveries were collected with time intervals of 100 ms using 488 nm excitation. After background correction and single normalization, FRAP curves were fitted using the Ellenberg fitting (47) with the help of FRAPAnalyser software (48):

$$I(t) = I_{\text{final}} \left(1 - (w^2(w^2 + 4\pi Dt)^{-1})^{1/2} \right) \quad (3)$$

Where $I(t)$ is the fluorescence intensity as a function of time, I_{final} the final intensity reached after complete recovery, w the width of the rectangular ROI, and D is the one-dimensional diffusion constant. Recovery half-time $t_{0.5}$ was calculated using the formula (49):

$$D = \frac{0.88w^2}{4t_{0.5}} \quad (4)$$

Image Analysis

Image analysis was performed using Fiji Image J (50). To quantify the immunofluorescence images of cells on microprinted spots, a custom image analysis algorithm was used. Shortly, spots were segmented based on an intensity threshold applied to the spots channel. Cells were segmented using an edge finding algorithm applied to the DIC image and a selection of objects based on size. Next, the segmentations of the spots and cells were combined resulting in masks for cellular parts covering the spots and cellular parts covering the surrounding area. These masks were subsequently used to measure the intensity of the fluorescent protein or immunostaining on the spots and the surrounding area. Enrichment of CD6 was quantified by measuring the ratio of the fluorescent intensity in parts of the cells covering the spots and the fluorescent intensity in parts of the cells covering the surrounding area coated with fibronectin. A value of 1 indicates no recruitment, while values higher or lower than 1 indicate recruitment or exclusion, respectively. In Jurkat CD6-GFP cells on SLBs, kymographs were created along the indicated lines using the *Dynamic Reslice* option under *Image>Stacks* in FIJI Image J for the indicated time periods. Co-localization of CD6-GFP and α CD3 during immunological synapse formation was determined using the JACoP plugin in FIJI Image J (51). A ROI in the central part of the cell-SLB interface was selected and the same size ROI was applied to each time point and to all cells analyzed. Co-localization was quantified by determining the Mander's Coefficient M1 by making use of appropriate thresholding which only included CD6- and α CD3-rich microclusters or the cSMAC. Also the Mander's Coefficient M2 (with same thresholding as for M1) and the Pearson Coefficient over time were determined. Relative CD6-GFP signal intensity in the same ROI over time was determined by calculating the integrated density of the total ROI and relating it to the integrated density at $t = 2$ min (start of immunological synapse formation). The fraction of cells forming an immunological synapse on SLBs upon Cytochalasin D treatment was determined by manual counting. Cells having formed an immunological synapse were defined as CD6-GFP positive cells, also visible in brightfield, on top of SLB (DiI-positive area), overlaying an α CD3 positive cluster. In bead experiments, CD6 enrichment was determined as the ratio between CD6-GFP fluorescence intensity of the membrane area of cell that was in contact with the bead and the fluorescence intensity in an equal sized ROI in the membrane of the cell at the opposite side of bead contact.

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism version 5.03. Data are presented as mean $\pm$ standard deviation for bar plots and median $\pm$ interquartile range for box plots. To compare two groups, a paired/unpaired *t*-test was applied. To compare three or more groups, one-way ANOVA with *post-hoc* Tukey's Multiple Comparison test or Kruskal–Wallis test with *post-hoc* Dunn's Multiple Comparison test was applied. Differences were considered statistically significant at $p < 0.05$.

RESULTS

CD6 and TCR/CD3 in Jurkat T Cells Co-localize Upon Ligation Through Micropatterned Antibody Spots

Although CD6 has been recognized as a TCR co-receptor, the nature of the interaction with the TCR/CD3 complex has not been resolved. To provide a biophysical characterization of the interplay of these receptors during IS formation, we first studied CD6 and TCR/CD3 (co-)localization using antibody spots created with microcontact printing (**Figure 1**) (52). Wildtype Jurkat T lymphoma cells were seeded on microprinted antibody spots (5 μ m in diameter) that were composed of 100% α CD6 or different concentrations of α CD3 (1–10–100%; diluted with mouse IgG2A-isotype control antibody) surrounded by fibronectin. Intracellular signaling through phospho-tyrosine (pTyr) was visualized by immunofluorescence staining on fixed cells (**Figure 1A**). Clustering of CD3 led to intracellular signaling as quantification of the pTyr fluorescence intensity levels in parts of the cells that covered the spots demonstrate a concentration-dependent increase on α CD3 spots (**Figure 1B**). Cells seeded on 1% α CD3 spots did not show any significant increase in pTyr levels compared to the 100% fibronectin (FN) spots (negative control) (**Figure 1B**). Next to clustering of CD3 also clustering of CD6 (using 100% α CD6 spots) induced T cell signaling; pTyr intensity on 100% α CD6 spots is comparable to the pTyr intensity on 10% α CD3 spots and significantly different from the intensity on 1% α CD3 and 100% FN spots. These results show that sufficient levels of cross-linking of CD3 or CD6 by microprinted antibody spots induce T cell signaling by increasing pTyr levels, an event generally leading to activation of Jurkat T cells.

To investigate the recruitment of CD6 upon cross-linking of the TCR/CD3 complex, we created Jurkat T cells stably expressing CD6-RFP or CD6-GFP. Total cell surface expression of CD6 as well as GFP and RFP expression in CD6-GFP cells, CD6-RFP cells and wildtype cells was determined with flow cytometry (**Supplementary Figure 1**). CD6-RFP Jurkat T cells were seeded onto the microprinted antibody spots (**Figures 1C,D**). As expected, confocal microscopy images show a strong recruitment of CD6-RFP to 100% α CD6 spots. Furthermore, a concentration-dependent recruitment of CD6-RFP was observed on 1–100% α CD3 antibody spots (**Figure 1C**). Quantification of CD6-RFP enrichment on the spots confirmed the concentration-dependent effect of α CD3 antibody in the spots on CD6-RFP enrichment. Also, CD6 enrichment to spots containing 100% α CD3 was comparable to that observed in the positive control, 100% α CD6 spots (**Figure 1D**). These results demonstrate that cross-linking of the TCR/CD3 complex induces recruitment of CD6 to spots. Consequently, we investigated whether cross-linking of CD6 also induces recruitment of the TCR/CD3 complex. To this end, wildtype Jurkat T cells were seeded on the microprinted antibody spots and stained for endogenous TCR/CD3 complex recruitment using a biotinylated α CD3 antibody and fluorescently labeled

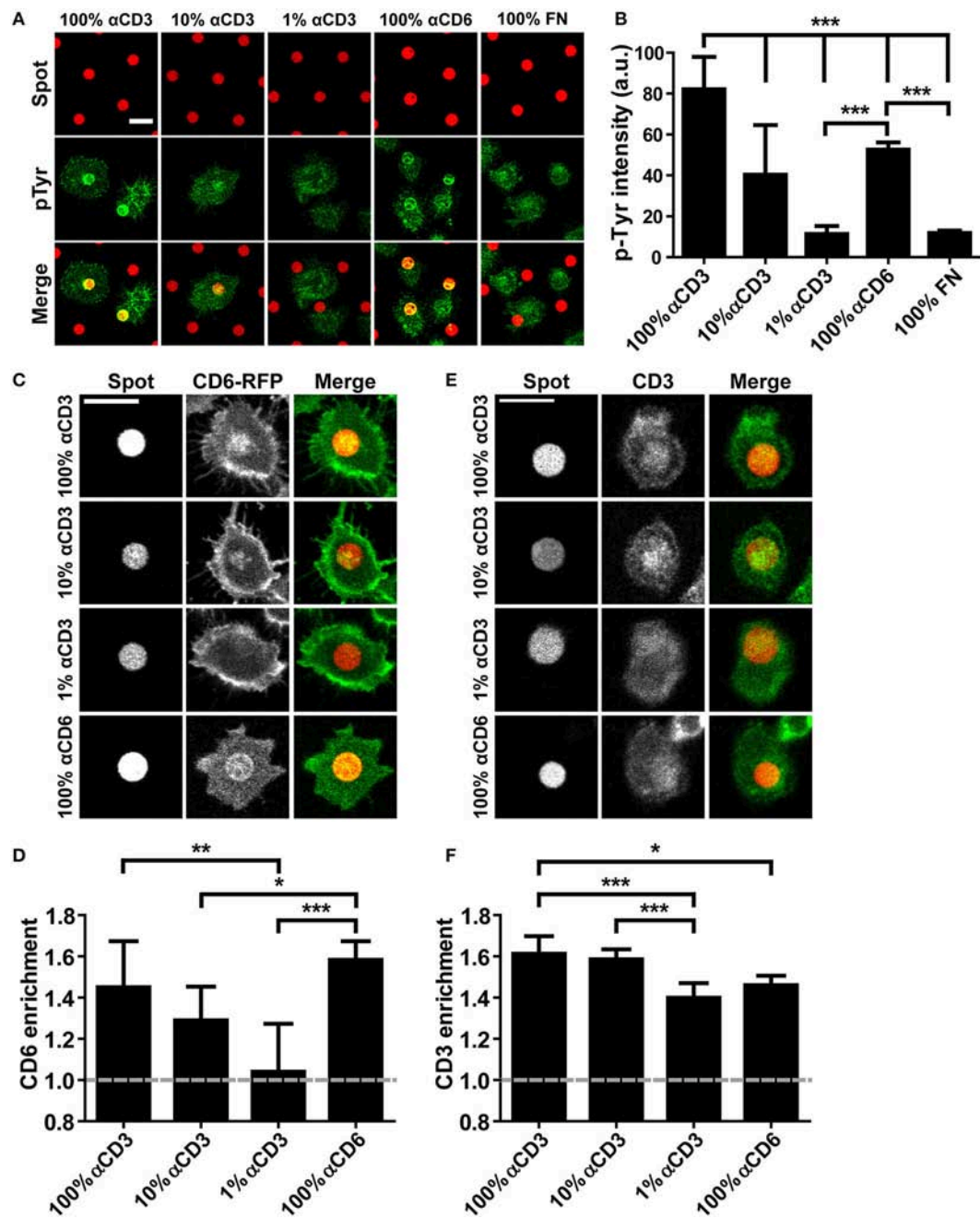


FIGURE 1 | CD6 and TCR/CD3 in Jurkat T cells co-localize upon ligation through micropatterned antibody spots. **(A,B)** Wildtype Jurkat T cells were seeded on micropatterned substrates containing 100, 10, or 1% αCD3 spots, 100% αCD6 spots or 100% fibronectin (FN) spots, surrounded by fibronectin, fixed after 15 min and stained for phospho-tyrosine. All spots were labeled with Alexa647, phospho-tyrosine was labeled with Alexa488. Representative confocal images are shown in **(A)**, quantification of phospho-tyrosine intensity in the spot area of $n = 10$ cells per condition is shown in **(B)**. Bars represent mean with SD. Statistical significance was tested with one-way ANOVA with *post-hoc* Tukey's Multiple Comparison test. **(C,D)** CD6-RFP Jurkat T cells were seeded on micropatterned substrates containing 100, 10, or 1% αCD3 spots, or 100% αCD6 spots, surrounded by fibronectin, and fixed after 15 min. All spots were labeled with Alexa647. Representative confocal images are shown in **(C)**, quantification of CD6 enrichment of $n = 10$ cells per condition is shown in **(D)**. CD6 enrichment is defined as the ratio between CD6-RFP intensity of parts of the cell on the spot vs. part of the cell covering the surrounding fibronectin. Bars represent mean with SD. Statistical significance was tested with Kruskal–Wallis test with *post-hoc* Dunn's Multiple Comparison test. **(E,F)** Wildtype Jurkat cells were seeded on micropatterned substrates containing 100, 10, or 1% αCD3 spots, or 100% αCD6 spots, surrounded by fibronectin, fixed after 15 min and stained for CD3. All spots were labeled with Alexa647, CD3 was labeled with TexasRed. Representative confocal images are shown in **(E)**, quantification of CD3 enrichment of $n = 10$ cells per condition is shown in **(F)**. Bars represent mean with SD. CD3 enrichment is defined as the ratio between CD3 intensity of parts of the cell on the spot vs. part of the cell covering the surrounding fibronectin. Statistical significance was tested with Kruskal–Wallis test with *post-hoc* Dunn's Multiple Comparison test. Scale bars represent 10 μm; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

NeutrAvidin (**Figures 1E,F**). As expected, the TCR/CD3 complex was recruited to α CD3 spots, even at concentrations as low as 1% α CD3. Vice versa, CD3 was also recruited to 100% α CD6 spots, suggesting that the TCR/CD3 complex interacts with and co-migrates with CD6 (**Figures 1E,F**).

CD3 Ligation on Micropatterned Antibody Spots Causes Immobilization of CD6

Next, we set out to investigate whether ligation of the TCR/CD3 complex on microprinted antibody spots influenced CD6 lateral mobility. To this end, CD6-GFP Jurkat T cells were seeded onto microprinted antibody spots composed of different concentrations of α CD3 (1–10–100%) (**Figure 2A**). To study CD6 mobility upon TCR/CD3 complex immobilization, fluorescence recovery after photobleaching (FRAP) of CD6-GFP was performed by bleaching circular 2.1 μ m regions of interest (ROIs) both on spots and on fibronectin-coated areas surrounding these spots (**Figure 2B**). At the interface between cell and antibody spot-covered surface, FRAP revealed partial immobilization of CD6-GFP on 10 and 100% α CD3 spots, but not on 1% α CD3 spots (**Figure 2C**); a CD6-GFP fraction of $\sim$ 30% was immobilized on 10 and 100% α CD3 spots, significantly different from the immobile CD6-GFP fraction on 1% α CD3 spots (appr. 10%), which was comparable to the immobile fraction on surrounding fibronectin-coated areas (**Figures 2E,F**). In comparison, FRAP outside antibody spot areas (on surrounding fibronectin-coated areas) showed unrestricted mobility of CD6-GFP with no effect of the α CD3 concentration within the spots (**Figures 2D,F**). The diffusion speed of the mobile CD6-GFP fraction was not affected by CD3 immobilization, as both on α CD3 spots and on surrounding fibronectin-coated areas recovery halftimes were similar (**Figures 2G,H**). These data indicate that a subpopulation of CD6 is immobilized upon CD3 ligation and confirm that CD6 at least partially interacts physically with the TCR/CD3 complex.

CD6 Is Co-transported With TCR/CD3 in Microclusters Toward the cSMAC of the Immunological Synapse Formed on α CD3-Containing SLBs

When a T cell engages contact with a stimulating antigen-presenting cell, the TCR/CD3 complex is transported to the center of the immunological synapse (IS) formed at the interface between these cells. CD6 has been previously shown to co-localize with the TCR/CD3 complex in the central supramolecular activation cluster (cSMAC) of this IS (29, 35). However, techniques exploited so far have only shown static co-localization of CD6 and TCR/CD3 complex at a fully matured IS. Therefore, to investigate the dynamics of the CD6-TCR/CD3 complex interplay during IS formation, we studied synapse formation in Jurkat T cells seeded on planar supported lipid bilayers (SLBs), a well-established and widely used system to study early steps of IS formation (1, 3, 6, 11, 42). SLBs containing biotinylated lipids were prepared by spin coating lipids directly from chloroform solutions onto glass coverslips (45). Subsequently, ATTO647-labeled, biotinylated α CD3 antibody was coupled to

the biotinylated lipids in the SLB via streptavidin, leading to free lateral diffusion of α CD3 antibody, confirmed by FRAP (data not shown).

To assess whether Jurkat T cells formed an IS on these α CD3-containing SLBs, wildtype cells were allowed to interact with and spread on the SLBs. After fixation, the α CD3 antibody in the lipid bilayer was visualized to localize TCR/CD3 complexes. Representative brightfield images overlaying α CD3 signal are shown in **Supplementary Figure 2**. Wildtype Jurkat T cells were stained for phospho-tyrosine, to visualize signaling, and for integrin LFA-1, a classical component of the peripheral supramolecular activation cluster (pSMAC) surrounding the cSMAC in the IS (7). Furthermore, LifeAct-GFP Jurkat T cells were seeded onto SLBs to visualize the actin cytoskeleton (**Figures 3A–C**). Total Internal Reflection Fluorescence (TIRF) microscopy images of α CD3 in SLBs show that Jurkat T cells formed a large central cluster (cSMAC) containing TCR/CD3 in contact with SLBs (**Figures 3A–C**, left panels). Clustering of TCR/CD3 through α CD3 engagement in SLBs was able to mediate signaling as shown by the pTyr staining that co-localized with the α CD3 antibody in SLBs (**Figure 3A**). Staining of LFA-1 confirmed the formation of a typical peripheral ring (pSMAC) surrounding the cSMAC (**Figure 3B**). Also, typical exclusion of actin from the cSMAC region was seen in LifeAct-GFP Jurkat T cells on SLBs (**Figure 3C**). Overall, these data indicate that SLBs containing α CD3 allowed IS formation in interacting Jurkat T cells.

Next, we investigated the dynamics of CD6-TCR/CD3 interplay during synapse formation. To this end, Jurkat cells expressing CD6-GFP were imaged during spreading on and engagement of contact with SLBs using live cell TIRF microscopy (**Figure 3D** and **Supplementary Video 1**). Within 2 min after initial contact of the cell with the SLB, TCR/CD3 microclusters could be observed that were radially transported from the cell periphery toward the center of the cell-SLB interface (**Figure 3D**, top row). After 5 min a large, bright, and stable TCR/CD3-rich central cluster, the cSMAC of the IS, was formed on the SLB. During cell spreading in the first 3 min, CD6-GFP in the plasma membrane spread out and formed a peripheral ring-like pattern (**Figure 3D**, middle row). Within this ring, microclusters containing CD6-GFP were present, co-localizing to α CD3 microclusters formed in the SLB (**Figure 3D**, bottom row; **Figure 3E**). Kymograph analysis of the cross section indicated in the merged image at timepoint 4 min in **Figure 3D** (during 3 min and 45 s to 6 min and 12 s after initiation of cell-SLB contact) revealed that these microclusters, containing both CD6 and TCR/CD3, were transported from the periphery toward the central region (cSMAC) of the IS (**Figure 3F**). After 4 min the CD6-GFP ring started to disappear as a result of constant transport of microclusters toward the cSMAC. Thereafter a large, bright cluster of CD6-GFP was visible at the center of the cell-SLB interface, which largely co-localized with the TCR/CD3-rich cSMAC (**Figure 3D**, middle and bottom row). Intensity and co-localization analysis of the central part of the cell-SLB interface was performed for multiple cells (representative ROI is shown in **Figure 3E**). CD6-GFP signal intensity increased in the center of the cell over time, indicating continuous recruitment of

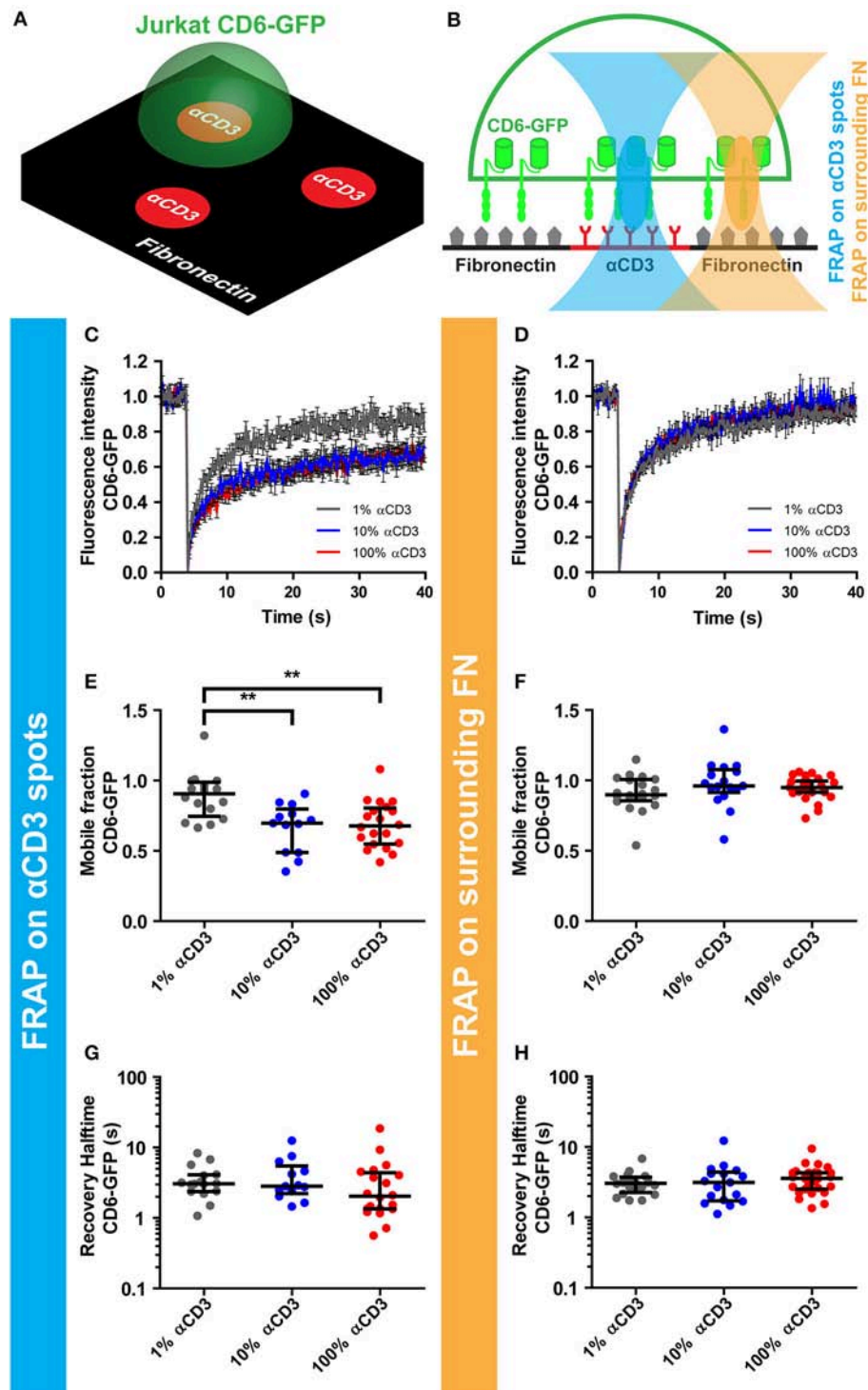
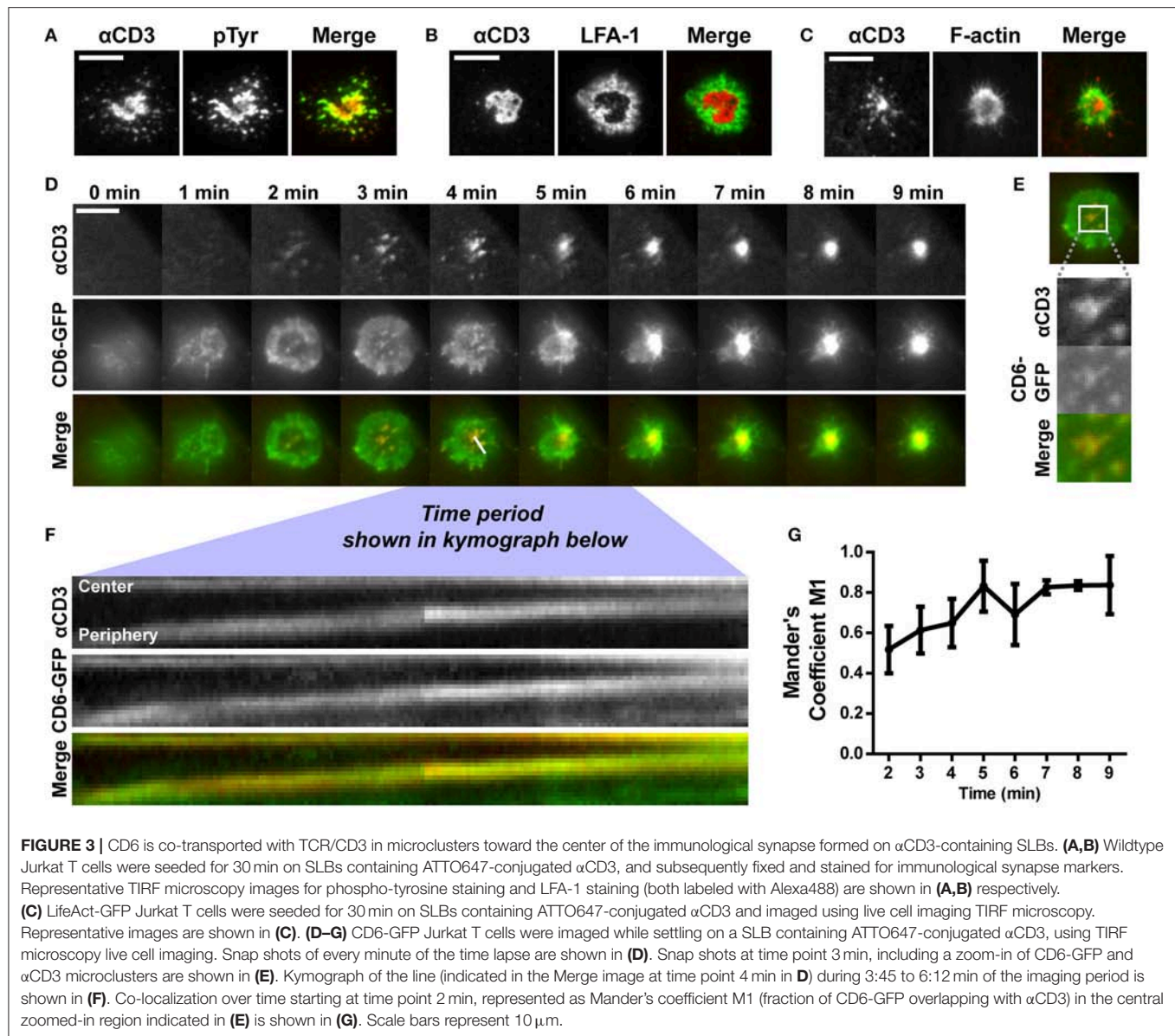


FIGURE 2 | CD3 ligation on micropatterned antibody spots causes immobilization of CD6. **(A–H)** CD6-GFP Jurkat T cells were seeded on micropatterned substrates containing 100, 10, or 1% αCD3 spots, created using microcontact printing, surrounded by fibronectin. All spots were labeled with Alexa647. Schematic representation of the set-up of the experiment and positioning of the FRAP region are shown in **(A,B)** respectively. **(C–H)** FRAP was performed on CD6-GFP covering the antibody spots and on CD6-GFP covering surrounding fibronectin. Fluorescence intensity of CD6-GFP in the FRAP region on the antibody spots or on the surrounding fibronectin is shown in **(C,D)** respectively. Curves represent the mean of ≥ 13 measurements $\pm$ SEM. Individual FRAP curves were fitted with a single exponential model and values for the mobile fraction and the recovery half time for each separate curve were determined. Mobile fraction values on antibody spots and on surrounding fibronectin are shown in **(E,F)** respectively. Lines indicate median with the interquartile range represented as black bars. Statistical significance was tested with one-way ANOVA with *post-hoc* Tukey's Multiple Comparison test. Recovery half-time values on antibody spots and on surrounding fibronectin are shown in **(G,H)** respectively. Lines indicate median with the interquartile range represented as black bars. $**p < 0.01$.



CD6-GFP to the cSMAC (**Supplementary Figure 3A**). Also, co-localization of CD6-GFP and TCR/CD3 increased as the fraction of CD6-GFP overlapping with α CD3 (Mander's coefficient M1) increased over time (**Figure 3G**), as well as the Pearson coefficient and Mander's coefficient M2 (fraction of α CD3 overlapping CD6-GFP) (**Supplementary Figures 3B,C**). Of note, engagement of CD6 did not seem to affect IS formation, as pre-treatment and incubation of CD6-GFP Jurkat T cells with soluble human ALCAM-Fc did not lead to a difference in the fraction of cells forming a typical cSMAC within 30 min after seeding on SLBs (**Supplementary Figure 4**). Taken together, these data indicate that a fraction of CD6 molecules in the T cell membrane constantly associate with the TCR/CD3 complex from the very early moment of SLB engagement, until the formation of the mature cSMAC, where CD6-GFP is continuously being

recruited. Thus, CD6 seems to be a member of the microclusters containing TCR/CD3, and is co-recruited with TCR/CD3 in these microclusters toward the IS.

Disruption of Actin Polymerization Inhibits TCR/CD3 and CD6 Co-transport Toward the cSMAC of the Immunological Synapse on α CD3-Containing SLBs

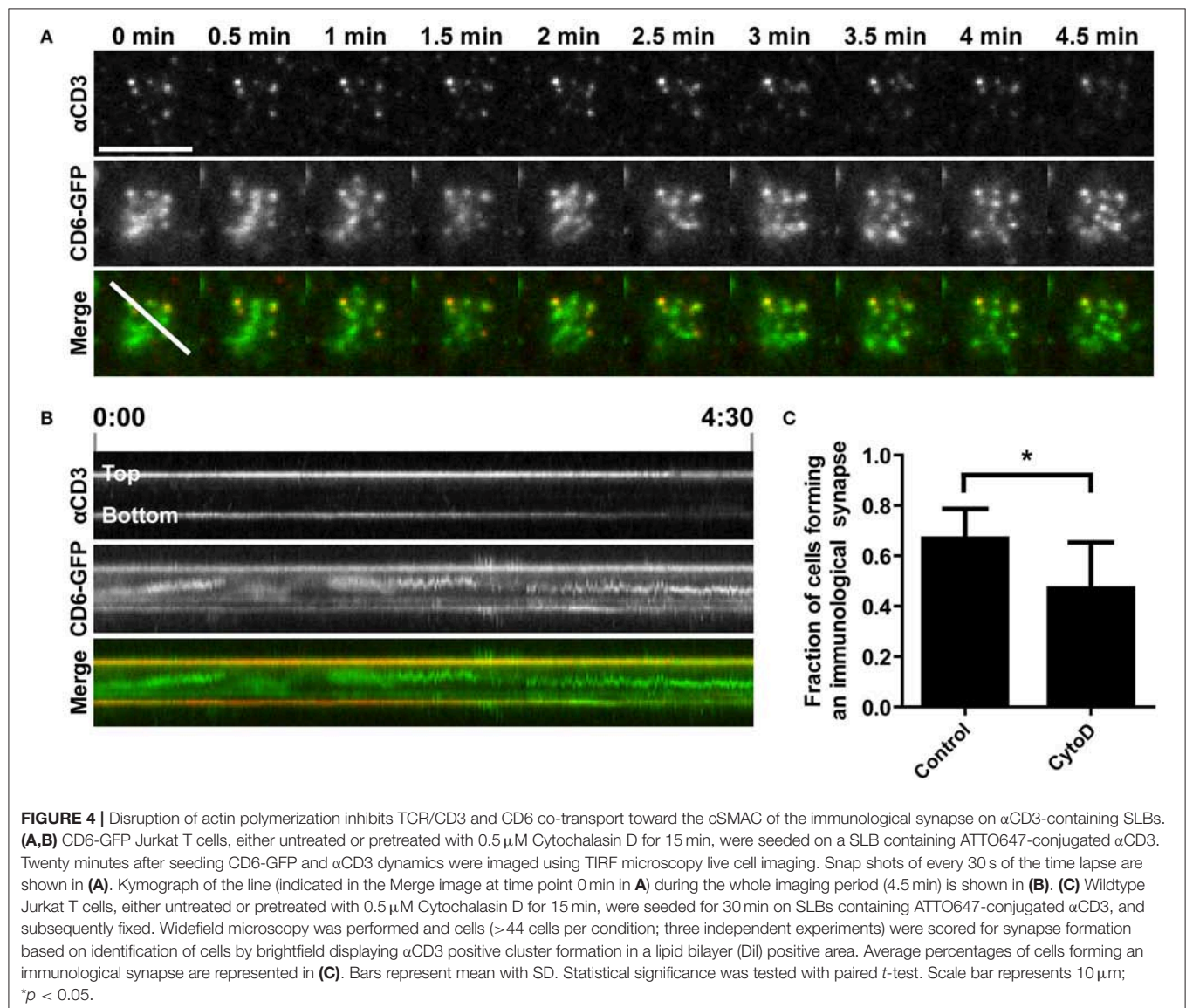
The actin cytoskeleton provides a dynamic mechanical framework to spatially organize the IS, and the radial transport of TCR/CD3 microclusters is dependent on retrograde actin flow (10, 11, 53). To investigate whether transport of CD6 toward the cSMAC also depends on an intact actin cytoskeleton, Jurkat CD6-GFP cells were treated with 0.5 μ M of the actin polymerization

inhibitor cytochalasin D (CytoD) for 15 min before allowing them to interact with α CD3-containing SLBs. CD6 and TCR/CD3 microcluster formation and transport were imaged by TIRF microscopy (Figure 4 and Supplementary Video 2). In cytochalasin D-treated cells, microclusters of both CD6 and the TCR/CD3 complex were still formed after inhibition of actin polymerization (Figure 4A). However, these clusters were static and not transported toward the center of the cell-SLB interface, as in untreated cells shown in Figure 3D. Indeed, kymograph analysis shows that the position of peripheral clusters in the cross section indicated in the merged image at timepoint 0 min in Figure 4A is stable over time, as represented by the horizontal line in both the α CD3 and the CD6 channel (Figure 4B). In addition, some CD6 microclusters did not co-localize with TCR/CD3 microclusters. Although not completely immobile, these clusters did not move toward the center of the contact (Figure 4B). Moreover, treatment of cells with CytoD resulted

in less Jurkat T cells forming a typical cSMAC within 30 min after SLB engagement and cell spreading (Figure 4C). This resulting difference may be an underestimation of the effect, as it is possible that CytoD-treated cells that did not engage the SLB at all have been washed away during fixation. In conclusion, these data demonstrate that the transport, but not the formation of CD6-TCR/CD3 microclusters clearly depends on actin polymerization.

Interaction With α CD3-Coated Beads Causes CD6 Clustering and Immobilization at Cell-Bead Interface

To better understand CD6 mobility in a cell-cell contact model, magnetic beads coated with α CD3 or with FN were added to CD6-GFP Jurkat T cells seeded on a FN-coated surface and CD6 enrichment at the cell-bead interface was



determined. Brightest point reconstructions of confocal image stacks of CD6-GFP show that CD6 was a threefold more enriched to α CD3-coated beads than to fibronectin-coated beads (**Figures 5A,B**). Next, CD6 mobility was assessed using FRAP. FRAP measurements on CD6-GFP were performed on cells incubated with magnetic α CD3 beads, either at the cell-bead interface (bead side) or at the opposing free side of the cell (no bead side) (**Figure 5C**; FRAP on cells with beads). Of note, in this set-up, diffusion of CD6-GFP was assessed in a vertically oriented membrane and therefore $2 \times 1 \mu\text{m}$ rectangular regions of interest (ROIs) were used for FRAP, in contrast to circular ROIs used on horizontal oriented membranes in **Figure 2**. As controls, CD6-GFP Jurkat T cells without beads, either untreated or incubated with soluble α CD3 were used for FRAP measurements (**Figure 5C**; FRAP on cells without beads). Resulting mobile fractions indicate that, as for CD6-GFP on 10 and 100% α CD3 antibody spots, a significant larger portion of the CD6-GFP population was immobilized at the cell-bead interface for cells in contact with α CD3-coated beads compared to CD6-GFP in the opposing side not in contact with a bead (**Figure 5D**). The mobile CD6-GFP fraction on the no bead site is comparable to that in untreated cells or cells treated with soluble α CD3. Again, the mobility of the mobile CD6-GFP fraction was not affected by interaction with the α CD3-coated bead, as recovery halftimes for all conditions did not differ significantly (**Figure 5E**). To determine the stability of this CD6-TCR/CD3 complex at the cell-bead interface, electromagnetic tweezers were used to displace the α CD3-coated bead through mechanical force (**Supplementary Figure 5**) (54). These data suggest that CD6 follows displacement of TCR/CD3 clusters and that the association between CD6 and TCR/CD3 complex is mechanically stable when exposed to mechanical forces of in the 200–900 pN range. Collectively these results confirm previous observations on microprinted antibody spots: cross-linking the TCR/CD3 complex by immobilized α CD3 results in immobilization of a significant fraction of CD6-GFP molecules, which strongly indicates a stable interaction between CD6 and the TCR/CD3 complex.

DISCUSSION

In this study, we applied different experimental techniques to characterize the interplay between CD6 and the TCR/CD3 complex. We show that CD6 and the TCR/CD3 complex are co-recruited to stable stimulatory clusters, both in Jurkat T cells seeded on antibody spots and in Jurkat T cells in contact with α CD3-coated beads. This association to TCR/CD3 applies to only a fraction of the CD6 population, as FRAP measurements on CD6-GFP (both in cells on α CD3 antibody spots or in cells in contact with α CD3-coated beads) indicate that more than half of the CD6-GFP population was still mobile. If the interaction was transient, a reduction in recovery time but no change in immobile fraction would have been expected. This partial association of CD6 with TCR/CD3 confirms previous reports by Gimferrer

and colleagues which showed a partial association using co-precipitation (35). Although substantial, the fully mobile and non-associated fraction CD6 of $\sim 70\%$ reported here may be an overestimation, as we made use of an over-expression model that most probably leads to an excess of CD6. This interaction between CD6 and TCR/CD3 seems mechanically rather stable, as we could show that CD6 follows displacement of TCR/CD3 by moving α CD3-coated magnetic beads with electromagnetic forces of 200–900 pN.

Next to recruitment to static ligands, we exploited SLBs where α CD3 could freely diffuse in the lateral plane. This setup allowed us to visualize CD6 dynamics during IS formation. We found that CD6 co-localizes with TCR microclusters on the Jurkat T cell membrane during IS formation. These CD6-TCR/CD3 microclusters were transported toward the cSMAC of the IS, which finally resulted in CD6-TCR/CD3 co-localization in the mature IS, as reported previously by us and others (29, 35). Since it has been shown that TCR signaling predominantly takes place in these microclusters that localize outside the cSMAC (1, 2, 12), the presence of CD6 in these microclusters suggests a role for CD6 in TCR receptor (co-)signaling. In our SLBs no ligand for CD6 was present; the co-localization of CD6 with TCR/CD3 microclusters we have demonstrated in this study is therefore independent of direct CD6 ligand binding. Therefore, although CD6-ALCAM interactions have been shown to localize to the cSMAC in stable T cell-DC interactions (29, 35), we cannot exclude that ligand binding affects the preceding CD6-TCR/CD3 co-localization in microclusters during IS formation and transport toward the cSMAC. Whether TCR/CD3 and CD6 interact directly or indirectly remains to be determined. Direct interaction between CD6 and TCR/CD3 is deemed unlikely, as the dimensions of receptor-ligand interactions differ; the optimal distance for TCR-pMHC is calculated to be 14–15 nm, whereas the binding distance between CD6 and ligand ALCAM would be probably larger than 21 nm (31).

Furthermore, in all set-ups we have used antibodies directed against CD3 to induce TCR/CD3 clustering and triggering. Although this is an artificial way of inducing T cell activation, it has been shown that stimulating CD3, without presence of an MHC-antigen complex, can sufficiently induce IS formation in Jurkat T cells (11). Furthermore, it has been shown that CD6 is phosphorylated on its cytoplasmic tail upon cross-linking of CD3 and CD2/CD3 co-cross-linking (34). TCR/CD3 complex triggering using α CD3 antibodies may result in differential downstream signaling than triggering with specific peptide-MHC complexes. As the association between CD6 and TCR/CD3 may depend on phosphorylation of CD3 and/or CD6 and could lead to different proteins interacting with CD3 and/or CD6, the type of molecule triggering the TCR/CD3 complex (α CD3 or pMHC complex) might modulate the CD6-TCR/CD3 interaction. Investigation of co-localization of CD6 cytoplasmic tail mutants with TCR/CD3 microclusters during IS formation would be able to shed more light on this question.

The actin cytoskeleton provides a dynamic mechanical framework to spatially organize the IS, and the radial transport of TCR microclusters depends on retrograde actin flow (10,

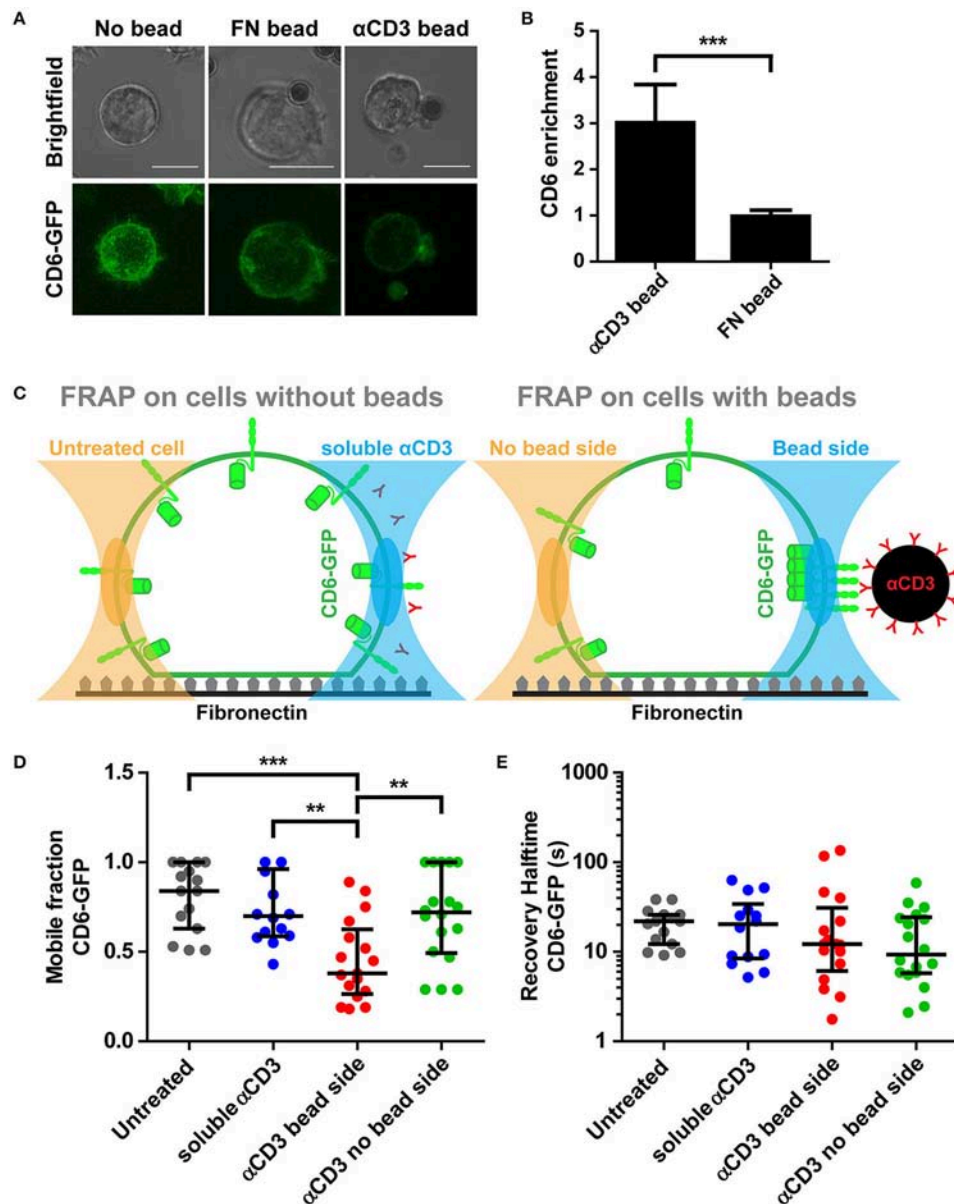


FIGURE 5 | Interaction with α CD3-coated beads causes CD6 clustering and immobilization at cell-bead interface. **(A,B)** CD6-GFP Jurkat T cells together with fibronectin (FN)-coated or α CD3-coated beads were seeded on a fibronectin-coated surface. Representative brightfield and confocal fluorescence images of live cell imaging are shown in **(A)**. CD6-GFP images are brightest point reconstructions of z-stacks, allowing display of CD6 distribution of the entire cell. Quantification of CD6 enrichment at cell-bead interface is shown in **(B)**. CD6 enrichment ($n = 10$ cells per condition) is determined as the ratio between the area of cell that is in contact with the bead and an equal area at the opposite side of bead contact. Bars represent mean with SD. Statistical significance was tested with unpaired t -test. **(C–E)** CD6-GFP Jurkat T cells incubated with FN-coated or α CD3-coated beads or soluble α CD3 were seeded on a fibronectin-coated surface. Schematic representation of FRAP regions is depicted in **(C)**. FRAP was performed on CD6-GFP in cells with beads, at parts of the cell not in contact or in contact with the bead (respectively left and right in right panel in **(C)**) and compared with cells without beads, either untreated or treated with soluble α CD3 antibodies (respectively left and right in left panel in **(C)**). Individual FRAP curves ($n \geq 14$ measurements per condition) were fitted with a single exponential model and values for the mobile fraction and the recovery half time for each separate curve were determined. Mobile fraction values and recovery half-time values of single FRAP curves for all conditions are shown in **(D,E)**, respectively. Lines indicate median with the interquartile range represented as black bars. Statistical significance was tested with one-way ANOVA with *post-hoc* Tukey's Multiple Comparison test. Scale bars represent 10 μ m; ** $p < 0.01$; *** $p < 0.001$.

11, 53). Also in our set-up actin was present in a peripheral ring on the intracellular side of the IS and excluded from the cSMAC. Furthermore, we found that the CD6-TCR/CD3 co-transport in microclusters toward the cell center depends on

actin polymerization. This suggests that CD6, similar to the TCR/CD3 complex, is linked to the actin cytoskeleton. Moreover, the peripheral ring-like pattern of CD6 we saw during the initial cell spreading is reminiscent of the F-actin pattern observed in

other studies on T cells forming an IS (11). The actin cytoskeleton itself might even provide the link between CD6 and TCR/CD3. Interestingly, CD6 has been shown to associate with the adaptor protein SLP-76, which is part of TCR microclusters (13, 33, 55). TCR-induced tyrosine phosphorylation of SLP-76 has been shown to be important in the recruitment of the proteins Nck and WASp to TCR microclusters for actin polymerization (56). However, CD6 association to the TCR/CD3 complex through SLP-76 cannot explain CD6-TCR/CD3 co-transport into the cSMAC, as it has been shown that SLP-76 (together with ZAP70) dissociates from TCR microclusters before these coalesce with the cSMAC, and localizes to unidentified perinuclear structures (13, 55).

Next to SLP-76, CD6 also interacts with the actin-binding adaptor protein syntenin-1 (57). Syntenin-1 has been implicated in functional asymmetry in T cells and actin polymerization and accumulation in T cell activation (58, 59). Presence of syntenin-1 is needed for CD3 accumulation at the cSMAC (59), and may provide the link between CD6 and the TCR/CD3 complex, in this way facilitating CD6-TCR/CD3 microcluster transport toward the cSMAC. Unlike SLP-76, syntenin-1 has been shown to localize to the cSMAC of the IS, where it co-localizes with CD6 and TCR/CD3 (57). Any possible link between CD6 and the TCR/CD3 via syntenin-1 would, however, be independent of the actin cytoskeleton, as the cSMAC is devoid of actin (11).

Detailed investigation of the organization of CD6, TCR/CD3, SLP-76 during IS formation using super-resolution imaging, such as Sherman and colleagues showed for the TCR, LAT, ZAP-70, and SLP-76 (60), could provide more insight into the organization of these TCR-CD6 microclusters and the exact role of SLP-76 and syntenin-1 in the interaction between these cell surface receptors. Next to that, super-resolution microscopy would also be able to shed light on the hitherto open question whether CD6 and TCR/CD3 associate directly or indirectly at the steady state level in the cell membrane of a resting T cell. New possible interaction partners of CD6 are still being identified and crucial molecules linking CD6 to the TCR/CD3 complex at the steady state level and/or during IS formation may therefore be still unknown at present (61).

Although the data presented here further substantiate the interplay between CD6 and TCR/CD3 and indicate that this co-recruitment already occurs in TCR microclusters prior to stable IS formation, it still remains a subject of debate whether CD6 signaling plays a stimulatory or inhibitory role in T cell activation. On the one hand, many studies employing monoclonal antibodies or soluble CD6 to target CD6 or its interaction with ALCAM have underlined the stimulatory role of CD6 in T cell activation and proliferation (23–29). On the other hand, the mere presence of CD6 in the T cell membrane has inhibitory effects on calcium response and IL-2 release in TCR-activated Jurkat T cells (30). Also, CD6 associates with family member CD5 (19), an established inhibitor of T cell signaling (62), which may in fact indirectly give CD6 its inhibitory capacities (31). It has been proposed that CD6 acts as a decoy receptor to capture downstream signaling molecules away from

the TCR signaling complex, as it localizes to the cSMAC of the IS, an area where TCR signaling is terminated through TCR endocytosis and degradation (31, 63). However, our data show that preceding formation of a stable mature IS, CD6 already co-localizes with TCR/CD3 microclusters, which are believed to be stimulating T cell activation. Still, CD6 may compose its own signaling hub independent of the TCR/CD3-LAT signalosome, as a proteomics study by Roncagalli and colleagues showed that LAT is dispensable for CD6-SLP-76 association (64). Because the CD6 gene has been shown to subject to alternative splicing upon T cell activation, the role of CD6 may alter during T cell-APC interaction, as one of the alternatively spliced forms has been shown to no longer translocate to the IS (65–67). In this study we have made use of full-length CD6 in our over-expression models. Therefore, investigation of the localization of alternatively spliced CD6 forms during IS formation, together with functional read-outs such as calcium fluxing and T cell proliferation, might provide more insight in the role of CD6 in microcluster formation and the mature IS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MM, AC, and CF designed the study. MM performed microcontact printing experiments. BJ, JW, and RB assisted with microcontact printing. SM performed lipid bilayer experiments. BJ assisted in cell culture, lipid bilayer experiments and image analysis. FC and JK performed magnetic tweezer and beads experiments. MM, SM, and FC analyzed data with support of JtR. SM, MM, AC, and CF wrote manuscript, with input from all authors.

ACKNOWLEDGMENTS

The authors thank dr. Geert van den Bogaart (Groningen Biomolecular Sciences and Biotechnology Institute, Groningen, the Netherlands) for assisting with the supported lipid bilayer preparation. Furthermore, the authors also thank the Microscopic Imaging Center (Radboud Institute for Molecular Life Sciences) for use of their microscopy facilities. CF was awarded with a Spinoza prize from The Netherlands Organization for Scientific Research (NWO). This research was supported by an intramural PhD fellowship from the Radboud University Medical Center awarded to SM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02333/full#supplementary-material>

REFERENCES

- Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med.* (2005) 202:1031–6. doi: 10.1084/jem.20051182
- Hashimoto-Tane A, Saito T. Dynamic regulation of TCR-microclusters and the microsynapse for T cell activation. *Front Immunol.* (2016) 7:255. doi: 10.3389/fimmu.2016.00255
- Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* (1999) 285:221–7. doi: 10.1126/science.285.5425.221
- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) 395:82–6. doi: 10.1038/25764
- Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, et al. Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol.* (2010) 28:79–105. doi: 10.1146/annurev-immunol-030409-101308
- Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* (2006) 25:117–27. doi: 10.1016/j.immuni.2006.04.010
- Dustin ML, Chakraborty AK, Shaw AS. Understanding the structure and function of the immunological synapse. *Cold Spring Harb Perspect Biol.* (2010) 2:a002311. doi: 10.1101/cshperspect.a002311
- Bunnell SC, Kapoor V, Triple RP, Zhang W, Samelson LE. Dynamic actin polymerization drives T cell receptor-induced spreading: a role for the signal transduction adaptor LAT. *Immunity* (2001) 14:315–29. doi: 10.1016/S1074-7613(01)00112-1
- Yi J, Wu XS, Crites T, Hammer JA III. Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells. *Mol Biol Cell* (2012) 23:834–52. doi: 10.1091/mbc.e11-08-0731
- Yu Y, Smoligovets AA, Groves JT. Modulation of T cell signaling by the actin cytoskeleton. *J Cell Sci.* (2013) 126(Pt 5):1049–58. doi: 10.1242/jcs.098210
- Kaizuka Y, Douglass AD, Varma R, Dustin ML, Vale RD. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc Natl Acad Sci USA.* (2007) 104:20296–301. doi: 10.1073/pnas.0710258105
- Courtney AH, Lo WL, Weiss A. TCR signaling: mechanisms of initiation and propagation. *Trends Biochem Sci.* (2017) 43:108–23. doi: 10.1016/j.tibs.2017.11.008
- Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol.* (2005) 6:1253–62. doi: 10.1038/ni1272
- Brownlie RJ, Zamoyska R. T cell receptor signalling networks: branched, diversified and bounded. *Nat Rev.* (2013) 13:257–69. doi: 10.1038/nri3403
- Mayer B, Funke I, Seed B, Riethmuller G, Weiss E. Expression of the CD6 T lymphocyte differentiation antigen in normal human brain. *J Neuroimmunol.* (1990) 29:193–202. doi: 10.1016/0165-5728(90)90162-G
- Bowen MA, Patel DD, Li X, Modrell B, Malacko AR, Wang WC, et al. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med.* (1995) 181:2213–20. doi: 10.1084/jem.181.6.2213
- Aruffo A, Melnick MB, Linsley PS, Seed B. The lymphocyte glycoprotein CD6 contains a repeated domain structure characteristic of a new family of cell surface and secreted proteins. *J Exp Med.* (1991) 174:949–52. doi: 10.1084/jem.174.4.949
- Braun M, Muller B, ter Meer D, Raffegerst S, Simm B, Wilde S, et al. The CD6 scavenger receptor is differentially expressed on a CD56 natural killer cell subpopulation and contributes to natural killer-derived cytokine and chemokine secretion. *J Innate Immun.* (2011) 3:420–34. doi: 10.1159/000322720
- Gimferrer I, Farnos M, Calvo M, Mittelbrunn M, Enrich C, Sanchez-Madrid F, et al. The accessory molecules CD5 and CD6 associate on the membrane of lymphoid T cells. *J Biol Chem.* (2003) 278:8564–71. doi: 10.1074/jbc.M209591200
- Enyindah-Asonye G, Li Y, Ruth JH, Spassov DS, Hebron KE, Zijlstra A, et al. CD318 is a ligand for CD6. *Proc Natl Acad Sci USA.* (2017) 114:E6912–21. doi: 10.1073/pnas.1704008114
- Saifullah MK, Fox DA, Sarkar S, Abidi SM, Endres J, Piktet J, et al. Expression and characterization of a novel CD6 ligand in cells derived from joint and epithelial tissues. *J Immunol.* (2004) 173:6125–33. doi: 10.4049/jimmunol.173.10.6125
- Patel DD, Wee SE, Whichard LP, Bowen MA, Pesando JM, Aruffo A, et al. Identification and characterization of a 100-kD ligand for CD6 on human thymic epithelial cells. *J Exp Med.* (1995) 181:1563–8. doi: 10.1084/jem.181.4.1563
- Gangemi RM, Swack JA, Gaviria DM, Romain PL. Anti-T12, an anti-CD6 monoclonal antibody, can activate human T lymphocytes. *J Immunol.* (1989) 143:2439–47.
- Swack JA, Gangemi RM, Rudd CE, Morimoto C, Schlossman SE, Romain PL. Structural characterization of CD6: properties of two distinct epitopes involved in T cell activation. *Mol Immunol.* (1989) 26:1037–49. doi: 10.1016/0161-5890(89)90068-0
- Osorio LM, Garcia CA, Jondal M, Chow SC. The anti-CD6 mAb, IOR-T1, defined a new epitope on the human CD6 molecule that induces greater responsiveness in T cell receptor/CD3-mediated T cell proliferation. *Cell Immunol.* (1994) 154:123–33. doi: 10.1006/cimm.1994.1062
- Osorio LM, Ordóñez C, Garcia CA, Jondal M, Chow SC. Evidence for protein tyrosine kinase involvement in CD6-induced T cell proliferation. *Cell Immunol.* (1995) 166:44–52. doi: 10.1006/cimm.1995.0006
- Osorio LM, Rottenberg M, Jondal M, Chow SC. Simultaneous cross-linking of CD6 and CD28 induces cell proliferation in resting T cells. *Immunology* (1998) 93:358–65. doi: 10.1046/j.1365-2567.1998.00442.x
- Hassan NJ, Barclay AN, Brown MH. Frontline: optimal T cell activation requires the engagement of CD6 and CD166. *Eur J Immunol.* (2004) 34:930–40. doi: 10.1002/eji.200424856
- Zimmerman AW, Joosten B, Torensma R, Parnes JR, van Leeuwen FN, Figdor CG. Long-term engagement of CD6 and ALCAM is essential for T-cell proliferation induced by dendritic cells. *Blood* (2006) 107:3212–20. doi: 10.1182/blood-2005-09-3881
- Oliveira MI, Gonçalves CM, Pinto M, Fabre S, Santos AM, Lee SF, et al. CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. *Eur J Immunol.* (2011) 42:195–205. doi: 10.1002/eji.201040528
- Santos RF, Oliveira L, Carmo AM. Tuning T cell activation: the function of CD6 at the immunological synapse and in T cell responses. *Curr Drug Target* (2016) 17:630–9. doi: 10.2174/1389450116666150531152439
- Castro MA, Nunes RJ, Oliveira MI, Tavares PA, Simoes C, Parnes JR, et al. OX52 is the rat homologue of CD6: evidence for an effector function in the regulation of CD5 phosphorylation. *J Leukocyte Biol.* (2003) 73:183–90. doi: 10.1189/jlb.0902437
- Hassan NJ, Simmonds SJ, Clarkson NG, Hanrahan S, Puklavec MJ, Bomb M, et al. CD6 regulates T-cell responses through activation-dependent recruitment of the positive regulator SLP-76. *Mol Cell Biol.* (2006) 26:6727–38. doi: 10.1128/MCB.00688-06
- Wee S, Schieven GL, Kiriha JM, Tsu TT, Ledbetter JA, Aruffo A. Tyrosine phosphorylation of CD6 by stimulation of CD3: augmentation by the CD4 and CD2 coreceptors. *J Exp Med.* (1993) 177:219–23. doi: 10.1084/jem.177.1.219
- Gimferrer I, Calvo M, Mittelbrunn M, Farnos M, Sarrias MR, Enrich C, et al. Relevance of CD6-mediated interactions in T cell activation and proliferation. *J Immunol.* (2004) 173:2262–70. doi: 10.4049/jimmunol.173.4.2262
- De Jager PL, Jia X, Wang J, de Bakker PI, Ottoboni L, Aggarwal NT, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. *Nat Genet.* (2009) 41:776–82. doi: 10.1038/ng.401
- Li Y, Singer NG, Whitbred J, Bowen MA, Fox DA, Lin F. CD6 as a potential target for treating multiple sclerosis. *Proc Natl Acad Sci USA.* (2017) 114:2687–92. doi: 10.1073/pnas.1615253114
- Aira LE, Hernandez P, Prada D, Chico A, Gomez JA, Gonzalez Z, et al. Immunological evaluation of rheumatoid arthritis patients treated with italizumab. *MAbs* (2016) 8:187–95. doi: 10.1080/19420862.2015.1105416
- Hernandez P, Moreno E, Aira LE, Rodriguez PC. Therapeutic targeting of CD6 in autoimmune diseases: a review of Cuban clinical studies with

- the antibodies IOR-T1 and itolizumab. *Curr Drug Target* (2016) 17:666–77. doi: 10.2174/1389450117666160201114308
40. Dogra S, Uprety S, Suresh SH. Itolizumab, a novel anti-CD6 monoclonal antibody: a safe and efficacious biologic agent for management of psoriasis. Expert opinion on biological therapy. (2017) 17:395–402. doi: 10.1080/14712598.2017.1279601
 41. Consuegra-Fernandez M, Lin F, Fox DA, Lozano F. Clinical and experimental evidence for targeting CD6 in immune-based disorders. *Autoimmun Rev*. (2018) 17:493–503. doi: 10.1016/j.autrev.2017.12.004
 42. Groves JT, Dustin ML. Supported planar bilayers in studies on immune cell adhesion and communication. *J Immunol Methods* (2003) 278:19–32. doi: 10.1016/S0022-1759(03)00193-5
 43. Riedl J, Crevenna AH, Kessenbrock K, Yu JH, Neukirchen D, Bista M, et al. Lifeact: a versatile marker to visualize F-actin. *Nat Methods* (2008) 5:605–7. doi: 10.1038/nmeth.1220
 44. van den Dries K, van Helden SFG, te Riet J, Diez-Ahedo R, Manzo C, Oud MM, et al. Geometry sensing by dendritic cells dictates spatial organization and PGE(2)-induced dissolution of podosomes. *Cell Mol Life Sci*. (2012) 69:1889–901. doi: 10.1007/s00018-011-0908-y
 45. Mennicke U, Salditt T. Preparation of solid-supported lipid bilayers by spin-coating. *Langmuir*. (2002) 18:172–7. doi: 10.1021/la025863f
 46. Phair RD, Gorski SA, Misteli T. Measurement of dynamic protein binding to chromatin *in vivo*, using photobleaching microscopy. *Methods Enzymol*. (2004) 375:393–414. doi: 10.1016/S0076-6879(03)75025-3
 47. Ellenberg J, Siggia ED, Moreira JE, Smith CL, Presley JF, Worman HJ, et al. Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J Cell Biol*. (1997) 138:1193–206. doi: 10.1083/jcb.138.6.1193
 48. Halavatyi AA, Nazarov PV, Al Tanoury Z, Apanasovich VV, Yatskou M, Friederich E. A mathematical model of actin filament turnover for fitting FRAP data. *Eur Biophys J*. (2010) 39:669–77. doi: 10.1007/s00249-009-0558-2
 49. Axelrod D, Koppel DE, Schlessinger J, Elson E, Webb WW. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys J*. (1976) 16:1055–69. doi: 10.1016/S0006-3495(76)85755-4
 50. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* (2012) 9:676–82. doi: 10.1038/nmeth.2019
 51. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. (2006) 224(Pt 3):213–32. doi: 10.1111/j.1365-2818.2006.01706.x
 52. Schwarzenbacher M, Kaltenbrunner M, Brameshuber M, Hesch C, Paster W, Weghuber J, et al. Micropatterning for quantitative analysis of protein-protein interactions in living cells. *Nat Methods* (2008) 5:1053–60. doi: 10.1038/nmeth.1268
 53. Ashdown GW, Burn GL, Williamson DJ, Pandzic E, Peters R, Holden M, et al. Live-cell super-resolution reveals F-actin and plasma membrane dynamics at the T cell synapse. *Biophys J*. (2017) 112:1703–13. doi: 10.1016/j.bpj.2017.01.038
 54. Shekhar S, Klaver A, Figdor CG, Subramaniam V, Kanger JS. Spatially resolved local intracellular chemical sensing using magnetic particles. *Sens Actuat B Chem*. (2010) 148:531–8. doi: 10.1016/j.snb.2010.05.006
 55. Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol*. (2002) 158:1263–75. doi: 10.1083/jcb.200203043
 56. Barda-Saad M, Braiman A, Titerence R, Bunnell SC, Barr VA, Samelson LE. Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat Immunol*. (2005) 6:80–9. doi: 10.1038/ni1143
 57. Gimferrer I, Ibanez A, Farnos M, Sarrias MR, Fenutria R, Rosello S, et al. The lymphocyte receptor CD6 interacts with syntenin-1, a scaffolding protein containing PDZ domains. *J Immunol*. (2005) 175:1406–14. doi: 10.4049/jimmunol.175.3.1406
 58. Zimmermann P, Tomatis D, Rosas M, Grootjans J, Leenaerts I, Degeest G, et al. Characterization of syntenin, a syndecan-binding PDZ protein, as a component of cell adhesion sites and microfilaments. *Mol Biol Cell* (2001) 12:339–50. doi: 10.1091/mbc.12.2.339
 59. Sala-Valdes M, Gordon-Alonso M, Tejera E, Ibanez A, Cabrero JR, Ursa A, et al. Association of syntenin-1 with M-RIP polarizes Rac-1 activation during chemotaxis and immune interactions. *J Cell Sci*. (2012) 125(Pt 5):1235–46. doi: 10.1242/jcs.094912
 60. Sherman E, Barr V, Manley S, Patterson G, Balagopalan L, Akpan I, et al. Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity* (2011) 35:705–20. doi: 10.1016/j.immuni.2011.10.004
 61. Hem CD, Ekornhol M, Granum S, Sundvold-Gjerstad V, Spurkland A. CD6 and Linker of activated T cells are potential interaction partners for T cell-specific adaptor protein. *Scand J Immunol*. (2017) 85:104–12. doi: 10.1111/sji.12513
 62. Tarakhovsky A, Kanner SB, Hombach J, Ledbetter JA, Muller W, Killeen N, et al. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* (1995) 269:535–7. doi: 10.1126/science.7542801
 63. Cemerski S, Das J, Giuriso E, Markiewicz MA, Allen PM, Chakraborty AK, et al. The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. *Immunity* (2008) 29:414–22. doi: 10.1016/j.immuni.2008.06.014
 64. Roncagalli R, Hauri S, Fiore F, Liang Y, Chen Z, Sansoni A, et al. Quantitative proteomics analysis of signalosome dynamics in primary T cells identifies the surface receptor CD6 as a Lat adaptor-independent TCR signaling hub. *Nat Immunol*. (2014) 15:384–92. doi: 10.1038/ni.2843
 65. Robinson WH, Neuman de Vegvar HE, Prohaska SS, Rhee JW, Parnes JR. Human CD6 possesses a large, alternatively spliced cytoplasmic domain. *Eur J Immunol*. (1995) 25:2765–9. doi: 10.1002/eji.1830251008
 66. Bowen MA, Whitney GS, Neubauer M, Starling GC, Palmer D, Zhang J, et al. Structure and chromosomal location of the human CD6 gene: detection of five human CD6 isoforms. *J Immunol*. (1997) 158:1149–56.
 67. da Gloria VG, Martins de Araujo M, Mafalda Santos A, Leal R, de Almeida SF, Carmo AM, et al. T cell activation regulates CD6 alternative splicing by transcription dynamics and SRSF1. *J Immunol*. (2014) 193:391–9. doi: 10.4049/jimmunol.1400038

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2D Kinetic Analysis of TCR and CD8 Coreceptor for LCMV GP33 Epitopes

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 18 June 2018

Accepted: 21 September 2018

Published: 15 October 2018

Citation:

Kolawole EM, Andargachew R, Liu B,
Jacobs JR and Evavold BD (2018) 2D
Kinetic Analysis of TCR and CD8
Coreceptor for LCMV GP33 Epitopes.
Front. Immunol. 9:2348.
doi: 10.3389/fimmu.2018.02348

The LCMV GP33 CD8 epitope has long been one of the most widely used antigens in viral immunology. Of note, almost all of the *in vitro* analyses of CD8 T cell responses to this epitope make use of an altered peptide ligand (APL) in which the cysteine from the original 9-mer peptide (KAVYNFATC) is substituted by a methionine at position 41 (KAVYNFATM). In addition, it is possible that the antigen processed during natural LCMV infection is an 11-mer peptide (KAVYNFATCGI) rather than the widely used 9-mer. Although previous affinity measurements using purified proteins for these antigen variants revealed minimal differences, we applied highly sensitive two dimensional (2D) biophysical based techniques to further dissect TCR interaction with these closely related GP33 variants. The kinetic analyses of affinity provided by the 2D micropipette adhesion frequency assay (2D-MP) and bond lifetime under force analyzed using a biomembrane force probe (BFP) revealed significant differences between 41M, 41C and the 11-mer 41CGI antigen. We found a hierarchy in 2D affinity as 41M peptide displayed augmented TCR 2D affinity compared to 41C and 41CGI. These differences were also maintained in the presence of CD8 coreceptor and when analysis of total TCR:pMHC and CD8:pMHC bonds were considered. Moreover, the three ligands displayed dramatic differences in the bond lifetimes generated under force, in particular the 41CGI variant with the lowest 2D affinity demonstrated a 15-fold synergistic contribution of the CD8 coreceptor to overall bond lifetime. Our analyses emphasize the sensitivity of single cell and single bond 2D kinetic measurements in distinguishing between related agonist peptides.

Keywords: TCR:pMHC, affinity, catch bond, GP33, LCMV, super agonist, immunodominance, CD8 T cell

INTRODUCTION

CD8⁺ cytotoxic T lymphocytes (CTLs), which recognize peptides presented by major histocompatibility complex (MHC) class I molecules, are critical for the antigen specific clearance of viral infections (1, 2). All CTL responses are dependent on recognition of the viral peptide, followed by sufficient triggering of the TCR to induce a cascade of signaling events (3). Thus, the initial interactions of TCR with pMHC are central to the recruitment of the adaptive immune arm of T cell mediated immunity. Consequently, the affinity of TCR for pMHC and other proximal parameters such as macromolecular orientation (4, 5), mechanosensing (6, 7), stability of the TCR:pMHC complex (8, 9), bond lifetime under force (9–11) and segregation of phosphatases from the T cell:APC synapse (9, 12–15) are critical in determining the efficiency of T cell differentiation and effector functions.

A major question that persists is how T cells can simultaneously possess a high level of specificity coupled with extreme sensitivity for as few as a single pMHC molecule (16, 17). This question has led to a number of models and technologies seeking to explain how TCR recognition of various pMHC complexes leads to such functionally different outcomes, giving rise to agonists and antagonist classifications (18–20). Affinity and T cell kinetics of TCR:pMHC can be acquired by surface plasmon resonance (SPR) which measures the receptor:ligand interaction in three dimensions (3D) (21), but this method lacks the sensitivity to measure the entire gamut of pMHC ligands, especially the lower affinity interactions. In contrast to using purified proteins for 3D measurements where the receptor:ligand interaction is isolated from cells, 2D based measures incorporate the proteins into the cellular membrane and are assessed at the cell-cell junctions, providing an added biological component to the interactions. More importantly, the 2D measurements possess increased levels of sensitivity to measure lower affinity and shorter bond lifetime interactions (6, 22, 23).

Lymphocytic choriomeningitis virus (LCMV) is one of the best characterized viral model systems in mice (24–26). LCMV has been key in impacting our overall understanding of T cell immunology responsible for many seminal findings including, but not limited to: peptide:MHC restriction, the kinetics of primary and memory T cell responses, viral epitope escape, T cell exhaustion and the role of PD-1, (27–30). An important feature is the existence of several well characterized strains conferring either a chronic (Clone 13 strain) or acute (Armstrong strain) viral infection (25, 31). During the response to LCMV infection, the majority of CD8⁺ CTLs are directed against three viral immunodominant H-2D^b MHC class I epitopes (in C57BL/6 mice); GP_{33–41}, GP_{276–286}, and NP_{396–404} (32, 33). Both the 11-mer GP_{276–286} and the 9-mer NP_{396–404} have a single optimal sequence. However, GP33 has been analyzed using several epitopes: the 9-mer 41M, GP_{33–41} (KAVYNFATC) (41C) and the 11 amino acid long GP_{33–43} (KAVYNFATCGI) (41CGI) (8).

The altered peptide ligand (APL) 9-mer 41M, was created by introducing a single methionine at the carboxy terminal end replacing the cysteine at position 41, (27, 34, 35). The terminal cysteine has been shown to form peptide dimers and has decreased stability in the MHC (27, 34–36). These data showed an increase in MHC class I binding from 41C to 41M (37). Therefore, most analyses of the LCMV GP33 D^b response have been analyzed with the more stable APL. Because of these changes in the peptide antigen used for the LCMV response, we sought to determine how the three immunodominant GP33 epitopes alter the 2D kinetics of TCR recognition of the respective antigens and the CD8 T cell functional response. In addition to the TCR:pMHC interaction we wanted to investigate the contribution of CD8 coreceptor to the TCR signaling. When blocked or in the absence of CD8 coreceptor, CD8⁺ T cells require longer and more substantial TCR engagement (38, 39). It has been suggested that CD8 binding helps weak ligands with low TCR:pMHC affinities (40). Furthermore, it has been demonstrated that CD8 binds sequentially following TCR:pMHC

engagement (41). Here, we demonstrate the distinct hierarchy of 2D kinetics for the three viral variants that has been absent in 3D kinetic analyses. In addition, we found the contribution of CD8 coreceptor to bond lifetime under force strikingly increases with the weaker 41CGI variant. Our work demonstrates that 2D analysis can sensitively distinguish differences in closely related agonist peptides.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute (NCI) and Charles River. P14 (Thy 1.2) TCR transgenic mice were housed and bred at the University of Utah. All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee at the University of Utah. C57BL6 Thy 1.1 congenic mice used as hosts for adoptive transfers were purchased from NCI. All mice were between 8 and 12 weeks of age.

Virus

Lymphocytic choriomeningitis virus (LCMV) Armstrong were kindly provided by Dr. Matthew Williams (University of Utah) and was made as described (42).

Peptides

GP33-41M (KAVYNFATM), GP33-41C (KAVYNFATC) and cognate GP33–43 (KAVYNFATCGI) peptides were synthesized at the University of Utah on the Prelude X peptide synthesizer (Protein Technologies).

Adoptive Cell Transfer

Naïve CD8⁺ T cells were isolated from spleens of P14 (Thy 1.2) transgenic mice using MACS CD8⁺ T cell magnetic separation kit (Miltenyi Biotec) and intravenously transferred to congenic C57BL/6 (Thy1.1) hosts. Mice were infected with 2×10^5 PFU of Armstrong by intraperitoneal (i.p.) injection 24 h after adoptive cell transfer.

LCMV INFECTIONS

8–12 weeks old C57BL/6 mice were injected i.p. with 2×10^5 pfu Armstrong and sacrificed at 8 days post infection. Spleens were harvested and stimulated with either KAVYNFATM (41M), KAVYNFATC (41C) or KAVYNFATCGI (41CGI) for 1 h and then intracellular cytokine staining was performed.

2D MICROPIPETTE ADHESION FREQUENCY ASSAY (2D-MP)

The relative 2D affinity of naïve P14 H2D^b GP33 specific CD8⁺ T cells was measured using the previously characterized 2D-MP (6, 43–47). In brief, RBC's were coated with Biotin-LC-NHS (BioVision) followed by streptavidin (Thermo Fisher Scientific) and either biotinylated pMHC GP_{33–41M} (KAVYNFATM), GP_{33–41C} (KAVYNFATC) or GP_{33–43} (KAVYNFATCGI) monomers. For relative 2D affinity measurements of TCR:pMHC

interaction, monomers with the D^b D227K mutation were used abrogating CD8 binding to MHC or without the D^b D227K mutation measuring normalized adhesion bonds of the TCR:pMHC:CD8 trimolecular interaction. All monomers were obtained from the NIH Tetramer Core Facility. In both sets of experiments the adhesion frequency between a single T cell and a ligand coated RBC aspirated on opposing pipettes was observed using an inverted microscope. An electronically controlled piezoelectric actuator repeated a T cell contact and separation cycle with the pMHC coated RBC 50 times while keeping contact area (A_c) and time (t) constant. Upon retraction of the T cell, adhesion (binding of TCR:pMHC) was observed as a distention of the RBC membrane, allowing for quantification of the adhesion frequency (Pa) at equilibrium. Surface pMHC (m_l) and TCR (m_r) densities were determined by flow cytometry using an anti-TCR β PE antibody (H57-597; BD Biosciences) and an anti-H2D^b antibody (clone:28-14-8; eBioscience) both at saturating concentrations along with BD QuantiBRITE PE beads for standardization (BD Biosciences). The calculation of molecules per area was determined by dividing the number of TCR and pMHC per cell by the respective surface areas. The relative 2D affinities were calculated using the following equation: $A_c K_a = -\ln [1 - P_a(1)] / m_r m_l$. Normalized adhesion frequency was calculated using the equation $[-\ln(1 - P_a(s)) / m_l (\text{pMHC})]$. Geometric means of all measured single cell affinities and normalized adhesion bonds are reported $\pm$ SEM. The centerpiece of our micropipette system is an Olympus IX71 inverted microscope equipped with fluorescence and a 100X oil immersion phase contrast and suspended on a TMC CleanBench vibration isolation table. The micropipettes are mounted onto the stage by adapters made by Narishige and are controlled by both fine and coarse micromanipulators. One micropipette is attached to the piezoelectric actuator via a Physik Instrumente P-840.1 piezo amplifier control module that is controlled by LabVIEW software on the imaging workstation. Micropipettes are held by Narishige HI-7 injection holders affixed by the heads of Narishige UT-2 universal joints (with joint removed) on custom mounts. Aspiration pressure of the micropipettes is maintained by Kontes water columns on height adjustable Velmex Unislide height adjusters. Micropipettes are produced on a Sutter Instruments P-1,000 pipette puller and finished using a Narishige MF-900 microforge.

Biomembrane Force Probe Assay (BFP)

Bond lifetime measurements under force were captured using the biomembrane force probe Assay (BFP). Procedures for coupling pMHC to glass beads have been described previously (10, 48). In brief, RBCs were first biotinylated with EZ-link NHS-PEG-Biotin (Thermo Fisher Scientific) and then reacted to streptavidin. Borosilicate beads were first cleaned, silanized, and then reacted to streptavidin-maleimide (Sigma-Aldrich, St. Louis, MO). Streptavidin beads were then coated with biotinylated pMHC either GP₃₃–41M (KAVYNFATM), GP₃₃–41C (KAVYNFATC) or GP₃₃–43 (KAVYNFATCGI) monomers and placed on the apex of an RBC that was aspirated onto a micropipette. This bead served as a force probe. The position of the edge of the bead was tracked by a high-resolution camera (1,600 frames/s) with

<3 nm displacement precision. The T cell of interest was brought into contact with the glass bead, then retracted a set distance and held by the computer-controlled piezoelectric actuator. The retraction and hold-phase generated a force on the TCR:pMHC bond, which can be altered based on the distance the T cell is retracted. The camera then recorded the time it took for the T cell to disengage the glass bead, which was visualized as the RBC retracted and the bead returned to its starting position. Repeated cycles (known as force-clamp cycles) can be carried out at a single force in order to generate an average bond lifetime between the TCR and pMHC complex. For an optimal response to antigen, the bond lifetime increases with increasing force before reaching a peak bond lifetime, which is typical of a catch bond physiology. By varying the force and measuring the bond lifetimes one can determine what type of bond occurred. Bond lifetimes were analyzed as described (47) using a customized package run by LabVIEW (National instruments) first described by Chen et al. (49). The BFP is built around a Carl Zeiss Axio Observer A1 inverted microscope with two Narishige three-axis hanging joystick oil micromanipulators that allow for precise control of the micropipettes. One micropipette is attached to a Physik Instrumente piezoelectric actuator with nanometer resolution that is controlled with software programed on LabVIEW. To aspirate cells onto the micropipettes, we have two Eppendorf CellTram Vario pressure pistons, an Eppendorf FemtoJet for ejection of the cells and an engineered hydrostatic force pressure system. For data capture and analysis, we have two Allied Vision Technology cameras, one high speed (1,600 fps) and one normal speed (32fps) allowing for general recording and viewing (normal) with simultaneous recording of Brownian motion to detect formation and lifetime of individual bonds (high speed). In addition, we have an Andor iXon EMCCD Camera with sensitivity to a single photon. This is all housed on a Newport Vibration isolation table in a vibration-free room (~100 sq ft). Twins screens on the computer allow for real time tracking of bond formation (LabVIEW left screen) and view of the micropipettes (right screen).

Intracellular Cytokine Staining

Splenocytes isolated from infected mice were plated at 2×10^6 cells per well in a 96-well plate and cytokine production was tested in response to either 41M, 41C or 41CGI peptides. Tested concentrations ranged from 1 μ M to 0.03 nM in various dilutions. Cells were incubated with peptide for 1 h at 37° Celsius in R10 media and washed before Brefeldin A was added (MP Biomedicals) and the cells incubated for another 4 h. R10 media was composed of RPMI 1640 (Mediatech), 10% heat inactivated FBS (Hyclone), 10 mM HEPES buffer (Mediatech), 2 mM L-glutamine (Mediatech), 50 μ M 2-mercaptoethanol (2ME) (Sigma), and 100 μ g/ml gentamicin (Mediatech). Additional samples were also cultured without peptide as a negative control and stimulation with a PMA (20 nM; Fisher Biotech) ionomycin (1 μ M; Sigma) combination was used as a positive control. Cells were then washed and stained with surface antibodies in the dark for 30 min on ice in FACS staining buffer composed of phosphate buffered saline (PBS) (Mediatech), 0.1% bovine serum albumin (BSA) (Fisher

Scientific), and 0.05% sodium azide (Sigma). Surface markers were stained with anti-CD90.2 FITC (53-2.1; Biolegend), anti-CD44 PerCP Cy5.5 (IM7; BD), anti-CD3 Brilliant Violet 605 (145.2C11; Biolegend) anti-CD4 Brilliant Violet 711 (RM4-5; Biolegend) and anti-CD8 Brilliant Violet 785 (53.6.7; Biolegend). Using Tonbo bioscience Fix/Perm kit, cells were fixed and permeabilized as per manufacturer's protocol. Intracellular antibody staining with anti-IFN γ APC-Cy7 (XMG1.2; BD), anti-TNF α PE-Cy7 (MP6-XT22; Biolegend), anti-IL-2 APC (JES6-5H4; BD), anti-Nur77 PE (12.14; ebioscience) and anti-IRF4 eFluor 450 (3E4; Invitrogen) antibodies was performed as per manufacturers' protocol in a permeabilization buffer for 30 min on ice. Cells were washed and kept on ice before being run on the LSRFortessa X-20 cell analyzer (Beckton Dickinson). All flow cytometry data were analyzed using FlowJo software (Treestar).

Statistics

Statistical significance of measured values was determined by Ordinary one-way ANOVA and Tukey's multiple comparison test using the Prism Software (GraphPad). Statistical significance indicated as ns = no significance, * $P > 0.05$, ** $P > 0.01$, *** $P > 0.001$, and **** $P > 0.0001$.

RESULTS

Truncation (41C) and Mutation (41M) of Position 41 of the Immunodominant GP33 Epitope (41CGI) Result in Augmented 2D Affinity

Previous surface plasmon resonance (SPR) 3D affinity data has shown the APL 41M and 41C to have similar 3D affinities, with K_D values of 17 μM and 45 μM (50), 9.1 μM , and 12.2 μM (51) and 2.3 μM and 3.5 μM respectively (52). More recently, the advantages of 2D based affinity measurements over 3D SPR analyses have been highlighted using FRET (53) and the mechanical 2D-MP assay (4, 6, 22). The embedment of the TCR and pMHC within their respective cell membranes and the use of live cells in such 2D based analyses give a physiologically relevant portrayal of the TCR's native environment and the 2D restricted interaction between this receptor and its ligand. Furthermore, 2D affinity correlates closely with the potency of CD4 and CD8 T cells (6, 22, 41, 44, 53–55) making 2D-MP a key biophysical parameter. In our experiments, we used the 2D-MP and measured the 2D TCR affinity of LCMV specific naïve TCR-transgenic (P14) CD8 T cells to the three immunodominant GP33 epitopes presented by a mutant D^b monomer (D227K mutation of the MHC I $\alpha 3$ domain that abolishes CD8 binding) (6, 56–59). We found that 41M had the highest 2D affinity as compared to wildtype 41C and 41CGI, with mean population affinities of 1.04E-03 μm^4 , 6.62E-04 μm^4 , and 1.55E-04 μm^4 respectively (Figures 1A). Furthermore, 2D-MP allowed us to take a single T cell of interest and probe against the three pMHCs. The data (Figure 1B) demonstrate the hierarchy of the three GP33 variants occurs at the level of each individual T cell. As a control, the order in which a given pMHC monomer was

tested against the same T cell was randomized. These differences in 2D affinity are independent of any inherent changes in peptide affinity for MHC. Interestingly, while all three pMHC complexes had relatively high 2D affinities (as compared to 2D measurements of OT-I with cognate pMHC) (55), 41M had a ~ 2 -fold higher affinity than 41C and ~ 9 -fold higher affinity than 41CGI.

CD8 Contribution to TCR:pMHC Binding Does Not Alter the 2D TCR Affinity Hierarchy to 41M, 41C, and 41CGI.

We next wanted to investigate the contribution of the CD8 co-receptor to the interaction between TCR and the pMHC complex. The affinity of CD8 is significantly lower than the affinity of TCR for pMHC (D^b) (5, 46) but it is thought to enhance binding to ligands by the lowest affinity TCRs or aid recognition in the presence of low dose antigen (40). One can use wild type pMHC monomers, (intact D^b -CD8 binding) to quantify the number of TCR:pMHC and CD8 to MHC bonds (41). Similar to our TCR:pMHC 2D affinity data, normalized adhesion bonds showed the same trend with 41M having a bond number higher than 41C which was higher still than 41CGI (mean bond numbers of 6.40E-01, 1.01E-01, and 2.93E-02 μm^2 respectively) (Figure 2A). Using the same analysis of 2D-MP as in Figure 1B, a single T cell was probed against the three viral variants and the highest to lowest total bond numbers were determined as: 41M > 41C > 41CGI (Figure 2B). Additionally, these data demonstrated that the CD8 co-receptor contribution for these relatively high affinity pMHCs was different across the three epitopes (Figure 2A). These data highlighted CD8's ability to contribute differently with each variant epitope, further revealing the differences between 41M, 41C, and 41CGI.

CD8 Coreceptor Binding Bolsters Bond Lifetime Under Force but Fails to Restore the 11-Mer 41CGI to a Bond Lifetime Comparable to Either 41C or 41M

Previously, using optical tweezer technology, it has been demonstrated that T cells generate piconewton (pN) force in response to agonist pMHC (60, 61). The BFP assay can also be used to apply force to single TCR:pMHC bonds. Under force, the TCR:pMHC interaction can be divided into two types of bonds: bonds that strengthen the interaction between TCR:pMHC as force is applied (a catch bond) or TCR:pMHC interactions that generate a bond that weakens as force increases (a slip bond) (9, 10, 61).

Here, we apply force to the TCR:pMHC bond with and without the contribution of CD8 for all three pMHC variants (Figures 3A–C). In each case, the P14 TCR exhibits a catch bond, which is intensified by the contribution of CD8 (Figure 3C). The bond lifetimes under force also revealed a hierarchy amongst the peptide antigens. In the absence of CD8, 41M had a peak bond lifetime of ~ 0.8 s (Figure 3A), which was similar to 41C with a lifetime of ~ 0.9 s (Figure 3A), both of which were significantly

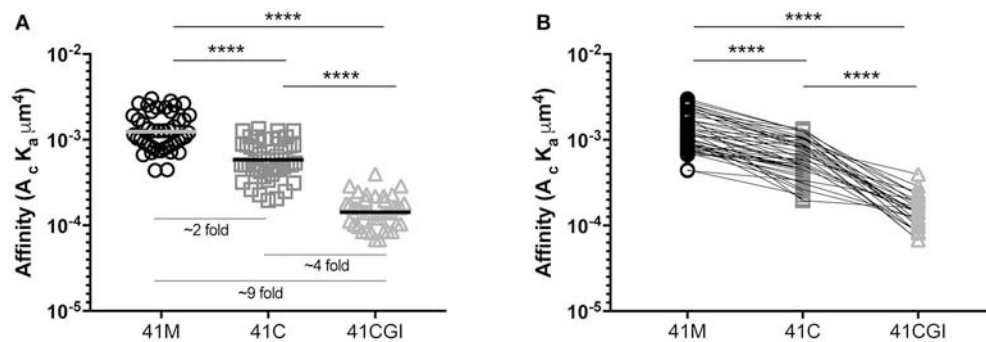


FIGURE 1 | Truncation (41C) and mutation (41M) of position 41 of the naturally processed immunodominant GP33 epitope (41CGI) result in augmented 2D affinity. 2D affinity of naive CD8⁺ P14 splenocytes tested to either D^b GP₃₃–41M, D^b GP₃₃–41C or D^b GP₃₃–41CGI monomers carrying the D^b D227K mutation (CD8-null). **(A)** shows the overall population mean affinity $\pm$ SEM while **(B)** shows the 2D affinity of a single P14 CD8⁺ T cell sequentially tested against each pMHC monomer (test sequence randomized from cell to cell). Statistical significance, **** $P > 0.0001$. Ordinary one-way ANOVA Tukey multiple comparison test. Each data point represents the affinity for one T cell to a given monomer. Data represents 3 individual experiments.

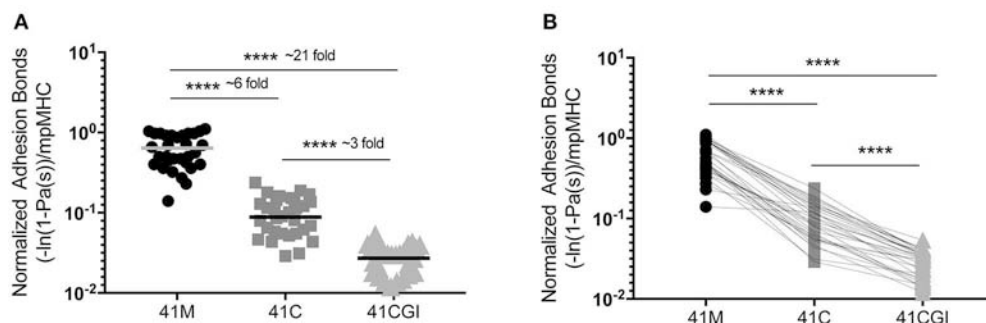


FIGURE 2 | The contribution of CD8 binding to TCR:pMHC does not alter the hierarchy of bond formation between dominant GP33 epitopes. Normalized adhesion bonds for the trimolecular interaction of TCR:pMHC:CD8. **(A)** Shows the overall normalized adhesion bond mean $\pm$ SEM of cells tested to 41M, 41C or 41CGI. **(B)** Shows the individual normalized adhesion bonds of each P14 CD8⁺ T cell tested to all three pMHC monomers in **(A)**. Statistical significance, **** $P > 0.0001$. Ordinary one-way ANOVA Tukey multiple comparison test. Each data point represents the normalized bonds for one T cell to a given monomer. Data represents 3 individual experiments.

higher than 41CGI which had the lowest bond lifetime of ~ 0.1 s (Figures 3A,B).

Liu et al. have revealed CD8 coreceptor to be critical for T cell mediated force generation and cell spreading (62). Therefore, we next wanted to investigate the previously uncharacterized CD8 coreceptor contribution to the TCR:pMHC complex under force. Thus, we generated force curves that measured the bond lifetime of TCR bound to CD8-intact pMHC monomers. Our data demonstrated that CD8 contribution significantly increased bond lifetime under force for all three peptide variants, with peak bond lifetimes showing a ~ 3 -fold increasing from ~ 0.8 to ~ 3.0 s (Figures 3A,C) for 41M while 41C showed a ~ 2 -fold increase from ~ 0.9 to ~ 1.9 s (Figures 3A,C). More interesting perhaps was the contribution of CD8 to TCR bond lifetimes under force with 41CGI, which exhibited a ~ 15 -fold increase from ~ 0.1 to ~ 1.5 s (Figures 3B,C).

Using the BFP assay, we and others have shown that the bond lifetime of a given TCR:pMHC pair changes when the applied force is varied (6, 9–11). Interestingly, we (10, 63) and others

(61, 64) have also shown that peak bond lifetime is often observed at ~ 10 pN for both CD4 and CD8 T cells (61). Additionally, Liu et al. have revealed that naïve T cells can naturally exert 12–19 pN of force on their TCRs within seconds of ligation (62). Therefore, we highlighted ~ 10 pN as the physiologically relevant point of comparison of the force curves generated with the three GP33 variant monomers. As such, we found that at 10 pN of force, 41C has a longer bond lifetime than 41M and dramatically more so than 41CGI (Figures 3D,E). However, in the presence of CD8, there is no significant difference between 41M and 41C (Figures 3F,G). These data highlight the dramatic contribution of CD8 coreceptor toward the weakest variant 41CGI.

2D Affinity and Bond Lifetime Under Force Are Indicative of Early T Cell Triggering and T Cell Function

Next, we determined the effect of the 2D kinetics on the T cell response. To assess whether TCR signal from either

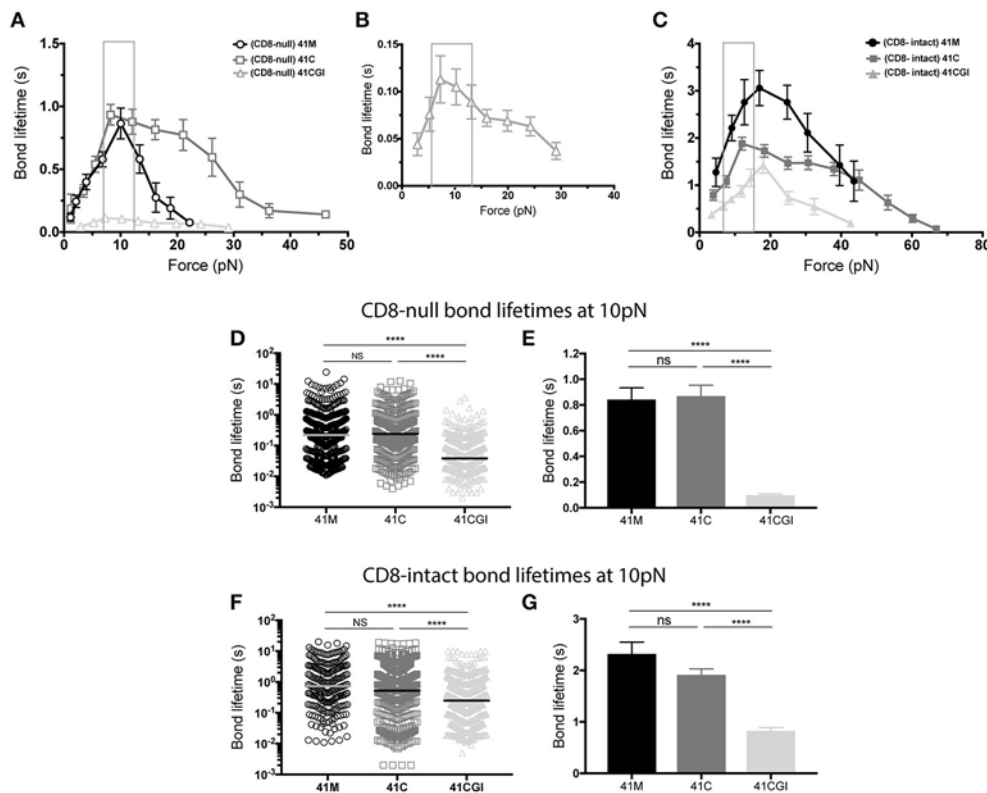


FIGURE 3 | The 11-mer 41CGI has dramatically reduced bond lifetime under force in comparison to 41C and 41M even with the contribution of CD8 coreceptor. Bond lifetime under force **(A)** shows bond lifetime curves for 41M (open circle), 41C (open square) and 41CGI (open triangle) with CD8-null pMHC **(B)** shows bond lifetime curve for 41CGI with CD8-null pMHC with a smaller scale. **(C)** Shows bond lifetime curves for 41M (closed circle), 41C (closed square) and 41CGI (closed triangle) with CD8-intact pMHC. CD8-null bond lifetimes at ~10 pN of force for all three variants shown as **(D)** a scatter plot or **(E)** a bar graph. CD8-intact bond lifetimes at ~10 pN of force for all three variants shown as **(F)** a scatter plot or **(G)** a bar graph. All force curves generated >1,000 bond lifetimes.

41M, 41C or 41CGI was perceived similarly, transgenic CD8+ P14 T cells were adoptively transferred into congenic hosts (**Figure 4A**) which were then infected with LCMV Armstrong a day later. Spleens were harvested at peak infection (D8) and cells were re-stimulated *ex vivo* with each variant peptide. The orphan nuclear hormone receptor, Nur77, has been shown to be rapidly upregulated in T cells stimulated with antigen via the TCR, but not by inflammatory stimuli (65), and has also been correlated with the strength of the TCR stimulus. Additionally, interferon regulatory factor-4 (IRF4) has been implicated in T cell differentiation and expansion and has been suggested to correlate with the stimulatory potency of a given pMHC (66–68). Our data demonstrated that a higher percentage of CD8+ T cells re-stimulated with 41M expressed Nur77 (**Figures 4B,E**) and have a higher MFI (peaking at 0.31 uM) than 41C and dramatically more so than 41CGI (**Figures 4C,D,F,G**). Similarly, 41C stimulated cells had significantly higher frequencies of Nur77+ CD8+ T cells than those stimulated with 41CGI (**Figures 4C,D**). However, while there was a difference in IRF4 MFI (**Figure 4H**) between 41M, 41C, and 41CGI when cells were stimulated at low peptide doses, we did not observe a difference between 41C and 41CGI at the highest peptide dose tested of 1 uM (**Figures 4I,J**). These data, while not factoring in peptide loading, indicated that

at equivalent peptide doses, ligand potency can correlate with TCR:pMHC 2D affinity (41M > 41C > 41CGI).

Where TCR signal transduction is detectable seconds after TCR engagement with pMHC, cytokine production is observed within hours. Using the same experimental design, we examined cytokine production for the three variants. We show that in response to 41M (which displayed the highest 2D affinity and perceived signal strength) cells also produced the most IFN γ (**Figures 5A,B**) and IL-2 (**Figure 5D**) than either 41C or 41CGI stimulated P14s. However, 41M and 41C activated cells produced similar amounts of TNF (**Figures 5C,E**). A significantly higher frequency of P14s were also double producers of IFN γ and TNF (**Figure 5F**) and triple producers of IFN γ , TNF and IL-2 (**Figure 5G**) with 41M stimulation than post activation with either the 41C or 41CGI peptides (**Figure 5H**).

We next wanted to investigate the impact of these peptide variants on the functional response of polyclonal CD8+ T cells. Thus, we infected C56BL/6 mice with LCMV Armstrong and splenocytes were harvested at peak infection then re-stimulated *ex vivo* with our peptide variants. Similar to our observation with monoclonal P14 cells, more polyclonal CD8 T cells exhibited increased frequencies of Nur77 expression upon stimulation with 41M as compared to 41C and 41CGI (**Figures 6A–D**) but

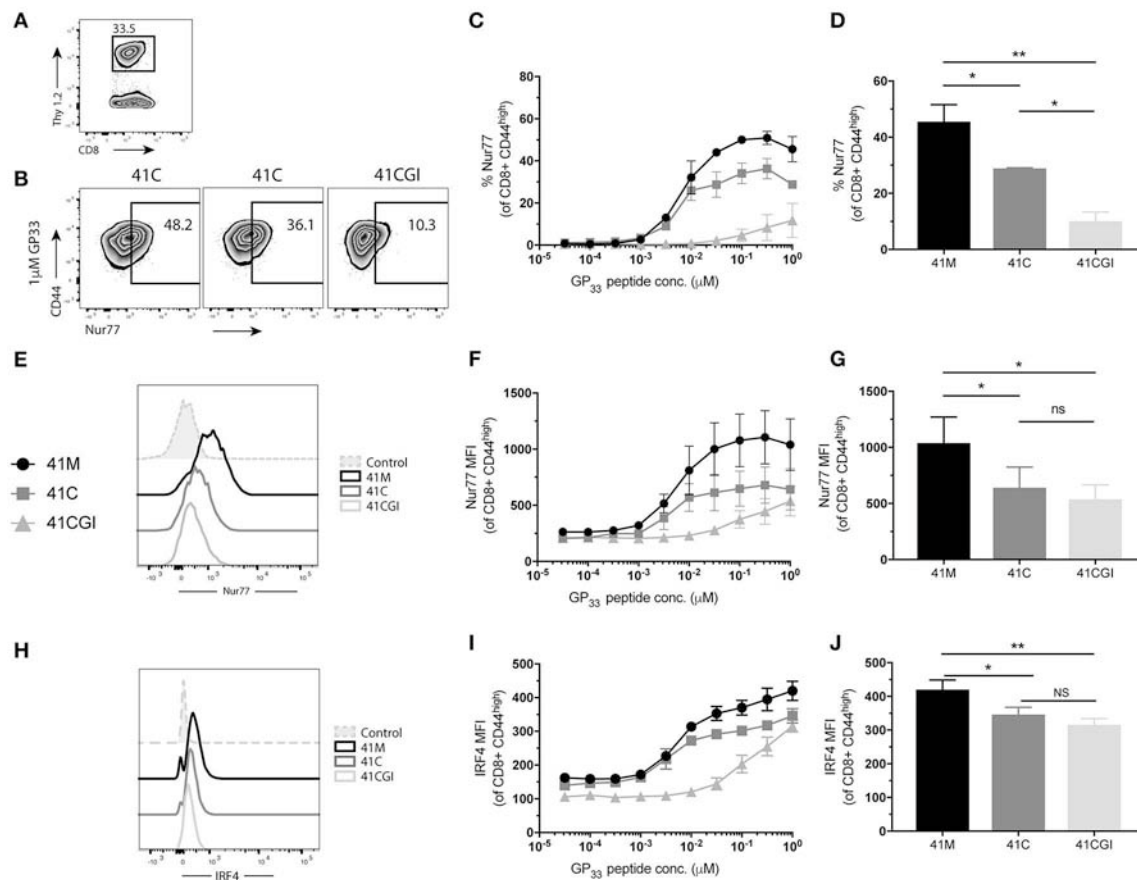


FIGURE 4 | 41M stimulated P14 CD8⁺ T cells perceive antigen more strongly than either 41C or 41CGI *ex vivo*. Naïve Thy1.2 P14 CD8⁺ T cells (1×10^4) were transferred into Thy1.1 congenic B6 hosts that were infected with LCMV Armstrong a day later. Splenocytes were harvested at D8 p.i. and re-stimulated for 1 h *ex vivo* with the three peptides at a range of doses. **(A)** Representative contour plot for P14⁺ Thy1.2 CD8⁺ T cell gating strategy. **(B)** Representative flow cytometry plot gating Nur77 staining (pre-gated on P14s (CD8⁺Thy1.2)) with 1 μ M peptide - left 41M, center 41C and right 41CGI. **(C)** Shows % Nur77⁺ at a range of doses. **(D)** Representative Nur77⁺ staining at 1 μ M for each peptide. **(E)** Nur77 MFI at a range of doses. **(F)** A representative histogram of Nur77 MFI staining using 1 μ M peptide. **(G)** Shows a representative experiment with Nur77 MFI staining at 1 μ M. **(H)** IRF4 MFI at a range of doses. **(I)** A representative histogram of IRF4 staining using 1 μ M peptide. **(J)** Shows a representative experiment with IRF4 MFI staining at 1 μ M. Data shown are representative of 3 experiments. $n = 3-4$ mice per experiment. Bar graphs with mean $\pm$ SEM. Statistical significance, ns = no significance, * $P > 0.05$, ** $P > 0.01$. Ordinary one-way ANOVA Tukey multiple comparison test.

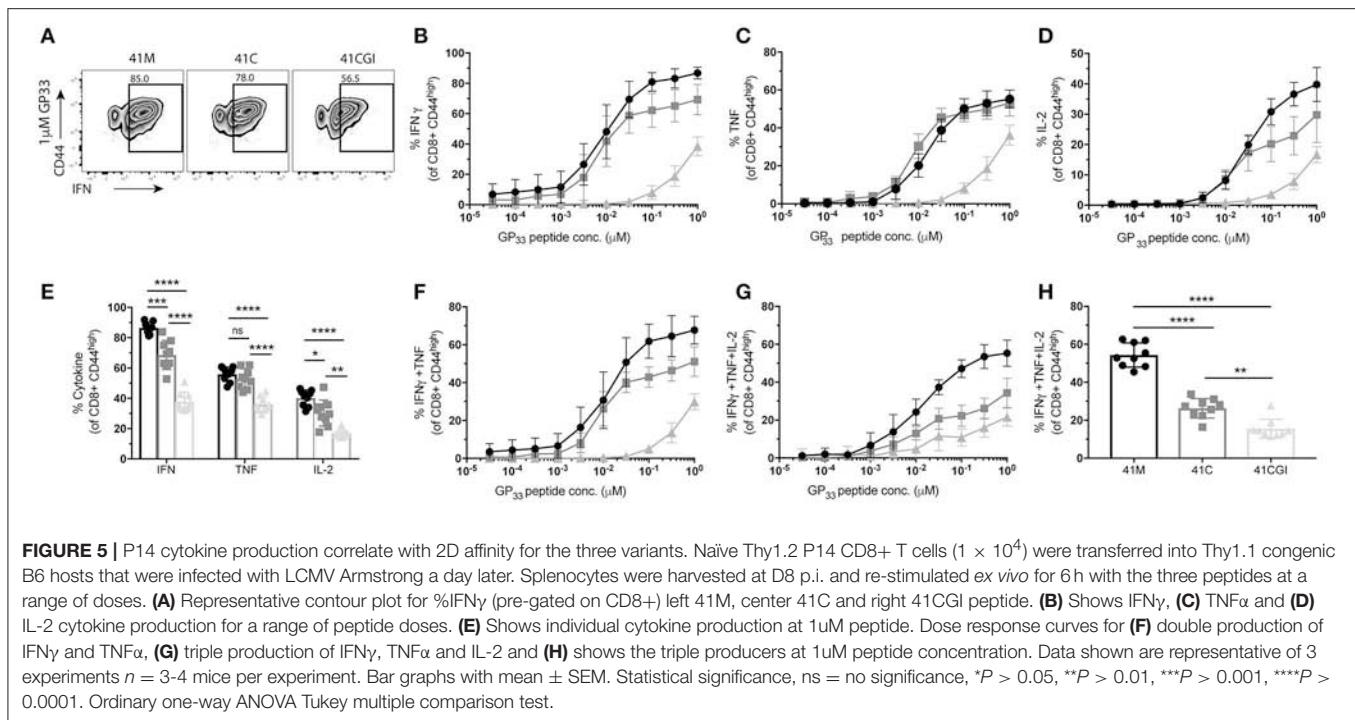
Nur77 MFI for 41C and 41CGI was not significantly different (Figure 6F). However, IRF4 expression revealed only a slight difference between 41M and 41C at the highest peptide dose of 1 μ M (Figures 6E–I).

DISCUSSION

The ability of an antigen specific TCR to be triggered and sufficiently induce activation related changes in the T cell that lead to clonal expansion and cytokine production is primarily dictated by early interactions with antigen. Identifying how TCR triggering equates to functional outcome and fate is still being investigated. Much of our understanding of TCR affinity for pMHC and functional outcomes stems from the use of APLs. For the most part, APLs are assumed to be of lower affinity for the TCR based on reduced functional responses. For many APLs exemplified by self and antagonist epitopes, SPR measurements lacked sensitivity to measure binding kinetics.

The OT-I OVA system and its ligands that display high to low functional responses have been used to dissect their direct influence on CD8 T cell effector functions and memory responses (55, 69–71). The 3D affinity for many of the OT-I APLs (71) has not been reported although we have analyzed several for 2D affinity (6, 55). Here, we build on the extensive knowledge of the GP33 immunodominant epitopes, evaluating the correlation between 2D kinetics of affinity and bond lifetime using three variant agonist antigens.

GP33 is documented as being one of the three immunodominant CD8 H2-D^b restricted epitopes for LCMV in C57BL/6 mice, along with GP_{276–286} and NP_{396–404}. Numerous studies have highlighted the contributing factors that confer immunodominance for a given peptide, namely stability of the peptide:MHC I complex, antigen processing and presentation, and TCR:pMHC I binding affinity (72–74). To this background we now overlay the contribution of 2D affinity and bond lifetime under force. Our data add insight and highlight the importance



and striking contrast between the most proximal step: TCR 2D affinity for pMHC and the ensuing TCR bond lifetime for pMHC under force. Furthermore, we emphasize the capability of 2D-MP to probe single receptor:ligand interactions and resolve subtle but significant changes in peptide binding that are independent of peptide loading.

Gairin et al. demonstrated, based on binding, that the 9-mer (41C) and the 11-mer (41CGI) are the immunodominant GP33 epitopes. In RMA-S MHC stability assays ($D^b K^b$), the 9-mer 41C was six times more efficient than 41CGI at inducing upregulation of H-2D^b. However, in competition experiments performed on the T-2D^b cell line, the 11-mer 41CGI was six times more efficient at competing for pMHC than the 9-mer 41C (8). The question is to what extent loading of MHC affects the T cell response and their biology. For example, 41M and 41C epitopes in our experiments had the same sensitivity to antigen, which would argue similar effective loading. In addition, others have reported biological differences in T cell responses and migration (interaction with APCs) that 3D affinity would not explain (51, 52). In the lymph node (LN), T cell motility has been labeled as having two distinct phases of behavior (36). Phase one type behavior consists of rapid T cell movements with multiple short sampling encounters with dendritic cells (DCs) before progression to a second phase comprised of stable and long lasting contacts with DCs (75). Henrickson et al. showed T cells interacting with 41M pulsed DCs transitioned more quickly to phase two than upon encountering 41C. We would suggest that the differences in 2D kinetics are integral to the different sampling phenotypes with 41C and 41M. In particular, one could envisage force being important to the biological migration observations to LFA-1 (76).

Several studies have reported the SPR derived 3D affinities for wildtype GP33 (41C) and the 41M mutation with ranges from 2 to 45 μ M (50–52). At these levels, there is probably no difference in P14 TCR affinity for 41C or 41M. For example, Boulter et al. show that 41C and 41CGI have very similar 3D affinities of 2.3 and 3.5 μ M, respectively. The 2D measurements displayed at least a 2-fold difference. We have previously identified 2-fold or greater differences in affinity can have profound changes in function (6, 55, 77–79), which would be consistent with the 41C giving larger responses as read out by Nur77, IFN γ and IL-2. Of note, one cannot directly compare 2D and 3D affinities as 3D affinities are measured in molarity and 2D affinities are measured in area. Instead, the bond lifetimes could provide a point of comparison. By SPR, 41C and 41M show bond lifetimes of ~ 0.5 s (51) which is similar to what we find by BFP (0.8–0.9s) for the TCR alone. A major difference in overall force occurs when the effect of CD8 is included as it increases the number of total bonds and the overall catch bond properties. These differences, which likely affect T cell biology, would not be apparent from the SPR assessment of P14 TCR for the respective GP33 peptides.

The crystal structures of several of the GP33 pMHC complexes have been reported with no major differences that explain our 2D kinetic findings (35). In general analysis of TCR:pMHC, crystal structures have failed to identify obvious factors for catch bond. TCR interactions that cause force are dynamic and occur optimally with CD8, Lck and the cell cytoskeleton (62). In recent collaborative work, it was found that the static crystal structures of pMHC with TCR alone did not identify the type of bond that will be formed under force. However, molecular dynamic simulations (MSD) could be used to add pulling force on the molecules and begin to identify key features (9).

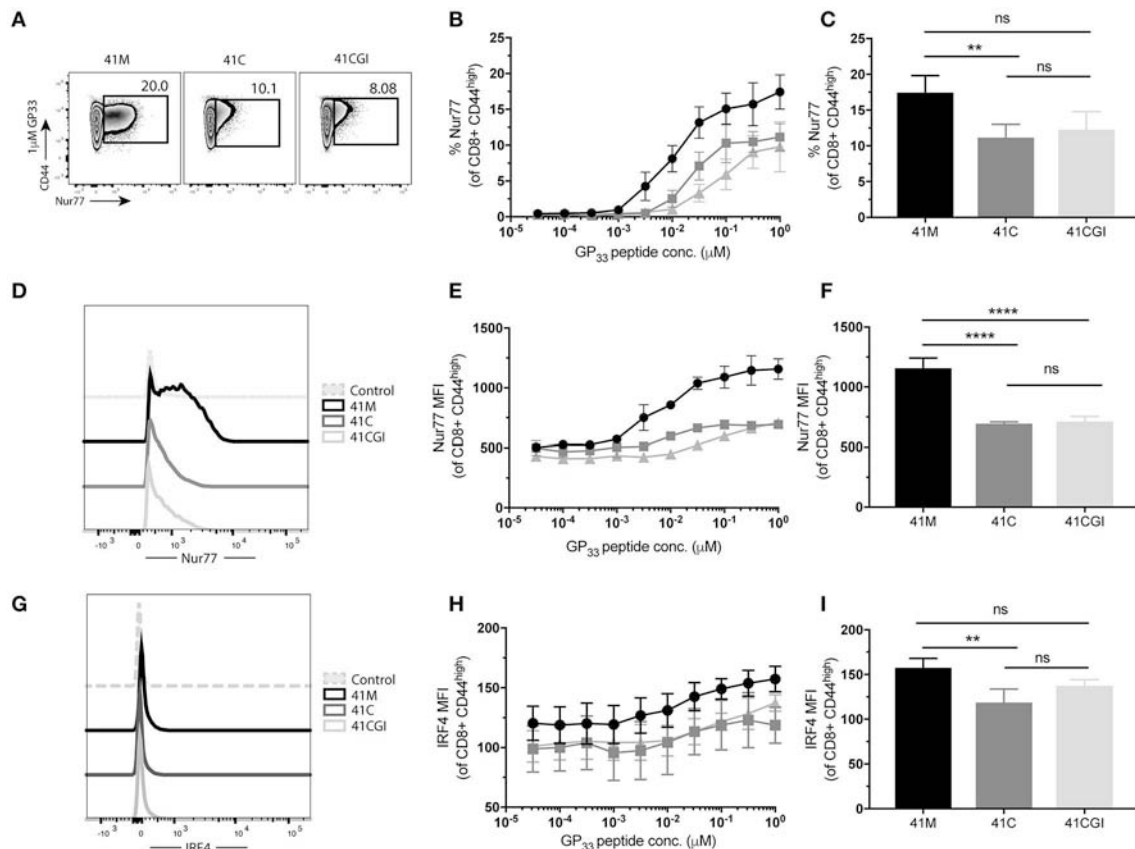


FIGURE 6 | Polyclonal CD8⁺ T cells recognize 41M more intensely than 41C or 41CGI. B6 mice were infected with LCMV Armstrong and splenocytes harvested at D8 p.i. and re-stimulated with either 41M, 41C or 41CGI *ex vivo* (A) Representative contour plot for nur77 (pre-gated on CD8⁺) for left 41M, center 41C, and right 41CGI peptide stimulations. (B) Shows %Nur77⁺ at a range of doses. (C) Representative %Nur77 staining at 1 μM for each peptide. (D) Representative histogram of Nur77 MFI staining at 1 μM for each peptide. (E) Shows Nur77⁺ MFI at a range of doses. (F) Representative Nur77⁺ MFI staining at 1 μM for each peptide. (G) A representative histogram of IRF4 staining using 1 μM peptide (H) IRF4⁺ MFI at a range of doses. (I) Shows a representative experiment with IRF4 MFI staining at 1 μM. Data shown are representative of 3 experiments $n = 3$ –4 mice per experiment. Bar graphs with mean $\pm$ SEM. Statistical significance, ns = no significance, ** $P > 0.01$. Ordinary one-way ANOVA Tukey multiple comparison test.

While 2D kinetics are not impacted by peptide loading as they are single molecule interactions, it is important to note that functional differences might be impacted by peptide loading. However Gairin et al. show that, based on binding, different assays give varying results on whether 41C or 41CGI is most stable (8). Our 2D kinetics clearly outline the hierarchy of GP33 epitopes and these data correlate with early signaling events. Using P14 adoptive transfer into congenic hosts studies wherein mice were injected with LCMV Armstrong and then re-stimulated *ex vivo* with our three peptides, we demonstrated that 41M stimulated P14 CD8 T cells give a stronger signal than 41C as measured by Nur77 upregulation. Additionally, both 41M and 41C stimulated cells induce significantly stronger signals than 41CGI stimulated cells. While Nur77 is often used as a readout of signal strength and indicative of functionality (80, 81), the type of CD8 function cannot clearly be deduced without further analysis. Using the same experimental design, we observed significantly diminished cytokine production in 41CGI stimulated P14 CD8 T cells as compared to 41M and 41C, and

the same hierarchy for P14 CD8 T cells producing IFN γ and IL-2 although 41M and 41C result in similar amounts of TNF α at the concentrations used here. Here we show 2D affinity to correlate with triple cytokine production in P14 transgenics. These findings are replicated using polyclonal CD8 T cells taken from D8 LCMV Armstrong infected mice re-stimulated with the different peptides. The 2D affinity distribution for a P14 monoclonal population spans a ~ 10 -fold range while the GP33 specific polyclonal CD8 T cell population can encompass a wider $\sim 1,000$ -fold affinity range (82, 83) but nevertheless the frequency hierarchy of triple cytokine producers in response to the three variants is preserved in both despite differences between monoclonal and polyclonal populations and any differences in peptide loading. Moreover, we have demonstrated that using 41M and its viral escape mutant 35A that while peptide loading is lower with 35A, in a polyclonal LCMV Armstrong infection 2D affinity is not significantly different (84).

Although T cell responses are aggregations of the TCR bond lifetimes, bond lifetime under force is a single molecule

measurement independent of peptide loading and is a critical parameter in determining T cell functionality (6, 10, 22). While 41CGI had the lowest 2D affinity of the three, it still had a relatively high 2D affinity. Given its high affinity, its low peak bond lifetime of ~ 0.1 s was somewhat surprising (Figures 3A,B). We have similarly recently analyzed the HLA B35-HIV epitope and found a high affinity TCR possessing a short bond lifetime that, in this case, showed slip bond characteristics. Together, this demonstrated that affinity and bond lifetime are not always directly correlated (9). Our data show that at 10pN of force, with or without CD8 contribution there is no significant difference in bond lifetime between 41M and 41C suggesting that any difference in functional responses between 41M and 41C based from assays in this study could be due to differences in 2D affinity.

Unlike the OT-I APL system where often the question is how 2D affinity differences elicit changes in the generation of effector and memory T cell populations, here the question is how the use of GP33 variants might change the perception of the GP33 epitope immunodominance within the CD8 response to LCMV. Our data clearly show that 41M elicits higher 2D affinity, increased number of total bonds with CD8 and longer bond lifetime under force as well as a more robust CD8 T cell response than both 41C and 41CGI. Super agonists are ligands for the TCR that stimulate the T cells more than the processed antigenic peptide (85). By our 2D affinity measures and functionality, 41M would be defined as a super agonist peptide variant as it possesses a prominent difference between 41CGI and the truncated 41C. This raises the question of whether using the super agonist 41M in lieu of 41C or 41CGI gives an accurate

interpretation of the efficacy of the GP33 targeted response relative to the other immunodominant CD8 LCMV epitopes (GP276 and NP396). Furthermore, our data highlight how the CD8 co-receptor engagement with TCR:pMHC can change with viral variants providing another point to consider. Lastly, our work demonstrates the power and sensitivity of 2D kinetic measurements in demonstrating how TCRs can determine subtle differences in related agonist ligands that can potentially lead to different functional outcomes.

AUTHOR CONTRIBUTIONS

EK and BE conceived the project and wrote the manuscript. EK and BL performed BFP experiments. EK and RA performed 2D-MP experiments. EK and JJ performed intracellular flow cytometry experiments. EK analyzed all data. BL and RA discussed findings and RA edited the manuscript.

FUNDING

This work was supported by NIH grant R01 AI096879.

ACKNOWLEDGMENTS

We thank the National Institute of Health Tetramer Core Facility for providing pMHC reagents and Matthew A. Williams for providing viral stocks. We also thank Catherine Gavile for helpful discussion and Linda Morrison for maintaining mouse colonies.

REFERENCES

- Zinkernagel RM, Doherty PC. H-2 compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded for in H-2K or H-2D. *J Exp Med.* (1975) 141:1427–36. doi: 10.1084/jem.141.6.1427
- Webster AD. Virus infections in primary immunodeficiency. *J Clin Pathol.* (1994) 47:965–7. doi: 10.1136/jcp.47.11.965
- Hudrisier D, Oldstone MBA, Gairin JE. The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2Db and H-2Kb molecules. *Virology* (1997) 234:62–73. doi: 10.1006/viro.1997.8627
- Adams JJ, Narayanan S, Liu B, Birnbaum ME, Kruse AC, Bowerman NA, et al. T cell receptor signaling is limited by docking geometry to peptide-major histocompatibility complex. *Immunity* (2011) 35:681–93. doi: 10.1016/j.immuni.2011.09.013
- Garcia KC, Scott CA, Brunmark A, Carbone FR, Peterson PA, Wilson IA, et al. CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature* (1996) 384:577–81. doi: 10.1038/384577a0
- Huang J, Zarnitsyna VI, Liu B, Edwards LJ, Jiang N, Evavold BD, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature* (2010) 464:932–6. doi: 10.1038/nature08944
- Lim TS, Mortellaro A, Lim CT, Hammerling GJ, Ricciardi-Castagnoli P. Mechanical Interactions between Dendritic Cells and T cells Correlate with T cell Responsiveness. *J Immunol.* (2011) 187:258–65. doi: 10.4049/jimmunol.1100267
- Gairin JE, Mazarguil H, Hudrisier D, Oldstone MB, Hudrisier D. Optimal lymphocytic choriomeningitis virus sequences restricted by H-2Db major histocompatibility complex class I molecules and presented to cytotoxic T lymphocytes. *J Virol.* (1995) 69:2297–305.
- Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell* (2018) 174:672–87.e27. doi: 10.1016/j.cell.2018.06.017
- Liu B, Chen W, Evavold BD, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* (2014) 157:357–68. doi: 10.1016/j.cell.2014.02.053
- Liu B, Chen W, Natarajan K, Li Z, Margulies DH, Zhu C. The cellular environment regulates in situ kinetics of T-cell receptor interaction with peptide major histocompatibility complex. *Eur J Immunol.* (2015) 45:2099–110. doi: 10.1002/eji.201445358
- Su X, Ditlev JA, Hui E, Xing W, Banjade S, Okrut J, et al. Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* (2016) 352:595–9. doi: 10.1126/science.aad9964
- Burroughs NJ, Lazic Z, Van Der Merwe PA. Ligand detection and discrimination by spatial relocation: A kinase-phosphatase segregation model of TCR activation. *Biophys J.* (2006) 91:1619–29. doi: 10.1529/biophysj.105.080044
- Carbone CB, Kern N, Fernandes RA, Hui E, Su X, Garcia KC, et al. *In vitro* reconstitution of T cell receptor-mediated segregation of the CD45 phosphatase. *Proc Natl Acad Sci USA.* (2017) 114:E9338–345. doi: 10.1073/pnas.1710358114
- Cordoba SP, Choudhuri K, Zhang H, Bridge M, Basat AB, Dustin ML, et al. The large ectodomains of CD45 and CD148 regulate their segregation from and inhibition of ligated T-cell receptor. *Blood* (2013) 121:4295–302. doi: 10.1182/blood-2012-07-442251
- George AJT, Stark J, Chan C. Understanding specificity and sensitivity of T-cell recognition. *Trends Immunol.* (2005) 26:653–9. doi: 10.1016/j.it.2005.09.011

17. Krogsgaard M, Davis MM. How T cells 'see' antigen. *Nat Immunol.* (2005) 6:239–45.
18. Germain RN, Stefanová I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu Rev Immunol.* (1999) 17:467–522.
19. Jameson SC, Bevan MJ. T cell receptor antagonists and partial agonists. *Immunity* (1995) 2:1–11. doi: 10.1016/1074-7613(95)90074-8
20. Kersh GJ, Allen PM. Essential flexibility in the T cell recognition of antigen. *Nature* (1996) 380:495–8.
21. Edwards LJ, Zarnitsyna VI, Hood JD, Evavold BD, Zhu C. Insights into T cell recognition of antigen: significance of two-dimensional kinetic parameters. *Front Immunol.* (2012) 3:86. doi: 10.3389/fimmu.2012.00086
22. Liu B, Zhong S, Malecek K, Johnson LA, Rosenberg SA, Zhu C, et al. 2D TCR-pMHC-CD8 kinetics determines T-cell responses in a self-antigen-specific TCR system. *Eur J Immunol.* (2014) 44:239–50. doi: 10.1002/eji.201343774
23. Zhong S, Malecek K, Johnson LA, Yu Z, Vega-Saenz de Miera E, Darvishian F, et al. T-cell receptor affinity and avidity defines antitumor response and autoimmunity in T-cell immunotherapy. *Proc Natl Acad Sci USA.* 110:6973–8. doi: 10.1073/pnas.1221609110
24. Klenerman P, Zinkernagel RM. What can we learn about human immunodeficiency virus infection from a study of lymphocytic choriomeningitis virus? *Immunol Rev.* (1997) 159:5–16. doi: 10.1111/j.1600-065X.1997.tb01003.x
25. Ahmed R, Salmi A, Butler LD, Chiller JM, Oldstone MBA. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med.* (1984) 160:521–40. doi: 10.1084/jem.160.2.521
26. Oldstone MB, Tishon A, Buchmeier MJ. Virus-induced immune complex disease: genetic control of C1q binding complexes in the circulation of mice persistently infected with lymphocytic choriomeningitis virus. *J Immunol.* (1983) 130:912–8.
27. Klenerman P, Zinkernagel RM. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* (1998) 394:482–5. doi: 10.1038/28860
28. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* (2006) 439:682–7. doi: 10.1038/nature04444
29. Murali-Krishna K, Altman JD, Suresh M, Sourdive DJD, Zajac AJ, Miller JD, et al. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* (1998) 8:177–87. doi: 10.1016/S1074-7613(00)80470-7
30. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJD, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med.* (1998) 188:2205–13. doi: 10.1084/jem.188.12.2205
31. Wherry EJ, Blattman JN, Murali-Krishna K, Most R, Van Der, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol.* (2003) 77:4911–3927. doi: 10.1128/JVI.77.8.4911-4927.2003
32. Oldstone MB, Lewicki H, Borrow P, Hudrisier D, Gairin JE. Discriminated selection among viral peptides with the appropriate anchor residues: implications for the size of the cytotoxic T-lymphocyte repertoire and control of viral infection. *J Virol.* (1995) 69:7423–9.
33. Gallimore A, Dumrese T, Hengartner H, Zinkernagel RM, Rammensee HG. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. *J Exp Med.* (1998) 187:1647–57.
34. Bachmann M, Speiser D, Ohashi PS. Functional management of an antiviral cytotoxic T-cell response. *J Virol.* (1997) 71:5764–8.
35. Tissot AC, Ciatto C, Mittl PRE, Grutter MG, Pluckthun A. Viral escape at the molecular level explained by quantitative T-cell receptor/peptide/MHC interactions and the crystal structure of a peptide/MHC complex. *J Mol Biol.* (2000) 302:873–85. doi: 10.1006/jmbi.2000.4501
36. Henrickson SE, Mempel TR, Mazo JB, Liu B, Artyomov MN, Zheng H, et al. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat. Immunol.* (2008) 9:282–91. doi: 10.1038/ni1559
37. van der Most RG, Murali-Krishna K, Whitton JL, Oseroff C, Alexander J, Southwood S, et al. Identification of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. *Virology* (1998) 240:158–67. doi: 10.1006/viro.1997.8934
38. Renard BV, Romero P, Vivier E, Malissen B, Luescher IF. Participation in TCR – ligand binding. *J Exp Med.* (1996) 184. doi: 10.1084/jem.184.6.2439
39. Arcaro A, Gregoire C, Bakker TR, Lucia B, Jordon M, Goffin L, et al. CD8beta endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56(lck) complexes. *J Exp Med.* (2001) 194:1485–95. doi: 10.1084/jem.194.10.1485
40. Laugel B, van den Berg HA, Gostick E, Cole DK, Wooldridge L, Boulter J, et al. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *J Biol Chem.* (2007) 282:23799–810. doi: 10.1074/jbc.M700976200
41. Jiang N, Huang J, Edwards LJ, Liu B, Zhang Y, Beal CD, et al. Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. *Immunity* (2011) 34:13–23. doi: 10.1016/j.immuni.2010.12.017
42. Kim C, Jay DC, Williams MA. Stability and function of secondary Th1 memory cells are dependent on the nature of the secondary stimulus. *J Immunol.* (2012) 189:2348–55. doi: 10.4049/jimmunol.1200244
43. Sabatino JJ, Huang J, Zhu C, Evavold BD. High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4⁺ T cell responses. *J Exp Med.* (2011) 208:81–90. doi: 10.1084/jem.20101574
44. Andargachew R, Martinez RJ, Elizabeth M, Evavold BD. CD4 T cell affinity diversity is equally maintained during acute and chronic infection. *J Immunol.* (2018) 201:19–30. doi: 10.4049/jimmunol.1800295
45. Martinez RJ, Andargachew R, Martinez HA, Evavold BD. Low-affinity CD4+ T cells are major responders in the primary immune response. *Nat Commun.* (2016) 7:13848. doi: 10.1038/ncomms13848
46. Huang J, Edwards LJ, Evavold BD, Zhu C. Kinetics of MHC-CD8 interaction at the T cell membrane. *J Immunol.* (2007) 179:7653–62. doi: 10.4049/jimmunol.179.11.7653
47. Evans E, Leung A, Heinrich V, Zhu C. Mechanical switching and coupling between two dissociation pathways in a P-selectin adhesion bond. *Proc Natl Acad Sci USA.* (2004) 101:11281–6. doi: 10.1073/pnas.0401870101
48. Chen Y, Liu B, Ju L, Hong J, Ji Q, Chen W, et al. Fluorescence biomembrane force probe: concurrent quantitation of receptor-ligand kinetics and binding-induced intracellular signaling on a single cell. *J Vis Exp.* (2015) 102:27–9. doi: 10.3791/52975
49. Chen W, Evans EA, McEver RP, Zhu C. Monitoring receptor-ligand interactions between surfaces by thermal fluctuations. *Biophys J.* (2008) 94:694–701. doi: 10.1529/biophysj.107.117895
50. Kerry SE, Buslepp J, Cramer LA, Maile R, Hensley LL, Nielsen AI, et al. Interplay between TCR affinity and necessity of coreceptor ligation: high-affinity peptide-MHC/TCR interaction overcomes lack of CD8 engagement. *J Immunol.* (2003) 171:4493–503. doi: 10.4049/jimmunol.171.9.4493
51. Tian S, Maile R, Collins EJ, Frelinger JA. CD8+ T cell Activation is governed by TCR-peptide/MHC affinity, not dissociation rate. *J Immunol.* (2007) 179:2952–60. doi: 10.4049/jimmunol.179.5.2952
52. Boulter JM, Schmitz N, Sewell AK, Godkin AJ, Bachmann ME, Gallimore AM. Potent T cell agonism mediated by a very rapid TCR/pMHC interaction. *Eur J Immunol.* (2007) 37:798–806. doi: 10.1002/eji.200636743
53. Huppa JB, Axmann M, Mortelmaier MA, Lillemeier BF, Newell EW, Brameshuber, et al. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature* (2010) 463:963–7. doi: 10.1038/nature08746
54. Rosenthal KM, Edwards LJ, Sabatino JJ, Hood JD, Wasserman HA, Zhu C, et al. Low 2-dimensional CD4 T cell receptor affinity for myelin sets in motion delayed response kinetics. *PLoS ONE* (2012) 7:e32562. doi: 10.1371/journal.pone.0032562
55. Krummey SM, Martinez RJ, Andargachew R, Liu D, Wagener M, Kohlmeier JE, et al. Low-affinity memory CD8+ T cells mediate robust heterologous immunity. *J Immunol.* (2016) 196:2838–46. doi: 10.4049/jimmunol.1500639

56. Potter T, Bluestone JA, Rajan T. A single amino acid substitution on the alpha3 domain of the H-2 class-I molecule abrogates reactivity with CTL. *J Exp Med.* (1987) 166:956–66.
57. Potter T, Rajan T, Dick RF, Bluestone JA. Substitution at the 227 of H-2 class-I molecules abrogates recognition by Cd8- dependent, but not CD8-independent, cytotoxic T lymphocytes. *Nature* (1989) 337:73–5.
58. Salter RD, Benjamin RJ, Wesley PK, Buxton SE, Garrett PJ, Clayberger C, et al. A binding site for the T-cell co-receptor CD8 on the alpha 3 domain of HLA-A2. *Nature* (1990) 345:41–6. doi: 10.1038/345041a0
59. Purbhoo MA, Boulter JM, Price DA, Vuidepot A, Houriga CS, Dunbar PR, et al. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor η chain. *J Biol Chem.* (2001) 276:32786–92 doi: 10.1074/jbc.M102498200
60. Kim ST, Takeuchi K, Sun ZJ, Touma M, Castro CE, Fahmy A, et al. The $\alpha\beta$ T cell receptor is an anisotropic mechanosensor. *J Biol Chem.* (2009) 284:31028–37. doi: 10.1074/jbc.M109.052712
61. Das DK, Feng Y, Mallis RJ, Li X, Keskin DB, Hussey RE, et al. Force-dependent transition in the T-cell receptor β -subunit allosterically regulates peptide discrimination and pMHC bond lifetime. *Proc Natl Acad Sci USA.* (2015) 112:1517–22. doi: 10.1073/pnas.1424829112
62. Liu Y, Blanchfield L, Ma VP, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci USA.* (2016) 113:5610–5. doi: 10.1073/pnas.1600163113
63. Hong J, Persaud SP, Horvath S, Allen PM, Evavold BD, Zhu Z. Force-regulated *in situ* TCR-peptide-bound MHC class II kinetics determine functions of CD4⁺ T cells. *J Immunol.* (2015) 195:3557–64. doi: 10.4049/jimmunol.1501407
64. Feng Y, Brazin KN, Kobayashi E, Mallis RJ, Reinherz EL, Lang M. Mechanosensing drives acuity of $\alpha\beta$ T-cell recognition. *Proc Natl Acad Sci USA.* (2017) 114:E8204–13 doi: 10.1073/pnas.1703559114
65. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in T_{reg} and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med.* (2011) 208:1279–89. doi: 10.1084/jem.20110308
66. Yao S, Buzo BF, Pham D, Jiang L, Taparowsky EJ, Kaplan MH, et al. Interferon regulatory factor 4 sustains CD8⁺ T cell expansion and effector differentiation. *Immunity* (2013) 39:833–45. doi: 10.1016/j.immuni.2013.10.007
67. Raczkowski F, Ritter J, Heesch K, Schumacher V, Guralnik A, Hocker L, et al. The transcription factor Interferon Regulatory Factor 4 is required for the generation of protective effector CD8⁺ T cells. *Proc Natl Acad Sci USA.* (2013) 110:15019–24. doi: 10.1073/pnas.1309378110
68. Man K, Miasari M, Shi W, Xin A, Henstridge DC, Preston S, et al. The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. *Nat Immunol.* (2013) 14:1155–65. doi: 10.1038/ni.2710
69. Knudson KM, Goplen NP, Cunningham CA, Daniels MA, Teixeira E. Low-affinity T cells are programmed to maintain normal primary responses but are impaired in their recall to low-affinity ligands. *Cell Rep.* (2013) 4:554–65 doi: 10.1016/j.celrep.2013.07.008
70. Daniels MA, Teixeira E, Gill J, Hausmann B, Roubaty D, Holmberg K, et al. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* (2006) 444:724–9. doi: 10.1038/nature05269
71. Zehn D, Lee SY, Bevan MJ. Complete but curtailed T-cell response to very low-affinity antigen. *Nature* (2009) 458:211–4. doi: 10.1038/nature07657
72. Gallimore A, Hengartner H, Zinkernagel R. Hierarchies of antigen-specific cytotoxic T-cell responses. *Immunol Rev.* (1998) 164:29–36. doi: 10.1111/j.1600-065X.1998.tb01205.x
73. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses 1. *Annu. Rev. Immunol.* (1999) 17:51–88. doi: 10.1146/annurev.immunol.17.1.51
74. Chen W, Antón LC, Bennink JR, Yewdell JW. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. *Immunity* (2000) 12:83–93.
75. Zheng H, Jin B, Henrickson SE, Perelson AS, von Andrian UH, Chakraborty AK. How antigen quantity and quality determine T-cell decisions in lymphoid tissue. *Mol Cell Biol.* (2008) 28:4040–51.
76. Jankowska KI, Williamson EK, Roy NH, Blumenthal D, Chandra V, Baumgart T, et al. Integrins modulate T cell receptor signaling by constraining actin flow at the immunological synapse. *Front Immunol.* (2018) 9:25. doi: 10.3389/fimmu.2018.00025
77. Merckenschlager J, Ploquin MJ, Eksmond U, Andargachew R, THornborn G, Filby A, et al. Stepwise B-cell-dependent expansion of T helper clonotypes diversifies the T-cell response. *Nat Commun.* (2016) 7:10281. doi: 10.1038/ncomms10281
78. Hood JD, Zarnitsyna VI, Zhu C, Evavold BD. Regulatory and T effector cells have overlapping low to high ranges in TCR affinities for self during demyelinating Disease. *J Immunol.* (2015) 195:4162–70. doi: 10.4049/jimmunol.1501464
79. Sanecka A, Yoshida N, Kolawole EM, Patel H, Evavold BD, Frickel E. T cell receptor-major histocompatibility complex interaction strength defines trafficking and CD103⁺ memory status of CD8 T cells in the Brain. *Front Immunol.* (2018) 9:1290. doi: 10.3389/fimmu.2018.01290
80. Au-Yeung BB, Zikherman J, Mueller JL, Ashouri JF, Matloubian Cheng DA, et al. A sharp T-cell antigen receptor signaling threshold for T-cell proliferation. *Proc Natl Acad Sci USA.* (2014) 111:E3679–88. doi: 10.1073/pnas.1413726111
81. Feng Y, van der Veen J, Shugay M, Putintseva EV, Osmanbeyoglu HU, Dikiy S, et al. A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. *Nature* (2015) 528:132–6. doi: 10.1038/nature16141
82. Blanchfield JL, Shorter SK, Evavold BD. Monitoring the dynamics of T cell clonal diversity using recombinant peptide:MHC technology. *Front Immunol.* (2013) 4:170. doi: 10.3389/fimmu.2013.00170
83. Martinez RJ, Evavold BD. Lower affinity T cells are critical components and active participants of the immune response. *Front Immunol.* (2015) 6:468. doi: 10.3389/fimmu.2015.00468
84. Shorter SK, Schnell FJ, McMaster SR, Pinelli DF, Andargachew R, Evavold BD. Viral escape mutant epitope maintains TCR affinity for antigen yet curtails CD8 T cell responses. *PLoS ONE* (2016) 11:e0149582. doi: 10.1371/journal.pone.0149582
85. Degano M, Garcia KC, Apostolopoulos V, Rudolph MG, Teyton L, Wilson IA. A functional hot spot for antigen recognition in a superagonist TCR/MHC complex. *Immunity* (2000) 12:251–61. doi: 10.1016/S1074-7613(00) 80178-8

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Integrating Immunology and Microfluidics for Single Immune Cell Analysis

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 29 May 2018

Accepted: 24 September 2018

Published: 16 October 2018

Citation:

Sinha N, Subedi N and Tel J (2018)
Integrating Immunology and
Microfluidics for Single Immune Cell
Analysis. *Front. Immunol.* 9:2373.
doi: 10.3389/fimmu.2018.02373

The field of immunoengineering aims to develop novel therapies and modern vaccines to manipulate and modulate the immune system and applies innovative technologies toward improved understanding of the immune system in health and disease. Microfluidics has proven to be an excellent technology for analytics in biology and chemistry. From simple microsystem chips to complex microfluidic designs, these platforms have witnessed an immense growth over the last decades with frequent emergence of new designs. Microfluidics provides a highly robust and precise tool which led to its widespread application in single-cell analysis of immune cells. Single-cell analysis allows scientists to account for the heterogeneous behavior of immune cells which often gets overshadowed when conventional bulk study methods are used. Application of single-cell analysis using microfluidics has facilitated the identification of several novel functional immune cell subsets, quantification of signaling molecules, and understanding of cellular communication and signaling pathways. Single-cell analysis research in combination with microfluidics has paved the way for the development of novel therapies, point-of-care diagnostics, and even more complex microfluidic platforms that aid in creating *in vitro* cellular microenvironments for applications in drug and toxicity screening. In this review, we provide a comprehensive overview on the integration of microsystems and microfluidics with immunology and focus on different designs developed to decode single immune cell behavior and cellular communication. We have categorized the microfluidic designs in three specific categories: microfluidic chips with cell traps, valve-based microfluidics, and droplet microfluidics that have facilitated the ongoing research in the field of immunology at single-cell level.

Keywords: immunoengineering, microfluidics, single-cell analysis, cellular heterogeneity, cellular communication

INTRODUCTION: IMMUNOENGINEERING

The human immune system recognizes myriads of environmental triggers and is highly flexible in generating a variety of signaling responses over time (1, 2). Several types of cells collaborate with antibodies and cytokines to generate an appropriate immune response (3). The spatial organization and migration of cells within tissues as well as the dynamic nature of cellular communication enhances the complexity of our immune system and determines the type of response (4–7). The nature and magnitude of an immune response is dependent on dynamic

molecular and cellular interactions where well-orchestrated cellular communication is the key factor to maintain it (8). The question arises whether all immune cells fight all pathogens and tumors similarly in order to leverage this broad flexibility and diversity. Even though there are multiple subsets of immune cells, with each subset responding to specific stimuli, responses are often initiated by individual cells within each subset and communicated in order to establish a more complex population level response (9). Stochastic expression of genes (influenced by a cellular microenvironment) or pre-defined molecular drivers (as in case of B-cell and T-cell receptors) are driving factors behind heterogeneity in the human immune system (9). Numerous studies over the last decades established that heterogeneity is a trademark of the human immune system (10, 11). The identification of heterogeneity requires systems beyond conventional biological methods like ELISA, western blot, and others that do not allow the required spatial and temporal manipulation of biological cells (12–14). Technologies such as microsystems and microfluidics, have allowed scientists to study the individual behavior of immune cells, identify signaling pathways, map distinct immune cell subsets, quantify secreted molecules and characterize the immune response under varied conditions (15–17). Research on immune cells with technology integration has contributed toward innovative immunotherapy-based treatment modalities with lower treatment-related toxicity and side effects (18–20). They provide better alternatives to more conventional treatment modalities e.g., chemotherapy, radiotherapy or targeted therapy. At the same time, microfluidic based models have assisted in the development of novel therapies, discovery of new drugs, and monitoring the clinical efficacy of new treatments (21–23). Microfluidic devices are also used for the isolation of circulating tumor cells from clinical samples for diagnosis, prognosis, and creation of patient-derived tumor models with the aim to develop and test personalized medicine (24–27). Furthermore, integration of assays, microarrays, and several sensor technologies has led to the development of several point-of-care devices for the early diagnosis of cancer by identification of cancer biomarkers (28, 29).

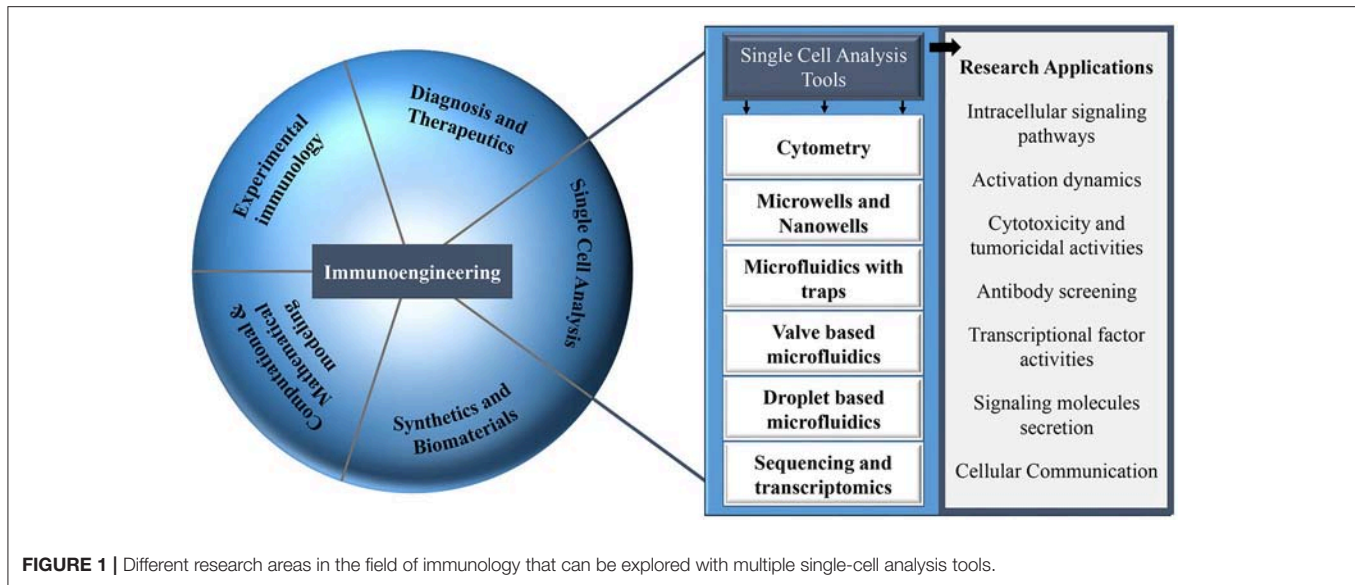
The majority of this type of research can be coined as immunoengineering. The term immunoengineering has been used since the seventies and covers several aspects in the field of immunology (30). Immunoengineering is an interdisciplinary and vast field of research comprising engineering methods and approaches that allow the modulation of the immune system and its responses: biomaterials, tissue engineering, protein engineering, synthetic biology, and drug delivery systems [Figure 1; (31–34)]. Immunoengineering includes the application of systems immunology to replicate complex immune microenvironment, *in vitro*, that aims to enhance our understanding of the human immune system for development of immunotherapy, the modulation of the human immune cells to boost their response against cancer (35–37). Recent

and most noteworthy examples of major discoveries within the field of immunoengineering, specially immunotherapy, are the development of chimeric antigen receptor T-cells and artificial antigen presenting cell systems (38–40). Moreover, immunoengineering also involves mathematical models that describe the functioning of the immune system, technologies to monitor and track the migration of immune cells and engineering tools to understand immune cell function at the systemic level in health and disease (41–43). The field of immunoengineering can, and has been described by various definitions, e.g., for the current issue in *Frontiers* “Application of systems immunology to engineering the tumor immunological microenvironment, aiming at predicting lymphocyte receptor’s recognition patterns. Building sophisticated mimetic *in vitro* models, for instance by means of optical and magnetic tweezers to develop novel immuno-oncotherapeutics paving the way toward personalized and predictive medicine.” The Center for Immunoengineering at Georgia Tech University defined this field as follows: “The field that applies engineering tools and principles to quantitatively study the immune system in health and disease, and to develop new therapies or improve existing therapies by precisely controlling and modulating a patient’s immune response.” The field of immunoengineering has been described in excellent reviews with a focus on engineering approaches to augment immunotherapy (44–48). In this review article, we highlight one aspect of immunoengineering and we particularly discuss various microfluidic and microsystems and focus on their advantages over conventional methods especially for decoding heterogeneous immune cell behavior and cellular interactions.

Single-Cell Technology

Immune cells, characterized by their heterogeneity, tend to differ in their behavior when in different societal contexts ranging from the single cell to the population level. Experiments performed at the population level average out the behavior of all the individual cells (49). Hence, bulk studies fail to provide a coherent understanding of the immune system by masking the phenotype, expressed genes, proteins or metabolites at single-cell level, and cellular communication between single immune cells (49, 50). The advent of single-cell technologies and the subsequent possibility to study the behavior of individual immune cells has uncovered various biological functions that were previously not detectable with bulk studies (51–53). For instance, Shalek et al. demonstrated the importance of paracrine communication for generation of immune response using single-cell analysis (54). Single-cell analysis enabled the investigation of maturation, activation, and signaling pathways of individual immune cells triggered by various environmental factors as well as intercellular communication between different immune cells (43, 55, 56). Additionally, this approach identified new immune cell subsets (57, 58). For instance, single cell transcriptomics, introduced a paradigm shift in the CD4⁺ T helper field and enabled the identification of multiple functionally distinct T helper cell subsets in addition to the two well-established subsets, Th1 and Th2 (59–62).

Abbreviations: IFN- γ , interferon gamma; IL, interleukin; mLSI, microfluidic large scale integration; NF- κ B, nuclear factor- κ B; NK cells, natural killer cells; PAIGE, protein assay via induced gene expression; PDMS, polydimethylsiloxane; TCR, T-cell receptor; TNF, tumor necrosis factor.



Single-cell technology requires isolation of individual cells from a population for multiple data extraction from each isolated cell in order to gain information on the genotype, phenotype, lineage, protein secretion, proliferation, activation, maturation, cytolytic activity, and intercellular communication (63). Single-cell analysis tools are currently investigated by various research groups worldwide and hold great promise in providing a comprehensive understanding of our immune system. After the isolation of individual immune cells, multiple experimental operations for DNA sequencing, RNA and protein expression profiling can be implemented to map the lineage and identify subsets of immune cells (12, 64, 65).

Amongst immunologists, flow and mass cytometry are well-established, high-throughput, and high-content single-cell analysis tools (66–68). Flow cytometers measure fluorescently labeled cells and mass cytometers use transition element isotopes for mapping the functional heterogeneity and phenotypes of different immune cells by quantification of multiple cytokines, chemokines, and surface protein markers of the individual cells (69, 70). One of the benefits of cytometry over other conventional methods is its potential to provide high-throughput analysis of thousands of single-cells and measure multiple parameters in a given time frame (71, 72). Further, with recent advancements, mass cytometry can acquire samples using laser ablation to improve the resolution of this technology and is known as imaging mass cytometry (73). Although cytometers are a powerful tool for single-cell analysis, spectral overlap and limited availability of antibodies labeled with isotopes for flow and mass cytometers, respectively are some of the drawbacks of this technology (13). Even though spectral overlap in conventional cytometry can be effectively mitigated by careful panel design, cytometers are still predominantly an end-point measurement tool that can only provide a snapshot in time and quantify static markers on cells to provide information on immune cell heterogeneity.

Microsystems for Single-Cell Analysis

The requirement for miniaturization of technological platforms has driven the development of several technologies such as microtiter plates (74). However, with microtiter plates, reaction volumes of immunological experiments have only been reduced from milliliters to microlitres. The problem of evaporation and capillary action in microtiter plate technology has hampered its further miniaturization (75). While microtiter plates cannot be further scaled down, the field of microsystems and microfluidics has played a key role in miniaturization to propel interdisciplinary research on single-cell analysis of immune cells.

Driven by the idea of scaling down, nanowells, in combination with microengraving, is a microsystems tool that was developed in the Love laboratory for single-cell analysis (76). Nanowells, made from polydimethylsiloxane (PDMS), contain features that have volumes in the order of nanolitres. When cells, from a bulk solution, are dispensed on the platform, individual cells settle down in each nanowell by gravity. Once the cells are isolated and activated, secreted molecules from the cells can be captured on functionalised glass slides (microengraving) or on the surface of nanowells and quantified by imaging cytometry or microscopy [Figure 2; (76, 77)]. Nanowell-based platforms with imaging cytometry and microarray analysis have been used for quantification of cytokines secreted by T-cells and observation of T-cell proliferation when activated with an array of ligands (CD80, major histocompatibility complex class II/peptide and intercellular adhesion molecule-1) or anti-CD3/CD28 (78). Nanowells can also be used for cell-pairing to study intercellular immune cell interactions and to monitor cytotoxic effector functions of immune cells (79). In 2017, An et al. presented their work on natural killer (NK) cells (80). In their study, they dynamically profiled the secretion of interferon gamma (IFN- γ) from single NK cells to map the phenotypic behavior of these cells based on their cytokine secretion pattern. Using this system,

they showed that CD56^{dim}CD16⁺ NK cells, when activated with phorbol 12-myristate 13-acetate and ionomycin, immediately secrete IFN- γ and that the secretion rate and amount of IFN- γ from these cells is dependent on the donor.

To study complex biological systems, it is essential that technology platforms replicate the cellular microenvironment accurately and also provide precise control over it. Microfluidic devices have been instrumental in providing automated platforms to perform all the essential functions for single-cell analysis of immune cells, on-chip. In the forthcoming sections, we discuss the contribution of microfluidics to the field of single-cell analysis, focused on immune cells, along with its advantages and disadvantages.

Microfluidics for Single-Cell Analysis

Over the last decade, microfluidics has made significant contributions to the field of single-cell analysis. This method allows cells to be monitored dynamically with a high degree of control over the cellular microenvironment (81, 82). These approaches have offered new information by creation of innovative conditions that are limited in conventional bulk methods. Microfluidic systems are being developed for applications in several areas such as protein purification and PCR on a drastically decreased scale (83, 84). Microfluidic chips are capable of accurately replicating *in vivo* biological environments and allow high-throughput analysis of cells (85). Microfluidics allows precise automation and control of analytical functions as well as manipulation of cells and their microenvironments with high resolution in both space and time (86, 87). With microfluidics, scientists can implement techniques and protocols for single-cell analysis through DNA sequencing, RNA expression, and protein quantification for understanding the mechanism of cell activation, proliferation, protein expression, motility and morphology, secretion, and cellular communication (88–91).

The ability to rapidly fabricate microfluidic devices in PDMS by soft lithography has greatly stimulated the development of several microfluidic designs (92). Besides being inexpensive, PDMS is biocompatible and permeable to gases, two properties that are a necessary for replication of artificial cellular microenvironments *in vitro* (93, 94). The flexibility of PDMS allows easy integration of membrane valves and pumps on more complex microfluidic designs to create an intricate network of microchannels wherein protocols can be realized in full automation with the help of programming software (95).

Microfluidic chip designs can be broadly classified in three categories: microfluidics with passive traps, valve-based microfluidics, and droplet microfluidics. With pros and cons of each design, in the field of immunology, microfluidics finds its applications in understanding immune cell behavior at single-cell level.

Microfluidic Chips With Cell Traps

Microfluidic chips with cell traps have been designed by multiple laboratories for single-cell studies (96, 97). In 2006, Di Carlo et al. designed a microfluidic chip with an array of hydrodynamic cell traps for analysis of enzyme kinetics in three different types

of cells (98). Later in 2009, Faley et al. presented their design of a microfluidic chip with multiple traps that was used to study signaling dynamics of isolated, individual, hematopoietic stem cells (99). Besides these studies, several groups have used hydrodynamic cell trap arrays for multiple applications in biology and chemistry (100–102). Of all these groups, the Voldman laboratory has extensively used a modified version of the hydrodynamic cell trap design for specifically studying immune cell interactions at single-cell level (103–106).

Cellular interactions play a vital role in establishing complex immune responses that originate from individual immune cells or immune cell subsets (107). Interactions between immune cells, if hampered, can cause several diseases (108). Hoehl et al. designed a single-layer microfluidic chip for parallel analysis of immune cell interactions at single-cell level (109). They used this design, integrated with weir like U-shaped traps, to pair two different cell types using a three-step loading protocol to obtain cell pairing efficiencies of more than fifty percent. The device was used for pairing murine T-cells with B cells to investigate the activation dynamics of T-cells. They demonstrated the presence of functional heterogeneity in the activation dynamics of OT-1 T-cells. Since OT-1 T-cell are reactive against ovalbumin, it is expected that all the T-cells will show similar activation profile when presented with the antigen. However, within the population of these cells, a variation in response was still observed even though all the cells bear the same identical T-cell receptor (TCR). Later, Dura et al. used this design to characterize the activation dynamics of CD8 T cells (OT-1 and TRP1) with different TCR affinities when paired with antigen presenting cells (104). This study showed that the variations in TCR affinity influences the secretion of cytokines by T-cells. The production of IFN- γ is strong for both low and high TCR affinity whereas the production of interleukin-2 (IL-2) reduces with reduction in the affinity. Dura et al. also used this chip to monitor cytotoxic effector functions of immune cells [Figure 3; (105)]. For this study, they modified the design of the cell traps to capture and pair NK92MI and K562 cells with high cell-pairing efficiency. The cytotoxic activity of NK cells was monitored by measuring the Ca²⁺ signaling for a day and further, the production of IFN- γ in NK cells, when activated with IL-2 and IL-18, was also quantified. Thirty-five percent of the cells showed cytotoxicity and 60% of the cell population produced IFN- γ over time, demonstrating cellular heterogeneity (105).

Besides applications in immune cell interactions, this design was also used for implementation of cell pairing and fusion protocols. In 2009, Skelley et al. paired fibroblasts, mouse embryonic stem cells, and myeloma cells, on-chip, to implement a more efficient electrical and chemical fusion protocol in comparison to the standard procedures (103). Further in 2014, Dura et al. modified the trap design to implement hydrodynamic and deformation based pairing and biologically, chemically, and electrically stimulated fusion of cells, on-chip (106). Kimmerling et al. in 2016 also used the principles of hydrodynamic trapping to design a microfluidic chip with an array of traps for the isolation of single murine leukemia cells, L1210, and primary CD8⁺ T-cells on chip (64). The isolated cells were cultured on-chip and,

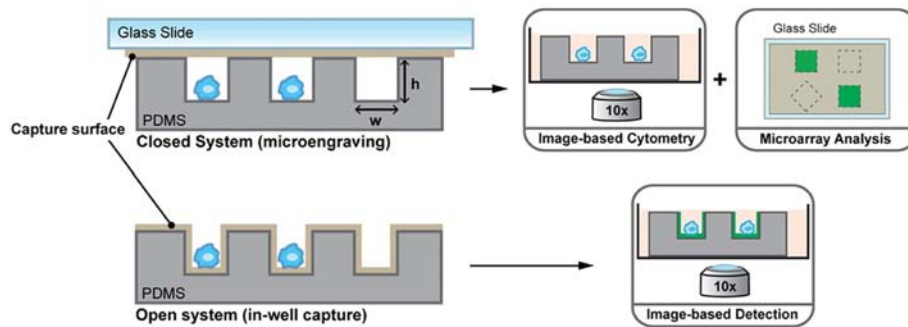


FIGURE 2 | Nanowell format array, developed at the Love laboratory, to understand activation dynamics of immune cells, probe cellular interactions, and quantify cytokine secretion. Figure adapted from Torres et al. (77).

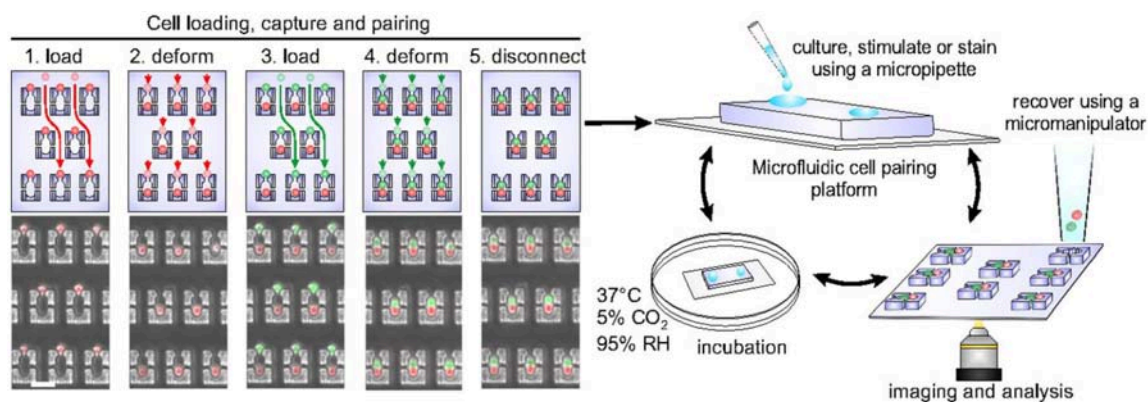


FIGURE 3 | Microfluidic chip, developed in the Voldman laboratory, with hydrodynamic based pillar-like traps. There are multiple variations of this design that allow different types of cells to be loaded and paired to monitor cellular interactions of different kinds. Figure adapted from Dura et al. (105).

after proliferation, these cells were released from the chip for investigating their transcriptomic profiles.

Microfluidic chips with cell traps have made significant contributions and have opened opportunities to investigate different immune cells to attain improved insights into cell-cell communication by allowing deterministic one to one cell pairing which is not easily attainable with other technologies.

Valve-Based Microfluidics: Microfluidic Large-Scale Integration Technology

Microfluidics large-scale integration (mLSI) is the integration of hundreds to thousands of pneumatic membrane valves, arrayed as fluidic multiplexers, on a microfluidic chip (95). Fluidic multiplexers, analogous to electronic multiplexers, allow complex manipulation of fluids with very small number of inputs. Valve-based microfluidic devices are made by aligning two separately cured PDMS layers with channels in such a way that the pneumatic membrane valves are formed when the channels in the two layers intersect each other orthogonally. These pneumatic membrane valves are “push down” when the control layer is on top of the flow layer and are “push up” when the alignment is reversed. The channels in the control layer are pressure driven and responsible for the actuation of pneumatic membrane

valves. The multiplexer on the chip helps in automation and parallelisation of experimental workflows and the pneumatic membrane valves on the chip help in fluid routing, metering, and control (110). mLSI integrated microfluidic devices are controlled using external units and can be programmed to operate for days, allowing them to monitor immune cell activity longitudinally (111).

mLSI devices are versatile and have been adapted for highly complex biological applications including cellular studies and genomic analysis (112–115). mLSI technology has been extensively used by the Quake lab for isolation of mRNA, synthesis of cDNA, and purification of DNA using fully-automated microfluidic chips (116, 117). Furthermore, they used a similar architecture for the ligation and transformation of genes with sample volumes of the order of a few nanolitres (118). In 2014, Ketterer et al. used a highly multiplexed microfluidic chip to develop a sensory system for quantification of metabolites from cellular samples (119). Blazek et al., from the same laboratory, implemented a proximity ligation assay on a fully automated microfluidic chip for analysis of phosphorylation kinetics in cells with high-throughput and parallel analysis (120, 121). The Maerkl lab also focuses on implementation of highly multiplexed and automated microfluidic designs for

characterization and quantification of transcriptional regulatory network and synthesis of genes and genome on microfluidic chips (122–124).

The generation of an immune response relies on environmental cues that are sensed by immune cells in their microenvironment (125). Signals received by cells within their microenvironment are not always continuous but often dynamic and can vary both in time, intensity, and concentration (126). More specifically, environmental cues can have variable amplitude, time of exposure, concentration gradient or can have pulsatile or sinusoidal variations (126). Among these variations, pulsatile modulation holds physiological relevance as several biological cues in our body such as hormones or cytokines are released with temporal variation that in turn affects the mode of action, downstream, of signaling molecules (127). In the human immune system pulsatile bursts of environmental signals dictate cellular heterogeneity and regulate the fate of transcription factors to influence the activation of genes and determine the phenotypic response of immune cells (126). Programmable mLSI based microfluidic devices make it possible to deliver pulsatile bursts of input stimuli to immune cells in a highly controlled fashion (128, 129). mLSI chips are capable of accurately mimicking the cellular microenvironment along with a reduction in extrinsic noise or cell-cell variability to generate synchronized immune cell responses at single-cell level.

In 2016, the Tay lab designed a fully-automated microfluidic device for studying the signaling dynamics of nuclear factor- κ B (NF- κ B) in macrophages [Figure 4A; (128)]. In their design they precisely replicated the dynamics of the immune cell microenvironment in a highly controlled manner and complete automation at single-cell level (128). NF- κ B is an important transcription factor that is responsible for production of cytokines and survival of immune cells (130). The activation and deactivation of NF- κ B shows oscillatory behavior and is popularly studied using microfluidics by providing immune cells with variable input stimuli (131–133). Junkin et al. used an mLSI based microfluidic system, which was integrated with a bead-based immunoassay, to investigate the transcription factor activity and quantify cytokine secretion in macrophages when stimulated with time variable inflammatory signals from the cellular microenvironment (128). The design comprised of 40, individually addressable, cell isolation chambers in which single immune cells were trapped using pillar like structures and each cell chamber was associated with its individual immunoassay unit. For the first time, this study showed the heterogeneous secretion profile of tumor necrosis factor (TNF) when single macrophages are simulated with dynamically variable input stimuli and that there is no correlation between the production of TNF and activation of NF- κ B. Very recently an immunoassay was patterned on the microfluidic chip using a modified version of the mechanically induced trapping of molecular interaction (MITOMI) method for trapping antibodies to quantify TNF secretion [Figure 4B; (129, 134)]. Since the microfluidic device is based on the mLSI technology, it was possible to completely automate the experimental workflow including the patterning of the surface for an immunoassay. In this work, they were able to stimulate cells with dynamically variable signaling molecules in a

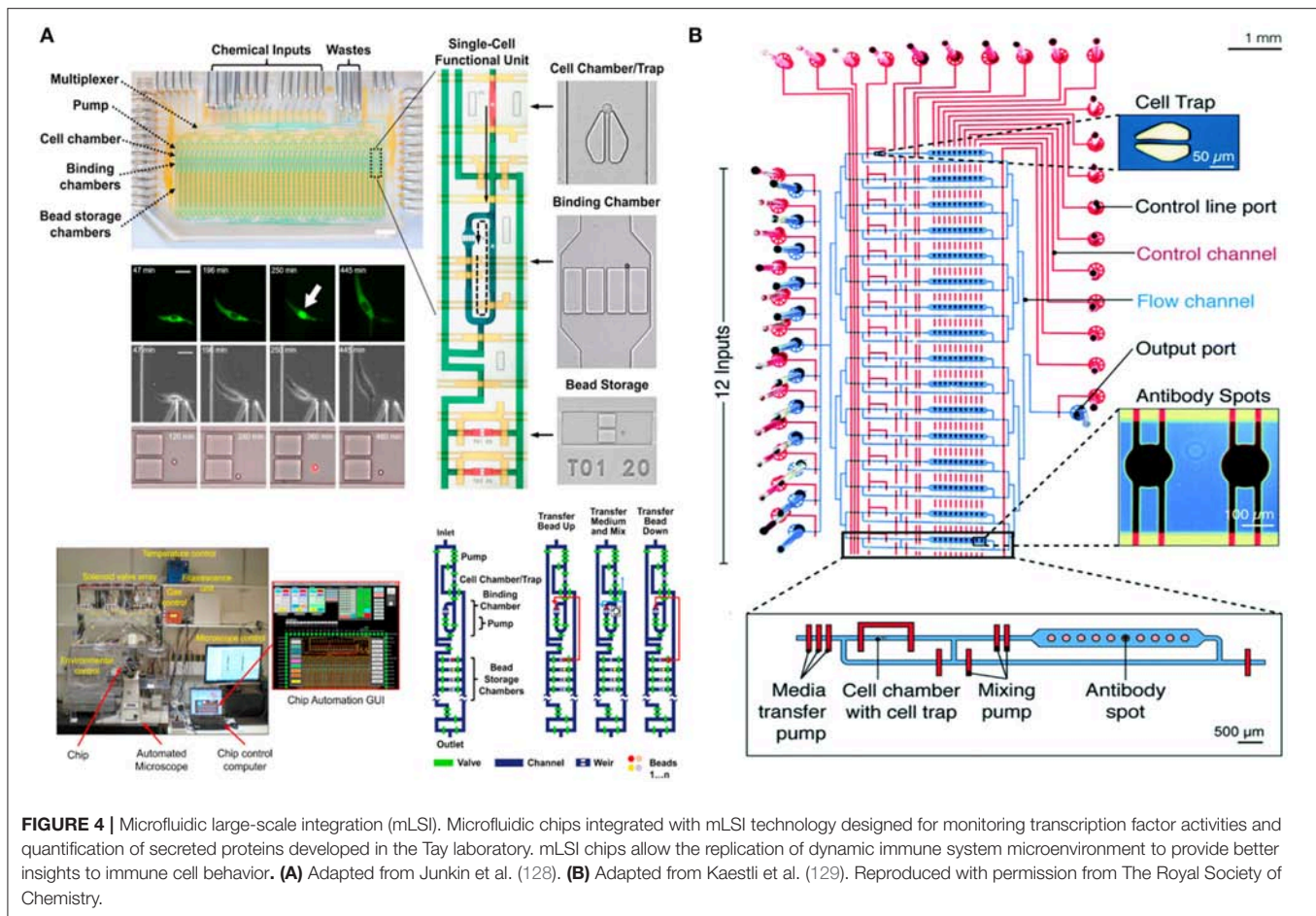
highly-precise and controlled manner as well as to monitor the activation of NF- κ B in real-time using automated microscopy (129). Earlier, Frank et al. presented a microfluidic device that was used to co-culture macrophages and fibroblasts on-chip (135). This co-culture platform enabled the interaction of single immune cells with populations of cells. They used the automated device to provide dynamic inputs of lipopolysaccharide to single macrophages and monitor the signal transmission of TNF, upon activation of NF- κ B, from single macrophages to a population of fibroblasts to replicate the initiation of the immune response. The experimental results of this work showed that an activated macrophage can spatiotemporally control the activation of NF- κ B in fibroblasts to demonstrate that inflammation in tissues is regulated by the dynamics of gene expression (135).

mLSI technology allows automation of functional steps, on-chip, giving researchers the freedom to implement multiple experimental functions that are required for single-cell analysis. The aforementioned examples demonstrate that this technology has made valuable contributions to accurately replicate the dynamics of the cellular microenvironment with high precision and control. These designs can be further implemented to dynamically investigate the behavior of different immune cells at single-cell level.

Droplet-Based Microfluidics

The idea to perform biological analysis in water-in-oil droplets was first published in the 1950s by Nossal and Lederberg (136). Since then droplet microfluidics has continued to fuel a growing body of research leading to multiple applications in fields of biology and chemistry (137–139). Droplet microfluidics has been widely implemented for high-throughput screening of biological and chemical reactions, single-cell analysis, genomics, and transcriptomics (140–144). It also finds applications in molecular detection, imaging, drug delivery, antibody screening, toxicity screening, and diagnostics (145–151). On a microfluidic chip, using two immiscible liquids, droplets, in one liquid phase, are generated in another liquid phase by breaking off either at a T-junction or flow-focusing junction (152, 153). In such a setup, passive generation of droplets relies on drag forces and viscous dissipation (154). Variations in channel geometries help to pair, trap, merge, mix, release, and split droplets (155). Pneumatic membrane valves, electrical forces, optical manipulations and acoustic waves are other alternatives for active production of droplets on microfluidic chips (156–159).

Droplet-based microfluidic platforms provide scientists with the ability to investigate immune cell behavior in complete isolation by creating a noise-free and controllable cellular microenvironment (160). Specifically, it allows to map immune cell subsets, quantify the secretion of signaling molecules from single cells, and investigate cellular communication. In 2015, Sarkar et al. demonstrated an array-based droplet device that allowed monitoring of nanolitre-sized droplets for T-cell activation longitudinally right from the onset of activation (161). Their results suggested that the activation of single T-cells is faster when cells come in contact with dendritic cells in comparison to other activation methods. Furthermore, they developed a method to probe into the potentially heterogeneous cytolytic behavior



of human NK cells (162). They demonstrated a 100% killing efficiency of NK cells, which is in contrast to earlier findings by various groups performed either in bulk or single cell (105, 163).

In order to quantify secreted molecules, cells are paired with functionalised beads or other sensing molecules to capture target analytes during incubation, prior to analysis (164). The droplet interface ensures that encapsulated cells are shielded from external factors that might influence their secretory behavior. Concurrently, this interface in combination with the small droplet volume, confines secreted molecules within the droplet resulting in increased sensitivity. Qiu et al. employed aptamer-based DNA sensors to quantify IFN γ secretion by encapsulating single T-cells in droplets followed by flow-cytometric and microscopic analysis [Figure 5A; (165)]. This study demonstrated the versatility of droplet microfluidics to be integrated with multiple detection methodologies. In another recent study, Eyer et al. used DropMap technology for phenotyping IgG secreting plasma cells at single-cell level [Figure 5B; (166)]. In this study, they paired antibody secreting cells with multiple paramagnetic functionalised nanoparticles that capture target antibodies in picolitre sized droplets. For the purpose of analysis, the generated droplets were immobilized in a glass observation chamber to measure fluorescence intensity of each droplet and to quantify secreted antibodies to map different

plasma cell phenotypes. With this technology it is possible to monitor and quantify antibody secretion by encapsulated cells in droplets real time.

Besides aqueous based droplets, hydrogel agarose can also be used to create droplets in oil phase, which allows washing steps and permits staining with antibodies within droplets by slow diffusion. This conceptual advantage of using hydrogel based droplets was exploited in the Huck laboratory, where agarose droplets were used for encapsulation of Jurkat T-cells to capture multiple cytokines on functionalised beads and used to demonstrate cellular heterogeneity and mapping cellular subsets [Figure 6; (167)]. Generally, for cytometry, droplets need to be broken to retrieve cells and beads. On the contrary, cells and beads encapsulated in hydrogels can be analyzed directly with flow cytometry, preventing loss of cells and saving significant amounts of time.

Recently, researchers have also implemented protocols for single-cell sequencing in droplet microfluidics (168). In 2015, Macosko et al. developed the Drop-seq technology where the transcriptomics of thousands of retinal cells were analyzed in droplets using barcoded microparticles (169). Later, the Abate lab also demonstrated the genomic sequencing of more than fifty thousand cells at single-cell level in agarose microgels (170). Single-cell sequencing allows researchers to

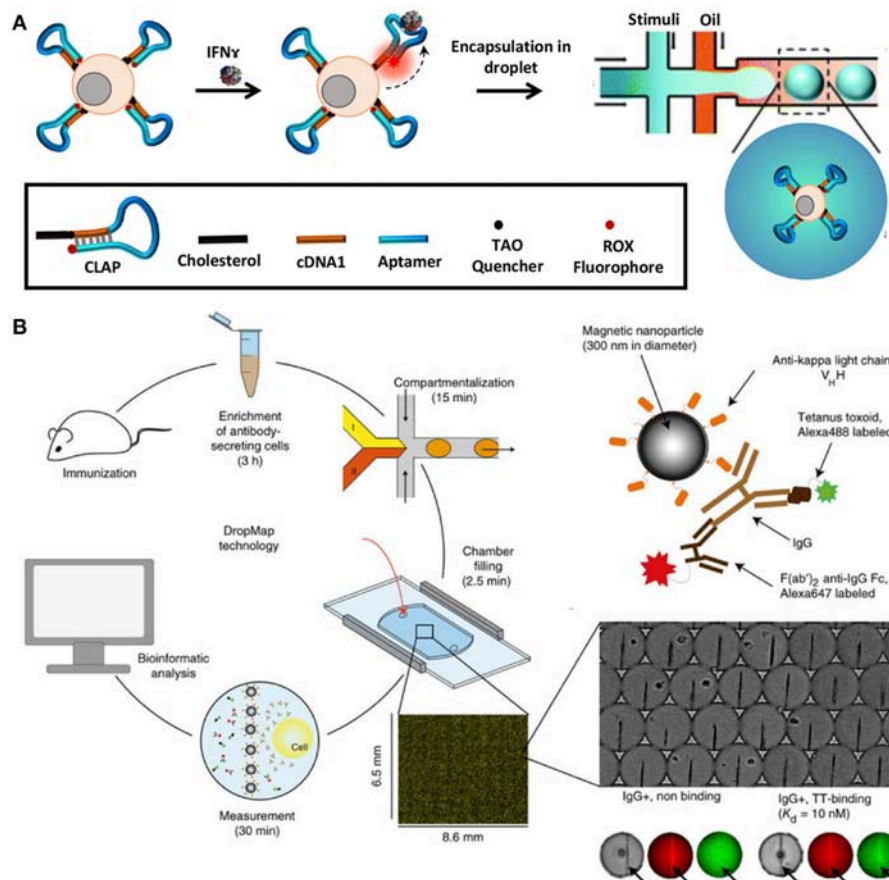


FIGURE 5 | Droplet microfluidics is a very versatile tool that allows single-cell analysis of immune cells in a noise-free environment. The cells are often paired with functionalized beads or sensors such as aptamer sensors for quantification of secreted molecules and proteins. Droplet microfluidics is combined with flow cytometry, mass cytometry, and automated microscopy for downstream analysis. **(A)** Adapted from Qiu et al. (165). **(B)** Adapted from Eyer et al. (166). Reproduced with permission from Springer Nature.

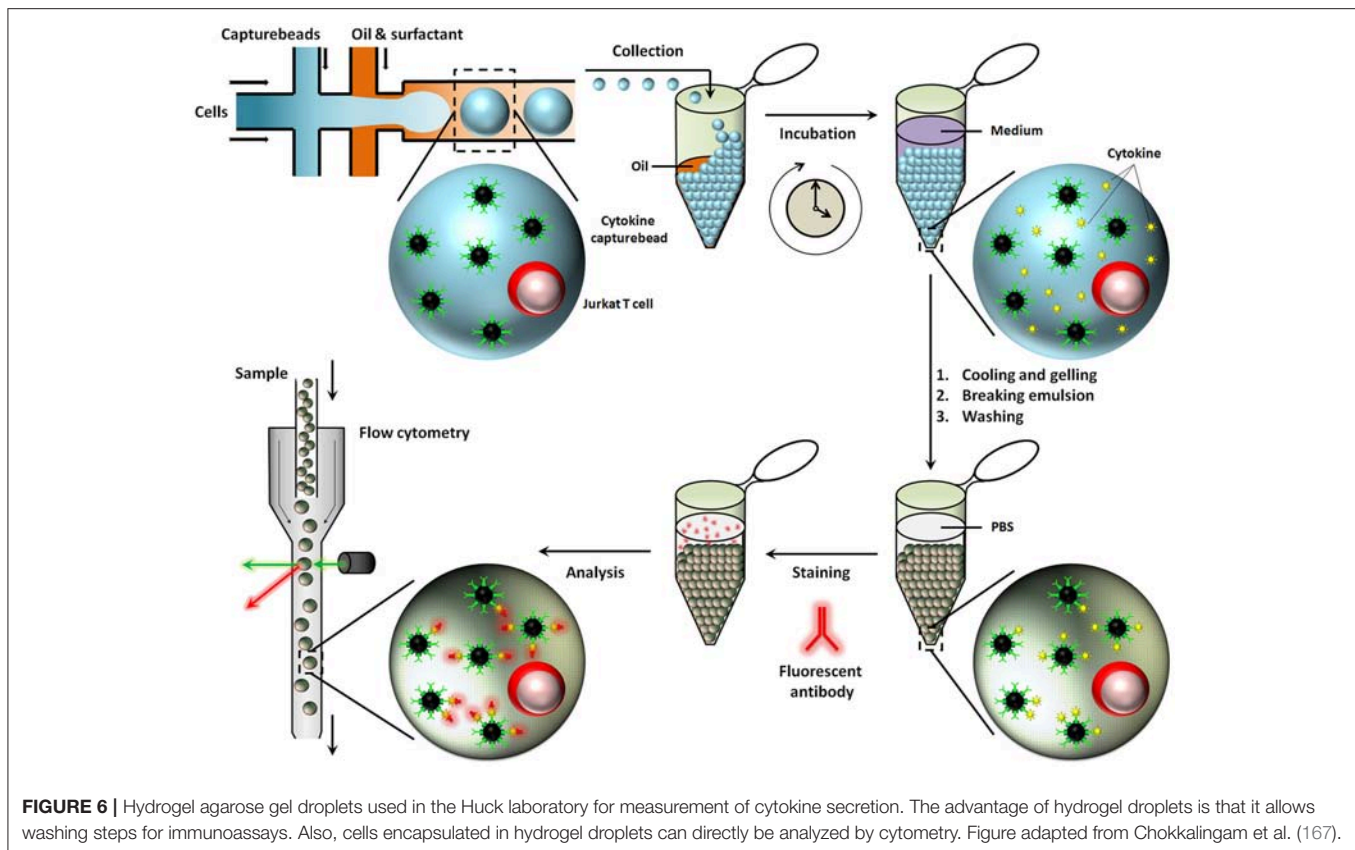
identify the differences in cellular behavior and understand the functionalities of individual cells, which assists in decoding immune cell heterogeneity (171). Genomic amplification for sequencing can be performed in droplet microfluidics with high accuracy and specificity in a massively parallel fashion (168). The work of Shahi et al. demonstrated the efficiency of droplet microfluidics to profile protein secretion by single immune cells using a high-throughput droplet-microfluidic barcoding technique, Abseq (172). This microfluidic device was integrated with functions to amplify DNA in nanolitre sized droplets to allow more than tens of thousands of cells to be analyzed in parallel.

Together, all these studies highlight the role of droplet microfluidics in single-cell analysis of immune cells. Droplet-based microfluidics is a highly versatile and flexible technology and is widely applicable in multiple realms of immunology. The ability to carry out high-throughput analysis of hundreds to thousands of individual immune cells and paired immune cells in a parallel manner makes droplet microfluidics a highly reliable and popular single-cell analysis tool.

Strengths and Weaknesses of Microfluidic Technologies

The motivation for miniaturization was driven by the requirement to acquire more information from single cells at higher resolution. Flow cytometric analysis allows for sampling cell populations in time but fails to provide dynamic information from single immune cells. Also, high costs of the equipment and infrastructure for mass cytometers often limits the usage of this technology. To compensate for the drawbacks of the cytometric analysis and gather more temporal information on the behavior of single immune cells, microsystems and microfluidics gained popularity. Innovation in microsystems and microfluidics facilitated the integration of numerous complex functions on-chip that were earlier not feasible or demanded a lot of manual labor. At the same time, the ability of these platforms to dynamically acquire information from immune cells and monitor immune cell activities real time made them popular among researchers.

Nanowells and microfluidic chips with hydrodynamic cell traps are the simplest examples of miniaturization that,



because of ease of fabrication and operation, are frequently used for decoding immune cell behavior and intercellular communication. These high-throughput analysis platforms allow both real-time and end-point measurements and can facilitate one to one cell pairing for decoding communication between immune cells, e.g., for monitoring cytotoxic cellular function. However, microfluidic chips with cell traps are more efficient in achieving desired pairing efficiencies in comparison to nanowells. These platforms are limited by their ability to replicate the dynamically variable immune cell microenvironment in which immune cells work. Also, for more efficient single-cell level analysis of immune cells it is essential that cells are isolated and analyzed in a noise-free environment to negate the effects of paracrine communication from neighboring cells.

Valve- and droplet-based microfluidics have realized the aforementioned key requirements and have been able to circumvent the drawbacks of other single-cell tools. Both these platforms have the ability to compartmentalize cells in a closed environment to understand cellular behavior with high sensitivity. One of the key advantages of programmable valve-based microfluidics is that it allows the replication of dynamic immune cell microenvironments with high precision for delivering input stimuli in forms of pulsatile bursts. Although the process of fabrication and experimental setup for such devices is fairly complex and time-consuming, automation and reproducibility compensates for the drawbacks of these designs (132). Also, the throughput of mLSI designs is often

low to medium, but has the capacity to be increased by scaling.

For high-throughput analysis of immune cells, droplet-based microfluidics is preferred. Easy to design and implement for multiple applications, it allows the isolation of single immune cells in droplets for analysis in an isolated system. Small compartment size and very low droplet volumes makes this system highly sensitive by preventing the loss of stimuli and secreted molecules from the system. Further, this system also facilitates encapsulation of multiple cells in a closed environment to understand immune cell communication, e.g., for cytotoxic behavior. While droplet microfluidics is a well-established tool in the single-cell analysis community, it often finds limited applications with use of primary, rare immune cells because of the difficulties faced during seeding of cells. Traditional cell seeding methods often lead to a loss of the cells because of attachment or sedimentation, and when cells are already rare in population, it is difficult to obtain high encapsulation rates (173). Encapsulation of cells in droplets is random and relies heavily on Poisson statistics (174). To overcome the limitations of Poisson statistics alternative cell seeding methods as well as use of external physical forces are required to ensure desired cell distribution in droplets (175, 176). The designs discussed in this review, each with their own set of advantages and disadvantages, have been widely implemented for several single cell studies to enhance our understanding of various immune cell functions (Table 1).

TABLE 1 | Table summarizing different single-cell analysis tools discussed in this review in terms of their advantages, disadvantages, and applications.

	Research applications	Advantages	Limitation	Commercial vendors	References
Cytometry	Cytokine and surface markers Signaling and activation dynamics Cytotoxicity Immunophenotyping Sorting	High throughput Quick to run (on the order of several hours) Ability to sort cells based on expression of molecules	Spectral overlaps Expensive Not possible to replicate dynamic cellular microenvironment Static snapshot over time	BD Biosciences, MilliporeSigma, Miltenyi Biotec, Thermo Fisher Scientific	(177), (178), (179)
MICROSYSTEMS					
Nano wells	Antibody and drug screening Activation dynamics Cytotoxicity Inter and intra cellular communication Cytokines and signaling molecules release	High-throughput Possibility to recover cells from the wells Simple to use Multiplexing and real time monitoring Quantitative analysis of proteins Possibility to pair multiple cells in each well	Seeding cells under the effect of gravity lowers the efficiency of cell loading Manual operation Limited control over the fluidic and cellular microenvironment Random distribution of cells in wells	μFluidix, microfluidic ChipShop	(76), (77), (79), (80), (163), (55), (78)
MICROFLUIDICS					
Trap-Based	Inter and intracellular communication Cytotoxicity Signaling and activation dynamics Cytokines and signaling molecules release	Efficient cell pairing and fusion Ability to monitor cellular behavior, from the point of cellular interaction and contact Multiplexing and real time monitoring High throughput	Stimulation of cells in bulk and not on-chip (Juxtacrine and paracrine interactions before loading the cells cannot be ruled out) Controlled on-chip stimulation is not possible Cells are not isolated from each other (The trapped cells and secreted molecules are not completely confined and this can influence the behavior of the neighboring cells by paracrine communication)	μFluidix, microfluidic ChipShop	(104), (105), (106), (96), (109)
Valve-based	Signaling and activation dynamics Cytokines and signaling molecules release Monitor transcription factors activity Migration	Complete automation Replication of dynamic cellular microenvironment Sensitive (addressable cell chambers with very small volume) Low sample volume Both real time monitoring and end-point analysis	Laborious and time-consuming fabrication Not portable (additional equipment for operation) Low to medium throughput	Fluidigm C1	(128), (129), (135), (16), (180), (181), (182)
Droplet-based	Inter and intracellular communication Cytokines and signaling molecules release Activation dynamics Antibody screening Cytotoxicity	High throughput Noise-free cellular microenvironment due to compartmentalization Both real time monitoring and end point analysis Multiple as well as single cell pairing Sensitive (low reaction volume) Small sample and reagent volume	Not possible to replicate dynamic cellular microenvironment Random encapsulation (follows Poisson Distribution) Difficult to incorporate washing steps	μFluidix, microfluidic ChipShop, Dolomite, Fluigent, 10X Genomics (Chromium Controller) BIORAD (ddSEQ)	(144), (162), (164), (166), (167), (161)

The applications described here are what is presented in this review as well as all the other potential applications of the design for immune cell analysis at single-cell level.

CONCLUSION AND FUTURE OUTLOOK

Single-cell analysis tools have played a major role in enhancing our understanding of the human immune system. Several research groups have focused on technology development to constantly provide novel design alternatives for biological studies. Thereby, it enabled to address complex immunological questions that were earlier not possible with conventional bulk methods and resulted in identification of heterogeneous immune cell behavior, discovery of new immune cell subsets, and understanding how single immune cells drive population responses. Single-cell analysis facilitated the design and development of new diagnostic tools, personalized medicines, and immunotherapies for treatment of cancer, immunosuppressive diseases, and autoimmune disorders (183–186). For example, single-cell studies recognized specific signaling pathways within individual immune cells that were suppressed in a tumor microenvironment (187). Identification of such immunosuppressive signaling pathways, molecules, and individual immune cells improves the design of treatment modalities aimed at targeting cells and activation of the suppressed signaling pathways to fight cancer, infectious- and auto-immune diseases (184, 188). For development of vaccines, it is critical to understand how specific antigens induce effective immunization. Novel vaccines with higher clinical efficacy can be developed using results from antibody screening and quantification experiments at single-cell level (149, 166). Also, quantification of signaling molecules at single-cell level provides information on new pathways for development of sensitive diagnostic tools that can provide faster and accurate results in comparison to traditional laboratory methods (189).

Developments in the field of microsystems and microfluidics have been ongoing for more than a decade and continues to grow. As our understanding of the human immune systems deepens, more questions arise to decode the complexity of our system. To cater to these questions, technology continues to evolve. The designs discussed in this review were limited to applications in single-cell analysis of immune cells. However, there are several established and on-going design developments in single-cell research for multiple biological applications that can be easily modified and implemented for better understanding of the immune system. As an example, the microfluidic droplet system published by Shembekar et al. can be modified for use with primary B-cells for antibody screening (149). Furthermore, a droplet microfluidic system integrated with Protein Assay via Induced Gene Expression (PAIGE) can be used for quantification of secretory molecules at single-cell level (190). Microfluidic chips can be also be used to study immunosurveillance and migration of immune cells, *in vitro* (191, 192). Finally, there are several other designs that can be integrated with different systems or modified for immune system related research (193–195).

There are multiple small and medium scale companies that have commercialized microfluidics, as individual components

or complete analytical system, to promote the integration of microfluidics in multiple laboratories for several single-cell analysis applications. Companies like Dolomite and Fluigent fabricate droplet microfluidic chips that can be bought and directly used in laboratories for research applications. These chips, however, still have to be integrated with downstream analytical methods. Other companies such as μ Fluidix and microfluidic ChipShop do provide professional facilities to fabricate different types of microfluidic devices as per the user requirements. Sphere Fluidics also provides completely integrated analytical solutions for single-cell research. Further, there are commercial systems integrated with microfluidics that provide complete analytical solutions to research problems. One such example is the C1, developed by FLUIDIGM, that is integrated with microfluidic circuits for transcriptomics at single-cell level to identify heterogeneity among immune cell population. This system provides a fully automated solution to implement experimental protocols with high precision and accuracy. The Chromium Controller by 10X Genomics allows profiling of immune cells and their repertoire at single-cell level with high automation and parallelisation. ddSEQ by BIORAD uses droplet microfluidics for isolation of single cells to provide sequencing solutions at single cell level. This commercial device has multiple applications including assessment of cellular heterogeneity, identification of cellular sub-populations, and functional analysis of immune cells. The aforementioned examples are just a few of the many companies that have commercialized microfluidic technology for research purposes.

Taken together, robust technology for decoding cell-cell or cell-pathogen interactions longitudinally and in great detail will revolutionize cell biology and the fields of immunology and cellular immunotherapy in particular. The impact of rapid expansion of single-cell analysis is evident in its great potential for numerous applications, including, but not limited to: cancer research, regenerative medicine, diagnostics, and synthetic biology. We believe that even though technology development might sometimes be an extended process, the ease and cost-effectiveness of microfluidics will boost the integration of this exciting technology in the portfolio of other single-cell assays used in cell biology and immunology related disciplines.

AUTHOR CONTRIBUTIONS

NSi, NSu, and JT conceived the manuscript and the writing of the manuscript and approved its final content.

ACKNOWLEDGMENTS

The authors thank Dr. Leda Klouda and Dr. Nina Tel-Karthaus for the critical reading of our manuscript. Furthermore, we acknowledge generous support by the Eindhoven University of Technology.

REFERENCES

- Janeway C A. How the immune system works to protect the host from infection: a personal view. *Proc Natl Acad Sci USA* (2001) 98:7461–8. doi: 10.1073/pnas.131202998
- Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* (2000) 406:782–7. doi: 10.1038/35021228
- Subramanian N, Torabi-Parizi P, Gottschalk RA, Germain RN, Dutta B. Network representations of immune system complexity. *Wiley Interdiscip Rev Syst Biol Med*. (2015) 7:13–38. doi: 10.1002/wsbm.1288
- Mueller SN, Zaid A, Carbone FR. Tissue-resident T cells: dynamic players in skin immunity. *Front Immunol*. (2014) 5:332. doi: 10.3389/fimmu.2014.00332
- Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+T cells to sites of CD4+T cell-dendritic cell interaction. *Nature* (2006) 440:890–5. doi: 10.1038/nature04651
- Cheroutre H, Huang Y. Crosstalk between adaptive and innate immune cells leads to high quality immune protection at the mucosal borders. *Adv Exp Med Biol*. (2013) 785:43–7. doi: 10.1007/978-1-4614-6217-0_5
- Rivera A, Siracusa MC, Yap GS, Gause WC. Innate cell communication kick-starts pathogen-specific immunity. *Nat Immunol*. (2016) 17:356–63. doi: 10.1038/ni.3375
- Xie J, Tato CM, Davis MM. How the immune system talks to itself: the varied role of synapses. *Immunol Rev*. (2013) 251:65–79. doi: 10.1111/imr.12017
- Satija R, Shalek AK. Heterogeneity in immune responses: from populations to single cells. *Trends Immunol*. (2014) 35:219–29. doi: 10.1016/j.it.2014.03.004
- Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* (2007) 27:393–405. doi: 10.1016/j.immuni.2007.08.007
- O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* (1998) 8:275–83. doi: 10.1016/S1074-7613(00)80533-6
- Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nat Rev Immunol*. (2018) 18:35–45. doi: 10.1038/nri.2017.76
- Chattopadhyay PK, Gierahn TM, Roederer M, Love JC. Single-cell technologies for monitoring immune systems. *Nat Immunol*. (2014) 15:128–35. doi: 10.1038/ni.2796
- Novo P, Dell'Aica M, Janasek D, Zahedi RP. High spatial and temporal resolution cell manipulation techniques in microchannels. *Analyst* (2016) 141:1888–1905. doi: 10.1039/C6AN00027D
- Kellogg RA, Tian C, Lipniacki T, Quake SR, Tay S. Digital signaling decouples activation probability and population heterogeneity. *Elife* (2015) 4:e08931. doi: 10.7554/eLife.08931
- Ma C, Fan R, Ahmad H, Shi Q, Comin-Anduix B, Chodon T, et al. A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat Med*. (2011) 17:738–43. doi: 10.1038/nm.2375
- Kirschbaum M, Jaeger MS, Duschl C. Correlating short-term Ca2+ responses with long-term protein expression after activation of single T cells. *Lab Chip* (2009) 9:3517–25. doi: 10.1039/b911865a
- Hemmer B, Nessler S, Zhou D, Kieseier B, Hartung HP. Immunopathogenesis and immunotherapy of multiple sclerosis. *Nat Clin Pract Neurol*. (2006) 2:201–11. doi: 10.1038/ncpneu0154
- Ma X, Hui H, Jin Y, Dong D, Liang X, Yang X, et al. Enhanced immunotherapy of SM5-1 in hepatocellular carcinoma by conjugating with gold nanoparticles and its *in vivo* bioluminescence tomographic evaluation. *Biomaterials* (2016) 87:46–56. doi: 10.1016/j.biomaterials.2016.02.007
- Li Y, Fang M, Zhang J, Wang J, Song Y, Shi J, et al. Hydrogel dual delivered celecoxib and anti-PD-1 synergistically improve antitumor immunity. *Oncoimmunology* (2016) 5:e1074374. doi: 10.1080/2162402X.2015.1074374
- Esch MB, King TL, Shuler ML. The role of body-on-a-chip devices in drug and toxicity studies. *Annu Rev Biomed Eng*. (2011) 13:55–72. doi: 10.1146/annurev-bioeng-071910-124629
- Hassell BA, Goyal G, Lee E, Sontheimer-Phelps A, Levy O, Chen CS, et al. Human organ chip models recapitulate orthotopic lung cancer growth, therapeutic responses, and tumor dormancy *in vitro*. *Cell Rep*. (2017) 21:508–16. doi: 10.1016/j.celrep.2017.09.043
- Lanz HL, Saleh A, Kramer B, Cairns J, Ng CP, Yu J, et al. Therapy response testing of breast cancer in a 3D high-throughput perfused microfluidic platform. *BMC Cancer* (2017) 17:709. doi: 10.1186/s12885-017-3709-3
- Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Utkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* (2007) 450:1235–9. doi: 10.1038/nature06385
- Chen H, Cao B, Sun B, Cao Y, Yang K, Lin YS, et al. Highly-sensitive capture of circulating tumor cells using micro-ellipse filters. *Sci Rep*. (2017) 7:610. doi: 10.1038/s41598-017-00232-6
- Khoo BL, Grecni G, Lim YB, Lee SC, Han J, Lim CT. Expansion of patient-derived circulating tumor cells from liquid biopsies using a CTC microfluidic culture device. *Nat Protoc*. (2018) 13:34–58. doi: 10.1038/nprot.2017.125
- Del Ben F, Turetta M, Celetti G, Piruska A, Bulfoni M, Cesselli D, et al. A method for detecting circulating tumor cells based on the measurement of single-cell metabolism in droplet-based microfluidics. *Angew Chemie* (2016) 55:8581–4. doi: 10.1002/anie.201602328
- Zheng G, Patolsky F, Cui Y, Wang WU, Lieber CM. Multiplexed electrical detection of cancer markers with nanowire sensor arrays. *Nat Biotechnol*. (2005) 23:1294–301. doi: 10.1038/nbt1138
- Chikkaveeraiah B V., Mani V, Patel V, Gutkind JS, Rusling JF. Microfluidic electrochemical immunoarray for ultrasensitive detection of two cancer biomarker proteins in serum. *Biosens Bioelectron*. (2011) 26:4477–83. doi: 10.1016/j.bios.2011.05.005
- Walford RL, Meredith PAJ, Cheney KE. Immunoengineering: prospects for correction of age-related immunodeficiency states. In: Makinodan T, Yunis E, editors. *Immunology and Aging. Comprehensive Immunology*. Vol. 1. Boston, MA: Springer (1977). p. 183–4.
- Singh A, Roy K. Immuno-engineering: the next frontier in therapeutics delivery. *Adv Drug Deliv Rev*. (2017) 114:1–2. doi: 10.1016/j.addr.2017.08.005
- Goldberg MS. Immunoengineering: how nanotechnology can enhance cancer immunotherapy. *Cell* (2015) 161:201–4. doi: 10.1016/j.cell.2015.03.037
- Xie YQ, Wei L, Tang L. Immunoengineering with biomaterials for enhanced cancer immunotherapy. *Wiley Interdiscip Rev Nanomedicine Nanobiotechnol*. (2018) 10:e1506. doi: 10.1002/wnan.1506
- Weiden J, Tel J, Figdor CG. Synthetic immune niches for cancer immunotherapy. *Nat Rev Immunol*. (2018) 18:212–9. doi: 10.1038/nri.2017.89
- Davis MM, Tato CM, Furman D. Systems immunology: just getting started. *Nat Immunol*. (2017) 18:725–32. doi: 10.1038/ni.3768
- Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. (2004) 10:909–15. doi: 10.1038/nm1100
- Papaioannou NE, Beniata OV, Vitsos P, Tsitsilonis O, Samara P. Harnessing the immune system to improve cancer therapy. *Ann Transl Med*. (2016) 4:261. doi: 10.21037/atm.2016.04.01
- Jackson HJ, Rafiq S, Brentjens RJ. Driving CAR T-cells forward. *Nat Rev Clin Oncol*. (2016) 13:370–83. doi: 10.1038/nrclinonc.2016.36
- Turtle CJ, Riddell SR. Artificial antigen-presenting cells for use in adoptive immunotherapy. *Cancer J*. (2011) 16:374–81. doi: 10.1097/PPO.0b013e3181eb33a6
- Eggermont LJ, Paulis LE, Tel J, Figdor CG. Towards efficient cancer immunotherapy: advances in developing artificial antigen-presenting cells. *Trends Biotechnol*. (2014) 32:456–65. doi: 10.1016/j.tibtech.2014.06.007
- Khailaie S, Bahrami F, Janahmadi M, Milanez-Almeida P, Huehn J, Meyer-Hermann M. A mathematical model of immune activation with a unified self-nonself concept. *Front Immunol*. (2013) 4:474. doi: 10.3389/fimmu.2013.00474
- Haessler U, Pisano M, Wu M, Swartz MA. Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19. *Proc Natl Acad Sci USA* (2011) 108:5614–9. doi: 10.1073/pnas.1014920108
- Junkin M, Tay S. Microfluidic single-cell analysis for systems immunology. *Lab Chip* (2014) 14:1246–60. doi: 10.1039/c3lc51182k

44. Jeanbart L, Swartz MA. Engineering opportunities in cancer immunotherapy. *Proc Natl Acad Sci USA* (2015) 112:14467–72. doi: 10.1073/pnas.1508516112
45. Swartz MA, Hirose S, Hubbell JA. Engineering approaches to immunotherapy. *Sci Transl Med.* (2012) 4:148rv9. doi: 10.1126/scitranslmed.3003763
46. Fesnak AD, June CH, Levine BL. Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat Rev Cancer* (2016) 16:566–81. doi: 10.1038/nrc.2016.97
47. Butler MO, Hirano N. Human cell-based artificial antigen-presenting cells for cancer immunotherapy. *Immunol Rev.* (2014) 257:191–209. doi: 10.1111/immr.12129
48. Delcassian D, Sattler S, Dunlop IE. T cell immunoengineering with advanced biomaterials. *Integr Biol.* (2017) 9:211–22. doi: 10.1039/c6ib00233a
49. Toriello NM, Douglas ES, Thaitrong N, Hsiao SC, Francis MB, Bertozzi CR, et al. Integrated microfluidic bioprocessor for single-cell gene expression analysis. *Proc Natl Acad Sci USA* (2008) 105:20173–8. doi: 10.1073/pnas.0806355106
50. Daojing Wang, SB. Single cell analysis: the new frontier in 'Omics.' *Trends Biotechnol.* (2010) 28:281–90. doi: 10.1016/j.tibtech.2010.03.002
51. Newell EW, Sigal N, Nair N, Kidd BA, Greenberg HB, Davis MM. Combinatorial tetramer staining and mass cytometry analysis facilitate T-cell epitope mapping and characterization. *Nat Biotechnol.* (2013) 31:623–9. doi: 10.1038/nbt.2593
52. Polikowsky HG, Wogtsland CE, Diggins KE, Huse K, Irish JM. Cutting edge: redox signaling hypersensitivity distinguishes human germinal center B cells. *J Immunol.* (2015) 195:1364–7. doi: 10.4049/jimmunol.1500904
53. Liu T, Yamaguchi Y, Shirasaki Y, Shikada K, Yamagishi M, Hoshino K, et al. Single-cell imaging of caspase-1 dynamics reveals an all-or-none inflammasome signaling response. *Cell Rep.* (2014) 8:974–82. doi: 10.1016/j.celrep.2014.07.012
54. Shalek AK, Satija R, Shuga J, Trombetta JJ, Gennert D, Lu D, et al. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. *Nature* (2014) 510:363–9. doi: 10.1038/nature13437
55. Zaretsky I, Polonsky M, Shifrut E, Reich-Zeliger S, Antebi Y, Aidelberg G, et al. Monitoring the dynamics of primary T cell activation and differentiation using long term live cell imaging in microwell arrays. *Lab Chip* (2012) 12:5007–15. doi: 10.1039/c2lc40808b
56. Wang J, Tham D, Wei W, Shin YS, Ma C, Ahmad H, Shi Q, et al. Quantitating cell-cell interaction functions with applications to glioblastoma multiforme cancer cells. *Nano Lett.* (2012) 12:6101–6. doi: 10.1021/nl302748q
57. De Rosa SC, Herzenberg LA, Herzenberg LA, Roederer M. 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med.* (2001) 7:245–8. doi: 10.1038/84701
58. Roncador G, Brown PJ, Maestre L, Hue S, Martínez-Torrecuadrada JL, Ling K-L, et al. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol.* (2005) 35:1681–91. doi: 10.1002/eji.200526189
59. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity* (2009) 30:616–25. doi: 10.1016/j.immuni.2009.04.009
60. Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol.* (2014) 35:61–8. doi: 10.1016/j.it.2013.10.004
61. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest.* (2009) 119:3573–85. doi: 10.1172/JCI40202
62. Gaublotte JT, Yosef N, Lee Y, Gertner RS, Yang L V, Wu C, et al. Single-cell genomics unveils critical regulators of Th17 cell pathogenicity. *Cell* (2015) 163:1400–12. doi: 10.1016/j.cell.2015.11.009
63. Yalcin A, Yamanaka YJ, Love JC. Analytical technologies for integrated single-cell analysis of human immune responses. *Methods Mol Biol.* (2012) 853:211–35. doi: 10.1007/978-1-61779-567-1_16
64. Kimmerling RJ, Lee Szeto G, Li JW, Genshaft AS, Kazer SW, Payer KR, et al. A microfluidic platform enabling single-cell RNA-seq of multigenerational lineages. *Nat Commun.* (2016) 7:10220. doi: 10.1038/ncomms10220
65. Jaitin DA, Kenigsberg E, Keren-Shaul H, Elefant N, Paul F, Zaretsky I, et al. Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. *Science* (2014) 343:776–9. doi: 10.1126/science.1247651
66. Ahn RS, Taravati K, Lai K, Lee KM, Nititham J, Gupta R, et al. Transcriptional landscape of epithelial and immune cell populations revealed through FACS-seq of healthy human skin. *Sci Rep.* (2017) 7:1343. doi: 10.1038/s41598-017-01468-y
67. Yan X, Wu C, Chen T, Santos MM, Liu CL, Yang C, et al. Cathepsin S inhibition changes regulatory T-cell activity in regulating bladder cancer and immune cell proliferation and apoptosis. *Mol Immunol.* (2017) 82:66–74. doi: 10.1016/j.molimm.2016.12.018
68. Korin B, Dubovik T, Rolls A. Mass cytometry analysis of immune cells in the brain. *Nat Protoc.* (2018) 13:377–91. doi: 10.1038/nprot.2017.155
69. Goetz C, Peng LJ, Aggeler B, Bonnevier J. Phenotyping CD4+ hTh2 cells by flow cytometry: Simultaneous detection of transcription factors, secreted cytokines, and surface markers. *Methods Molecul Biol.* (2017) 1554:175–84. doi: 10.1007/978-1-4939-6759-9_10
70. Lin D, Maecker HT. Mass cytometry assays for antigen-specific T cells using CyTOF. *Methods Mol Biol.* (2018) 1678:37–47. doi: 10.1007/978-1-4939-7346-0_3
71. Dhoble AS, Bekal S, Dolatowski W, Yanz C, Lambert KN, Bhalerao KD. A novel high-throughput multi-parameter flow cytometry based method for monitoring and rapid characterization of microbiome dynamics in anaerobic systems. *Bioresour Technol.* (2016) 220:566–71. doi: 10.1016/j.biortech.2016.08.076
72. Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. *Trends Immunol.* (2012) 33:323–32. doi: 10.1016/j.it.2012.02.010
73. Giesen C, Wang HAO, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat Methods* (2014) 11:417–22. doi: 10.1038/nmeth.2869
74. Clausell-Tormos J, Lieber D, Baret JC, El-Harrak A, Miller OJ, Frenz L, et al. Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms. *Chem Biol.* (2008) 15:427–37. doi: 10.1016/j.chembiol.2008.04.004
75. Dove A. Drug screening - beyond the bottleneck. *Nat Biotechnol.* (1999) 17:859–63. doi: 10.1038/12845
76. Love JC, Ronan JL, Grotenbreg GM, Van Der Veen AG, Ploegh HL. A microengraving method for rapid selection of single cells producing antigen-specific antibodies. *Nat Biotechnol.* (2006) 24:703–7. doi: 10.1038/nbt1210
77. Torres AJ, Hill AS, Love JC. Nanowell-based immunoassays for measuring single-cell secretion: characterization of transport and surface binding. *Anal Chem.* (2014) 86:11562–9. doi: 10.1021/ac4030297
78. Torres AJ, Contento RL, Gordo S, Wucherpennig KW, Love JC. Functional single-cell analysis of T-cell activation by supported lipid bilayer-tethered ligands on arrays of nanowells. *Lab Chip* (2013) 13:90–9. doi: 10.1039/c2lc40869d
79. Yamanaka YJ, Berger CT, Sips M, Cheney PC, Alter G, Love JC. Single-cell analysis of the dynamics and functional outcomes of interactions between human natural killer cells and target cells. *Integr Biol.* (2012) 4:1175–84. doi: 10.1039/c2ib20167d
80. An X, Sendra VG, Liadi I, Ramesh B, Romain G, Haymaker C, et al. Single-cell profiling of dynamic cytokine secretion and the phenotype of immune cells. *PLoS ONE* (2017) 12:e0181904. doi: 10.1371/journal.pone.0181904
81. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature* (2014) 507:181–9. doi: 10.1038/nature13118
82. Young EWK, Beebe DJ. Fundamentals of microfluidic cell culture in controlled microenvironments. *J Chem Soc.* (2010) 39:1036–48. doi: 10.1039/B813328J/Analyst
83. Zhu Q, Qiu L, Xu Y, Li G, Mu Y. Single cell digital polymerase chain reaction on self-priming compartmentalization chip. *Biomicrofluidics* (2017) 11:014109. doi: 10.1063/1.4975192
84. Rodríguez-Ruiz I, Babenko V, Martínez-Rodríguez S, Gavira JA. Protein separation under a microfluidic regime. *Analyst* (2018) 143:606–19. doi: 10.1039/c7an01568b

85. Kim D, Wu X, Young AT, Haynes CL. Microfluidics-based in vivo mimetic systems for the study of cellular biology. *Acc Chem Res.* (2014) 47:1165–73. doi: 10.1021/ar4002608
86. Yi C, Li CW, Ji S, Yang M. Microfluidics technology for manipulation and analysis of biological cells. *Anal Chim Acta* (2006) 560:1–23. doi: 10.1016/j.aca.2005.12.037
87. Meyvantsson I, Beebe DJ. Cell culture models in microfluidic systems. *Annu Rev Anal Chem.* (2008) 1:423–9. doi: 10.1146/annurev.anchem.1.031207.113042
88. Deng Y, Zhang Y, Sun S, Wang Z, Wang M, Yu B, et al. An integrated microfluidic chip system for single-cell secretion profiling of rare circulating tumor cells. *Sci Rep.* (2014) 4:7499. doi: 10.1038/srep07499
89. Hultström J, Manneberg O, Dopf K, Hertz HM, Brismar H, Wiklund M. Proliferation and viability of adherent cells manipulated by standing-wave ultrasound in a microfluidic chip. *Ultrasound Med Biol.* (2007) 33:145–51. doi: 10.1016/j.ultrasmedbio.2006.07.024
90. Diercks AH, Ozinsky A, Hansen CL, Spotts JM, Rodriguez DJ, Aderem A. A microfluidic device for multiplexed protein detection in nano-liter volumes. *Anal Biochem.* (2009) 386:30–5. doi: 10.1016/j.ab.2008.12.012
91. Lecault V, White AK, Singhal A, Hansen CL. Microfluidic single cell analysis: from promise to practice. *Curr Opin Chem Biol.* (2012) 16:381–90. doi: 10.1016/j.cbpa.2012.03.022
92. Sia SK, Whitesides GM. Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis* (2003) 24:3563–76. doi: 10.1002/elps.200305584
93. Kane RS, Takayama S, Ostuni E, Ingber DE, Whitesides GM. Patterning proteins and cells using soft lithography. *Biomaterials* (1999) 20:2363–76. doi: 10.1016/S0142-9612(99)00165-9
94. Bhagat AAS, Jothimuthu P, Papautsky I. Photodefinable polydimethylsiloxane (PDMS) for rapid lab-on-a-chip prototyping. *Lab Chip* (2007) 7:1192–7. doi: 10.1039/b704946c
95. Thorsen T, Maerkl SJ, Quake SR, Hadd AG, Jacobson SC, Ramsey JM, et al. Microfluidic large-scale integration. *Science* (2002) 298:580–4. doi: 10.1126/science.1076996
96. Kobel S, Valero A, Latt J, Renaud P, Lutolf M. Optimization of microfluidic single cell trapping for long-term on-chip culture. *Lab Chip* (2010) 10:857–63. doi: 10.1039/b918055a
97. Zhou Y, Basu S, Wohlfahrt KJ, Lee SF, Klenerman D, Laue ED, Seshia AA. A microfluidic platform for trapping, releasing and super-resolution imaging of single cells. *Sensors Actuators, B Chem.* (2016) 232:680–91. doi: 10.1016/j.snb.2016.03.131
98. Di Carlo D, Aghdam N, Lee LP. Single-cell enzyme concentrations, kinetics, and inhibition analysis using high-density hydrodynamic cell isolation arrays. *Anal Chem.* (2006) 78:4925–30. doi: 10.1021/ac060541s
99. Faley SL, Copland M, Wlodkowic D, Kolch W, Seale KT, Wikswo JP, et al. Microfluidic single cell arrays to interrogate signalling dynamics of individual, patient-derived hematopoietic stem cells. *Lab Chip* (2009) 9:2659–64. doi: 10.1039/b902083g
100. Gossett DR, Weaver WM, Ahmed NS, Di Carlo D. Sequential array cytometry: multi-parameter imaging with a single fluorescent channel. *Ann Biomed Eng.* (2011) 39:1328–34. doi: 10.1007/s10439-010-0199-8
101. Huebner A, Bratton D, Whyte G, Yang M, deMello AJ, Abell C, et al. Static microdroplet arrays: a microfluidic device for droplet trapping, incubation and release for enzymatic and cell-based assays. *Lab Chip* (2009) 9:692–8. doi: 10.1039/B813709A
102. Li ZG, Liu AQ, Klaseboer E, Zhang JB, Ohl CD. Single cell membrane poration by bubble-induced microjets in a microfluidic chip. *Lab Chip* (2013) 13:1144–50. doi: 10.1039/c3lc41252k
103. Skelley AM, Kirak O, Suh H, Jaenisch R, Voldman J. Microfluidic control of cell pairing and fusion. *Nat Methods* (2009) 6:147–52. doi: 10.1038/nmeth.1290
104. Dura B, Dougan SK, Barisa M, Hoehl MM, Lo CT, Ploegh HL, et al. Profiling lymphocyte interactions at the single-cell level by microfluidic cell pairing. *Nat Commun.* (2015) 6:5940. doi: 10.1038/ncomms6940
105. Dura B, Servos MM, Barry RM, Ploegh HL, Dougan SK, Voldman J. Longitudinal multiparameter assay of lymphocyte interactions from onset by microfluidic cell pairing and culture. *Proc Natl Acad Sci USA* (2016) 113:E3599–608. doi: 10.1073/pnas.1515364113
106. Dura B, Liu Y, Voldman J. Deformability-based microfluidic cell pairing and fusion. *Lab Chip* (2014) 14:2783–90. doi: 10.1039/c4lc00303a
107. Valeev NV, Hundhausen C, Umezawa Y, Kotov NV, Williams G, Clop A, et al. A systems model for immune cell interactions unravels the mechanism of inflammation in human skin. *PLoS Comput Biol.* (2010) 6:e1001024. doi: 10.1371/journal.pcbi.1001024
108. Ng CT, Snell LM, Brooks DG, Oldstone MBA. Networking at the level of host immunity: immune cell interactions during persistent viral infections. *Cell Host Microbe* (2013) 13:652–64. doi: 10.1016/j.chom.2013.05.014
109. Hoehl MM, Dougan SK, Ploegh H, Voldman J. Massively parallel microfluidic cell-pairing platform for the statistical study of immunological cell-cell interactions. In: *15th International Conference on Miniaturized Systems for Chemistry and Life Sciences* (Seattle, WA).
110. Araci IE, Brisk P. Recent developments in microfluidic large scale integration. *Curr Opin Biotechnol.* (2014) 25:60–8. doi: 10.1016/j.copbio.2013.08.014
111. Fidalgo LM, Maerkl SJ. A software-programmable microfluidic device for automated biology. *Lab Chip* (2011) 11:1612–9. doi: 10.1039/c0lc00537a
112. Taylor RJ, Falconnet D, Niemisto A, Ramsey SA, Prinz S, Shmulevich I, et al. Dynamic analysis of MAPK signaling using a high-throughput microfluidic single-cell imaging platform. *Proc Natl Acad Sci USA* (2009) 106:3758–63. doi: 10.1073/pnas.0813416106
113. Fan HC, Wang J, Potanina A, Quake SR. Whole-genome molecular haplotyping of single cells. *Nat Biotechnol.* (2011) 29:51–9. doi: 10.1038/nbt.1739
114. Gomez-Sjoberg R, Leyrat AA, Pirone DM, Chen CS, Quake SR. Versatile, fully automated, microfluidic cell culture system. *Anal Chem.* (2007) 79:8557–63. doi: 10.1021/ac071311w
115. Zhong JF, Chen Y, Marcus JS, Scherer A, Quake SR, Taylor CR, et al. A microfluidic processor for gene expression profiling of single human embryonic stem cells. *Lab Chip* (2008) 8:68–74. doi: 10.1039/b712116d
116. Marcus JS, Anderson WF, Quake SR. Microfluidic single-cell mRNA isolation and analysis. *Anal Chem.* (2006) 78:3084–9. doi: 10.1021/ac0519460
117. Hong JW, Studer V, Hang G, Anderson WF, Quake SR. A nanoliter-scale nucleic acid processor with parallel architecture. *Nat Biotechnol.* (2004) 22:435–9. doi: 10.1038/nbt951
118. Hong JW, Chen Y, Anderson WF, Quake SR. Molecular biology on a microfluidic chip. *J Phys Condens Matter* (2006) 18:S691–701. doi: 10.1088/0953-8984/18/S14
119. Ketterer S, Hövermann D, Guebli RJ, Bartels-Burgahn F, Riewe D, Altmann T, et al. Transcription factor sensor system for parallel quantification of metabolites on-chip. *Anal Chem.* (2014) 86:12152–8. doi: 10.1021/ac503269m
120. Blazek M, Betz C, Hall NM, Reth M, Zengerle R, Meier M. Proximity ligation assay for high-content profiling of cell signalling pathways on a microfluidic chip. *Mol Cell Proteomics* (2013) 12:3898–907. doi: 10.1074/mcp.M113.032821
121. Blazek M, Santisteban TS, Zengerle R, Meier M. Analysis of fast protein phosphorylation kinetics in single cells on a microfluidic chip. *Lab Chip* (2015) 15:726–34. doi: 10.1039/c4lc00797b
122. Blackburn MC, Petrova E, Correia BE, Maerkl SJ. Integrating gene synthesis and microfluidic protein analysis for rapid protein engineering. *Nucleic Acids Res.* (2015) 44:e68. doi: 10.1093/nar/gkv1497
123. Volpetti F, Garcia-Cordero J, Maerkl SJ. A microfluidic platform for high-throughput multiplexed protein quantitation. *PLoS ONE* (2015) 10:e0117744. doi: 10.1371/journal.pone.0117744
124. Geertz M, Shore D, Maerkl SJ. Massively parallel measurements of molecular interaction kinetics on a microfluidic platform. *Proc Natl Acad Sci USA* (2012) 109:16540–5. doi: 10.1073/pnas.1206011109
125. Pollard JW. Opinion: tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* (2004) 4:71–8. doi: 10.1038/nrc1256
126. Sumit M, Takayama S, Linderman JJ. New insights into mammalian signaling pathways using microfluidic pulsatile inputs and mathematical modeling. *Integr Biol.* (2017) 9:6–21. doi: 10.1039/C6IB00178E
127. Martinez-Corral R, Garcia-Ojalvo J. Modeling cellular regulation by pulsatile inputs. *Curr Opin Syst Biol.* (2017) 3:23–9. doi: 10.1016/j.coisb.2017.03.003

128. Junkin M, Kaestli AJ, Cheng Z, Jordi C, Albayrak C, Hoffmann A, Tay S. High-content quantification of single-cell immune dynamics. *Cell Rep.* (2016) 15:411–22. doi: 10.1016/j.celrep.2016.03.033
129. Kaestli AJ, Junkin M, Tay S. Integrated platform for cell culture and dynamic quantification of cell secretion. *Lab Chip* (2017) 17:4124–33. doi: 10.1039/C7LC00839B
130. Bonizzi G, Karin M. The two NF- κ B activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* (2004) 25:280–8. doi: 10.1016/j.it.2004.03.008
131. Lee REC, Qasaimeh MA, Xia X, Juncker D, Gaudet S. NF- κ B signalling and cell fate decisions in response to a short pulse of tumour necrosis factor. *Sci Rep.* (2016) 6:39519. doi: 10.1038/srep39519
132. Kellogg RA, Gómez-Sjöberg R, Leyrat AA, Tay S. High-throughput microfluidic single-cell analysis pipeline for studies of signaling dynamics. *Nat Protoc.* (2014) 9:1713–26. doi: 10.1038/nprot.2014.120
133. Tay S, Hughey JJ, Lee TK, Lipniacki T, Quake SR, Covert MW. Single-cell NF-B dynamics reveal digital activation and analogue information processing. *Nature* (2010) 466:267–71. doi: 10.1038/nature09145
134. Rockel S, Geertz M, Maerkl SJ. MITOMI: a microfluidic platform for *in vitro* characterization of transcription factor-DNA interaction. *Methods Mol Biol.* (2012) 786:97–114. doi: 10.1007/978-1-61779-292-2_6
135. Frank T, Tay S. Automated co-culture system for spatiotemporal analysis of cell-to-cell communication. *Lab Chip* (2015) 15:2192–200. doi: 10.1039/C5LC00182J
136. Nossal GVJ, Lederberg J. Antibody production by single cells. *Nature* (1958) 181:1419–20.
137. Jakiela S, Kaminski TS, Cybulski O, Weibel DB, Garstecki P. Bacterial growth and adaptation in microdroplet chemostats. *Angew Chemie* (2013) 52:8908–11. doi: 10.1002/anie.201301524
138. Abalde-Cela S, Taladriz-Blanco P, De Oliveira MG, Abell C. Droplet microfluidics for the highly controlled synthesis of branched gold nanoparticles. *Sci Rep.* (2018) 8:2440. doi: 10.1038/s41598-018-20754-x
139. Kim SC, Clark IC, Shahi P, Abate AR. Single-Cell RT-PCR in microfluidic droplets with integrated chemical lysis. *Anal Chem.* (2018) 90:1273–9. doi: 10.1021/acs.analchem.7b04050
140. Chen Q, Utech S, Chen D, Prodanovic R, Lin J-M, Weitz DA. Controlled assembly of heterotypic cells in a core-shell scaffold: organ in a droplet. *Lab Chip* (2016) 16:1346–9. doi: 10.1039/C6LC00231E
141. Nuti N, Verboket PE, Dittich PS. Multivesicular droplets: a cell model system to study compartmentalised biochemical reactions. *Lab Chip* (2017) 17:3112–9. doi: 10.1039/C7LC00710H
142. Küster SK, Pabst M, Zenobi R, Dittich PS. Screening for protein phosphorylation using nanoscale reactions on microdroplet arrays. *Angew Chem.* (2015) 54:1671–5. doi: 10.1002/anie.201409440
143. Klein AM, Mazutis L, Akartuna I, Tallapragada N, Veres A, Li V, Peshkin L, et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. *Cell* (2015) 161:1187–201. doi: 10.1016/j.cell.2015.04.044
144. Debs BE, Utharala R, Balyasnikova IV, Griffiths AD, Merten CA. Functional single-cell hybridoma screening using droplet-based microfluidics. *Proc Natl Acad Sci USA* (2012) 109:11570–5. doi: 10.1073/pnas.1204514109
145. Guan Z, Zou Y, Zhang M, Lv J, Shen H, Yang P, et al. A highly parallel microfluidic droplet method enabling single-molecule counting for digital enzyme detection. *Biomicrofluidics* (2014) 8:014110. doi: 10.1063/1.4866766
146. Park JW, Na SC, Nguyen TQ, Paik SM, Kang M, Hong D, et al. Live cell imaging compatible immobilization of *Chlamydomonas reinhardtii* in microfluidic platform for biodiesel research. *Biotechnol Bioeng.* (2015) 112:494–501. doi: 10.1002/bit.25453
147. Pessi J, Santos HA, Miroshnyk I, Joukoiliruusi, Weitz DA, Mirza S. Microfluidics-assisted engineering of polymeric microcapsules with high encapsulation efficiency for protein drug delivery. *Int J Pharm.* (2014) 472:82–7. doi: 10.1016/j.jipharm.2014.06.012
148. Shuga J, Zeng Y, Novak R, Lan Q, Tang X, Rothman N, et al. Single molecule quantitation and sequencing of rare translocations using microfluidic nested digital PCR. *Nucleic Acids Res.* (2013) 41:e159. doi: 10.1093/nar/gkt613
149. Shembekar N, Hu H, Eustace D, Merten CA. Single-cell droplet microfluidic screening for antibodies specifically binding to target cells. *Cell Rep.* (2018) 22:2094–106. doi: 10.1016/j.celrep.2018.01.071
150. Rakszewska A, Tel J, Chokkalingam V, Huck WT. One drop at a time: toward droplet microfluidics as a versatile tool for single-cell analysis. *NPG Asia Mater* (2014) 6:e133. doi: 10.1038/am.2014.86
151. Churski K, Kaminski TS, Jakiela S, Kamysz W, Baranska-Rybak W, Weibel DB, et al. Rapid screening of antibiotic toxicity in an automated microdroplet system. *Lab Chip* (2012) 12:1629–37. doi: 10.1039/c2lc21284f
152. Garstecki P, Fuerstman MJ, Stone HA, Whitesides GM. Formation of droplets and bubbles in a microfluidic T-junction—scaling and mechanism of break-up. *Lab Chip* (2006) 6:437–446. doi: 10.1039/b510841a
153. Seo M, Paquet C, Nie Z, Xu S, Kumacheva E. Microfluidic consecutive flow-focusing droplet generators. *Soft Matter* (2007) 3:986–92. doi: 10.1039/b700687j
154. Pit AM, Duits MHG, Mugele F. Droplet manipulations in two phase flow microfluidics. *Micromachines* (2015) 6:1768–93. doi: 10.3390/mi6111455
155. Chen X, Ren CL. A microfluidic chip integrated with droplet generation, pairing, trapping, merging, mixing and releasing. *RSC Adv.* (2017) 7:16738–50. doi: 10.1039/C7RA02336G
156. Padmanabhan S, Misteli T, DeVoe DL. Controlled droplet discretization and manipulation using membrane displacement traps. *Lab Chip* (2017) 17:3717–24. doi: 10.1039/C7LC00910K
157. Lorenz RM, Edgar JS, Jeffries GDM, Chiu DT. Microfluidic and optical systems for the on-demand generation and manipulation of single femtoliter-volume aqueous droplets. *Anal Chem.* (2006) 78:6433–9. doi: 10.1021/ac060748l
158. Franke T, Abate AR, Weitz DA, Wixforth A. Surface acoustic wave (SAW) directed droplet flow in microfluidics for PDMS devices. *Lab Chip* (2009) 9:2625–7. doi: 10.1039/b906819h
159. Chiang MY, Hsu YW, Hsieh HY, Chen SY, Fan SK. Constructing 3D heterogeneous hydrogels from electrically manipulated prepolymer droplets and crosslinked microgels. *Sci Adv.* (2016) 2:e1600964. doi: 10.1126/sciadv.1600964
160. Collins DJ, Neild A, deMello A, Liu A-Q, Ai Y. The Poisson distribution and beyond: methods for microfluidic droplet production and single cell encapsulation. *Lab Chip* (2015) 15:3439–59. doi: 10.1039/C5LC00614G
161. Sarkar S, Motwani B, Sabhachandani P, Cohen N, Konry T. T cell dynamic activation and functional analysis in nanoliter droplet microarray. *J Clin Cell Immunol.* (2015) 6:334. doi: 10.4172/2155-9899.1000334
162. Sarkar S, Sabhachandani P, Ravi D, Potdar S, Purvey S, Beheshti A, et al. Dynamic analysis of human natural killer cell response at single-cell resolution in B-Cell Non-Hodgkin Lymphoma. *Front Immunol.* (2017) 8:1736. doi: 10.3389/fimmu.2017.01736
163. Guldevall K, Brandt L, Forslund E, Olofsson K, Frisk TW, Olofsson PE, et al. Microchip screening platform for single cell assessment of NK cell cytotoxicity. *Front Immunol.* (2016) 7:119. doi: 10.3389/fimmu.2016.00119
164. Konry T, Dominguez-Villar M, Baecher-Allan C, Hafler DA, Yarmush ML. Droplet-based microfluidic platforms for single T cell secretion analysis of IL-10 cytokine. *Biosens Bioelectron.* (2011) 26:2707–10. doi: 10.1016/j.bios.2010.09.006
165. Qiu L, Wimmers F, Weiden J, Heus HA, Tel J, Figgdor CG. A membrane-anchored aptamer sensor for probing IFN γ secretion by single cells. *Chem Commun.* (2017) 53:8066–9. doi: 10.1039/c7cc03576d
166. Eyer K, Doineau RCL, Castrillon CE, Briseño-Roa L, Menrath V, Mottet G, et al. Single-cell deep phenotyping of IgG-secreting cells for high-resolution immune monitoring. *Nat Biotechnol.* (2017) 35:977–82. doi: 10.1038/nbt.3964
167. Chokkalingam V, Tel J, Wimmers F, Liu X, Semenov S, Thiele J, et al. Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. *Lab Chip* (2013) 13:4740–4. doi: 10.1039/c3lc50945a
168. Hosokawa M, Nishikawa Y, Kogawa M, Takeyama H. Massively parallel whole genome amplification for single-cell sequencing using droplet microfluidics. *Sci Rep* (2017) 7:5199. doi: 10.1038/s41598-017-05436-4
169. Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* (2015) 161:1202–14. doi: 10.1016/j.cell.2015.05.002
170. Lan F, Demaree B, Ahmed N, Abate AR. Single-cell genome sequencing at ultra-high-throughput with microfluidic droplet barcoding. *Nat Biotechnol.* (2017) 35:640–6. doi: 10.1038/nbt.3880

171. Eberwine J, Sul JY, Bartfai T, Kim J. The promise of single-cell sequencing. *Nat Methods* (2014) 11:25–7. doi: 10.1038/nmeth.2769
172. Shahi P, Kim SC, Haliburton JR, Gartner ZJ, Abate AR. Abseq: ultrahigh-throughput single cell protein profiling with droplet microfluidic barcoding. *Sci Rep.* (2017) 7:44447. doi: 10.1038/srep44447
173. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA. Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protoc.* (2013) 8:870–91. doi: 10.1038/nprot.2013.046
174. Wu L, Chen P, Dong Y, Feng X, Liu BF. Encapsulation of single cells on a microfluidic device integrating droplet generation with fluorescence-activated droplet sorting. *Biomed Microdevices* (2013) 15:553–60. doi: 10.1007/s10544-013-9754-z
175. Kemna EWM, Schoeman RM, Wolbers F, Vermes I, Weitz DA, van den Berg A. High-yield cell ordering and deterministic cell-in-droplet encapsulation using Dean flow in a curved microchannel. *Lab Chip* (2012) 12:2881–7. doi: 10.1039/c2lc00013j
176. Chung MT, Núñez D, Cai D, Kurabayashi K. Deterministic droplet-based co-encapsulation and pairing of microparticles: Via active sorting and downstream merging. *Lab Chip* (2017) 17:3664–71. doi: 10.1039/c7lc00745k
177. Bendall SC, Simonds EF, Qiu P, Amir EAD, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* (2011) 332:687–96. doi: 10.1126/science.1198704
178. Qiu P, Simonds EF, Bendall SC, Gibbs KD, Bruggner RV, Linderman MD, et al. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. *Nat Biotechnol.* (2011) 29:886–93. doi: 10.1038/nbt.1991
179. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods* (2006) 3:361–8. doi: 10.1038/nmeth872
180. Bao XR, Fraser IDC, Wall EA, Quake SR, Simon MI. Variability in G-protein-coupled signaling studied with microfluidic devices. *Biophys J.* (2010) 99:2414–22. doi: 10.1016/j.bpj.2010.08.043
181. Frank T, Tay S. Flow-switching allows independently programmable, extremely stable, high-throughput diffusion-based gradients. *Lab Chip* (2013) 13:1273–81. doi: 10.1039/c3lc41076e
182. Singhal A, Haynes CA, Hansen CL. Microfluidic measurement of antibody-antigen binding kinetics from low-abundance samples and single cells. *Anal Chem.* (2010) 82:8671–9. doi: 10.1021/ac101956e
183. Armbrrecht L, Gabernet G, Kurth F, Hiss JA, Schneider G, Dittrich PS. Characterisation of anticancer peptides at the single-cell level. *Lab Chip* (2017) 17:2933–40. doi: 10.1039/c7lc00505a
184. Krieg C, Nowicka M, Guglietta S, Schindler S, Hartmann FJ, Weber LM, et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat Med.* (2018) 24:144–53. doi: 10.1038/nm.4466
185. Wiegand A, Spindler J, Hong FF, Shao W, Cyktor JC, Cillo AR, et al. Single-cell analysis of HIV-1 transcriptional activity reveals expression of proviruses in expanded clones during ART. *Proc Natl Acad Sci USA* (2017) 114:E3659–68. doi: 10.1073/pnas.1617961114
186. Golumbeanu M, Cristinelli S, Rato S, Munoz M, Cavassini M, Beerenwinkel N, et al. Single-cell RNA-seq reveals transcriptional heterogeneity in latent and reactivated HIV-infected cells. *Cell Rep.* (2018) 23:942–50. doi: 10.1016/j.celrep.2018.03.102
187. Khandelwal N, Breinig M, Speck T, Michels T, Kreutzer C, Sorrentino A, et al. A high-throughput RNAi screen for detection of immune-checkpoint molecules that mediate tumor resistance to cytotoxic T lymphocytes. *EMBO Mol Med.* (2015) 7:450–63. doi: 10.15252/emmm.201404414
188. Marçais A, Walzer T. An immunosuppressive pathway for tumor progression. *Nat Med.* (2018) 24:260–1. doi: 10.1038/nm.4508
189. Hou H, Zhao Y, Li C, Wang M, Xu X, Jin Y. Single-cell pH imaging and detection for pH profiling and label-free rapid identification of cancer-cells. *Sci Rep.* (2017) 7:1759. doi: 10.1038/s41598-017-01956-1
190. Abbaspourrad A, Zhang H, Tao Y, Cui N, Asahara H, Zhou Y, et al. Label-free single-cell protein quantification using a drop-based mix-and-read system. *Sci Rep.* (2015) 5:12756. doi: 10.1038/srep12756
191. Schwarz J, Bierbaum V, Merrin J, Frank T, Hauschild R, Bollenbach T, et al. A microfluidic device for measuring cell migration towards substrate-bound and soluble chemokine gradients. *Sci Rep.* (2016) 6:36440. doi: 10.1038/srep36440
192. Jain NG, Wong EA, Aranyosi AJ, Boneschansker L, Markmann JF, Briscoe DM, et al. Microfluidic mazes to characterize T-cell exploration patterns following activation *in vitro*. *Integr Biol.* (2015) 7:1423–31. doi: 10.1039/c5ib00146c
193. Eduati F, Utharala R, Madhavan D, Neumann UP, Longerich T, Cramer T, et al. A microfluidics platform for combinatorial drug screening on cancer biopsies. *Nat Commun.* (2018) 9:2434. doi: 10.1038/s41467-018-04919-w
194. Garcia-Cordero JL, Nembrini C, Stano A, Hubbell JA, Maerkl SJ. A high-throughput nanoimmunoassay chip applied to large-scale vaccine adjuvant screening. *Integr Biol.* (2013) 5:650–8. doi: 10.1039/c3ib20263a
195. Woodruff K, Maerkl SJ. Microfluidic module for real-time generation of complex multimolecule temporal concentration profiles. *Anal Chem.* (2018) 90:696–701. doi: 10.1021/acs.analchem.7b04099

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Role of Mechanotransduction and Tension in T Cell Function

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T cell migration from blood to, and within lymphoid organs and tissue, as well as, T cell activation rely on complex biochemical signaling events. But T cell migration and activation also take place in distinct mechanical environments and lead to drastic morphological changes and reorganization of the acto-myosin cytoskeleton. In this review we discuss how adhesion proteins and the T cell receptor act as mechanosensors to translate these mechanical contexts into signaling events. We further discuss how cell tension could bring a significant contribution to the regulation of T cell signaling and function.

Keywords: T cell, mechanotransduction, tension, adhesion, migration, TCR, actin cytoskeleton, signaling

OPEN ACCESS

Edited by:

Mario Mellado,

Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:

Ronen Alon,

Weizmann Institute of Science, Israel
Miguel Vicente-Manzanares,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 21 August 2018

Accepted: 26 October 2018

Published: 15 November 2018

Citation:

Rossy J, Laufer JM and Legler DF
(2018) Role of Mechanotransduction
and Tension in T Cell Function.
Front. Immunol. 9:2638.
doi: 10.3389/fimmu.2018.02638

INTRODUCTION

To mount a proper adaptive immune response and establish immune memory, T cells carry out many distinct cellular processes. In a simplified view, these processes can be grouped in three categories. (a) The adhesion cascade, during which circulating T cells exit the blood flow to roll, adhere and eventually extravasate through the endothelial cell layer. (b) Migration, on the wall of blood or lymph vessels, within lymph nodes and inflamed or cancerous tissues. And (c), activation, which primes naïve T cells and triggers cytotoxicity and cytokine secretion from effector cells. The molecular interactions and signaling pathways associated with T cell activation (1), migration through venular walls (2) and T cell migration in general (3) have been extensively characterized and are comprehensively described in these recent reviews. But the emergence of novel biophysical approaches has allowed to shine light on a previously neglected aspect of these processes: they all generate mechanical stimuli.

During the adhesion cascade, the blood flow applies an external shear stress on T cells binding and migrating on and through endothelial cells (2). T cell migration in tissues is driven by morphological changes, constantly fluctuating actin polymerization and molecular motors-driven contractions, which all generate internal mechanical tension (4). It goes the same with T cell activation, which involves a tight contact between T cells and antigen-presenting cells or target cells, acto-myosin contractions and a sustained actin retrograde flow (5). Adding to the multiplicity of these mechanical contexts, T cells interact with substrates displaying various and changing stiffness (6) and with adhesion molecules that are either diffusive or firmly anchored to cortical actin (7). Hence, the idea that force plays an essential role in the T cell-mediated immune response has matured from an exciting hypothesis to a well-established field of T cell biology (8–11).

In this review we first focus on demonstrated mechanotransduction events in T cells. We discuss how adhesion proteins—selectins and integrins—and the T cell receptor (TCR) act as mechanosensors during the adhesion cascade and during T cell activation, respectively. In the second part of the review, we get inspiration from other cell types and systems to picture how cell tension might contribute to the cellular signaling that regulates T cell migration and activation.

SHEAR FORCE: A KEY PLAYER DURING T CELL ROLLING AND ARREST ON THE ENDOTHELIUM

In search for their cognate antigen, T cells circulate between peripheral tissues and secondary lymphoid tissues, thereby exploiting a network of blood and lymphatic vessels (12). T cells circulating in the blood enter lymph nodes through high endothelial venules (HEVs). Before they can extravasate through HEVs, T cells first need to roll, arrest and finally adhere to the vessel walls (2, 13). Forces derived from the blood flow play a decisive role in this adhesion cascade, contributing both to the initial capture by selectins and to the firm integrin-mediated arrest preceding extravasation (**Figure 1**).

Rolling on HEVs is mediated by fast on and off rates interactions between selectins on T cells and their ligands displayed by the endothelium. Pioneering work using atomic force microscopy (AFM) in combination with flow-chambers revealed that selectin-ligand interactions form catch bonds—molecular interactions whose dissociation rate decreases with force, see **Glossary** at the end of the article—when subjected to low shear force generated by the blood flow (11, 14, 15). Thus, a mechanotransduction process, driven by a conformational change in the selectin headpiece, prolongs the life time of the bond between selectins and their ligand and thereby gives rise to enhanced cell adhesion under flow conditions (**Figure 1A**).

T cell tethering and rolling eventually leads to arrest and firm adhesion on endothelial cells, which is driven by heterodimeric integrins and their ligands and which also requires low force from the blood flow (2, 13, 16). Remarkably, integrin adhesiveness is increased very shortly after T cells make contact with endothelial cells, through a multistep process during which force plays an essential role (17). The first step in integrin-mediated adhesion is activation by signals coming from selectins and chemokine receptors. In a certain way, this first step prepares integrin to bear tensile forces, as it (a) increases integrin affinity for immobilized ligands on the extracellular side and (b) strengthened integrin-actin cytoskeleton connection on the intracellular side through the recruitment of talin and kindlin to the intracellular integrin tail (17, 18). Indeed, integrin activation by chemokines alone is not sufficient to trigger adhesiveness, which is achieved only by the effect of shear force from the blood flow (19). Integrins bound to immobilized ligand on one side and firmly anchored to the actin cytoskeleton on the other side are pulled into a high affinity, open conformation by the low force of the shear flow (**Figure 1B**). This force-mediated reorganization of integrin conformation eventually allows stable bonds with ligands at the surface of endothelial cells to support T cell immobilization.

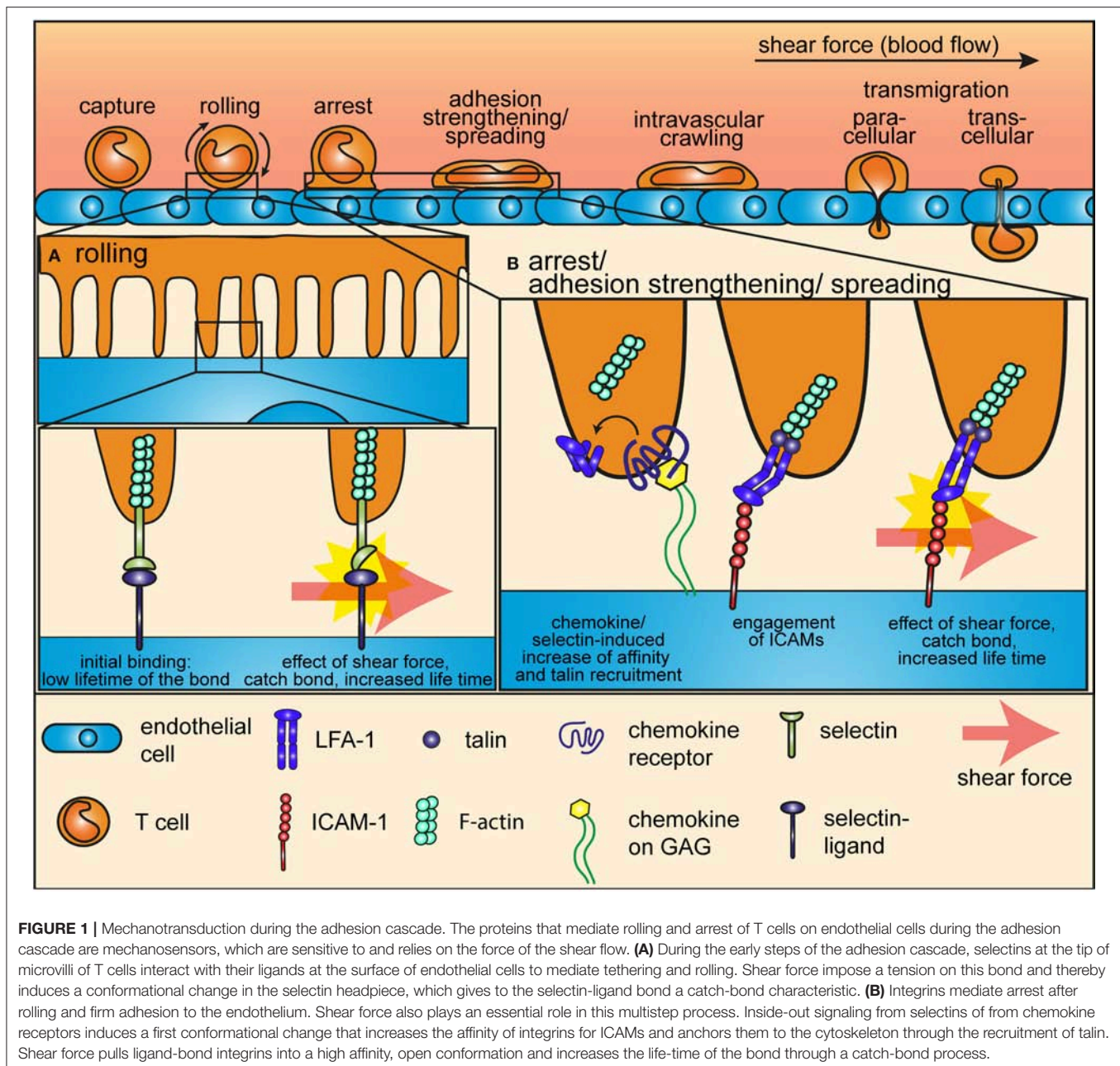
T CELL MIGRATION: STEERING TOWARD STIFFNESS

After adhesion and extravasation through endothelial cells, T cells adopt a motile behavior to reach antigen-presenting cells in lymph nodes or inflamed tissues. As described in an excellent recent review, the link between the actin cytoskeleton,

adhesion modules and the extracellular matrix is highly dynamic and allows cells to convert the mechanical properties of their environment into signaling (20). In the context of migration, this can result in durotaxis—the ability of cells to migrate toward stiffer substrates. Durotaxis is another way how mechanotransduction could potentially contribute to T cell functions. Typical targets of T cells, such as (a) cancer cells that can be softer than normal cells (21); (b) tumors, that are stiffer than normal tissue because of high collagen density and crosslinking (22, 23), or (c) antigen-presenting cells (6) have specific stiffness properties. Changes in extracellular matrix stiffness of specific tissues are generally associated with disease progression (24). Neutrophils, whose amoeboid type of migration is similar to that of T cells, spread more and migrate slower but more persistently and exert stronger traction forces on stiffer substrates (25, 26). Like neutrophils, T cell migration on ICAM-1 coated surfaces is also influenced by substrate rigidity. Indeed, it has been recently shown that T cells migrate faster on stiffer substrates (27).

T CELL ACTIVATION NEEDS FORCE

Contact of a migrating T cell with a target cell or an antigen-presenting cell displaying a cognate antigen result in activation and arrest and in the formation of an immunological synapse (1, 28). In this paragraph, we will discuss in detail how mechanotransduction plays an essential role in this process. By demonstrating that T cell activation with antigen-coated beads requires the beads to be larger than 4 μm , Mescher provided the first hint that the generation of tension over a significant scale is indispensable for T cell activation (29). The first mechanosensor model for TCR was published quite some time later, in a study demonstrating that the binding of an immobilized agonist antibody to CD3 ϵ induces a torque in the structure of the TCR-CD3 complex. Non-activating antibodies however, need to be conjugated to a bead and pulled tangentially to the receptor using optical tweezers to induce a similar activating response (30, 31). By suggesting that the migration-related movement of T cells engaging a cognate peptide at the surface of antigen-presenting cells induces tangential forces on TCR, this study is also an important reminder that T cells are actually migrating and under tension when they find their cognate antigen. Mechanosensing cells or proteins can sense and react to externally applied mechanical stimuli, without actively contributing to the force that is at the source of the stimulus. For instance, in the case of a cell submitted to shear stress. This can be termed passive mechanosensing (32), in contrast to active touch sensing (mentioned further in this review), where the mechanosensor is actively involved in the mechanical stimulus it is sensitive to, a bit like poking a mango to determine if it is ripe or not. Cell motility generates cell tension and thereby might lead to passive mechanosensing as migration-related forces are transferred onto the TCR-CD3 complex (**Figure 2A**). Similarly, formation of the immunological synapse leads to activation of the integrin LFA-1 and to tight adhesion to immobilized ICAM-1 on antigen-presenting cells (33), as well as, acto-myosin



contractions (34) and cytoskeletal tensions [(35), **Figure 2B**]. Hence, transition from migration to activation upon engagement of a cognate peptide represents a mechanical signal that is very likely to result in passive mechanosensing by TCR. Interestingly, TCR engagement promotes local actin polymerization around the receptor itself (35), in a way that reminds of the signal-dependent and talin-mediated anchorage of integrins to the actin cytoskeleton during the adhesion cascade. This means that TCR is further anchored to the underlying cortical actin cytoskeleton upon activation, which could very well make it more susceptible to respond to mechanical stimuli. Along this line, it is now well-established that T cells, like many other cells, engage in the “active touch sensing” described by Kobayashi and Sokabe (32)

by actively pushing and pulling on the substrate they adhere to in order to interrogate its stiffness (**Figure 2B**). Within the first tens of seconds of TCR triggering on a biomembrane force probe setup, T cells engage in a sequence of pushing and pulling forces even in the absence of LFA-1 engagement (36). Traction force microscopy (TFM) on polyacrylamide gels further confirmed that antibody activation of CD3 leads to acto-myosin-mediated pulling forces, which originate at the cell edge and are directed toward the cell center (37). Another TFM study on micropillars determined that these centripetal forces are generated through the binding of TCR to activating ligands, further suggesting that integrins are not the mechanosensor at play during T cell activation (38). These forces are in the range of 100 pN, which

is lower than the nanonewton forces observed during epithelial cells migration (39). Of note, phosphorylation of the early TCR signaling kinase Lck takes place on the side of the pillars facing the cell edge, suggesting that TCR signaling is triggered where the tension is highest and strengthening the idea that TCR works better when it is under tension (38). The surface of T cells is covered with microvilli, whose tips are enriched with TCR [(40, 41), **Figure 2A**]. These microvilli extend and retract while T cells scan antigen-presenting cells and it is likely that the first step of antigen recognition on antigen-presenting cells is mediated by TCR located on stretched microvilli. This raises the possibility that active touch sensing might already be involved in the very early stages of T cell activation, as TCR at the tip of microvilli is subjected to specific forces resulting from the scanning of antigen-presenting cells. But forces applied on TCR at the tip of microvilli are also likely to be reduced by the elastic nature of these projections, which can act as shock absorbers, for instance in the context of the adhesion cascade (42). Further investigations are required to determine if TCR at the tip of microvilli is put under tension due to the exploratory character of these projections, or if on the contrary, the force on TCR is dissipated through a shock absorber effect. Finally, forces imposed on TCR located on collapsed microvilli will be very different once the immunological synapse is fully established.

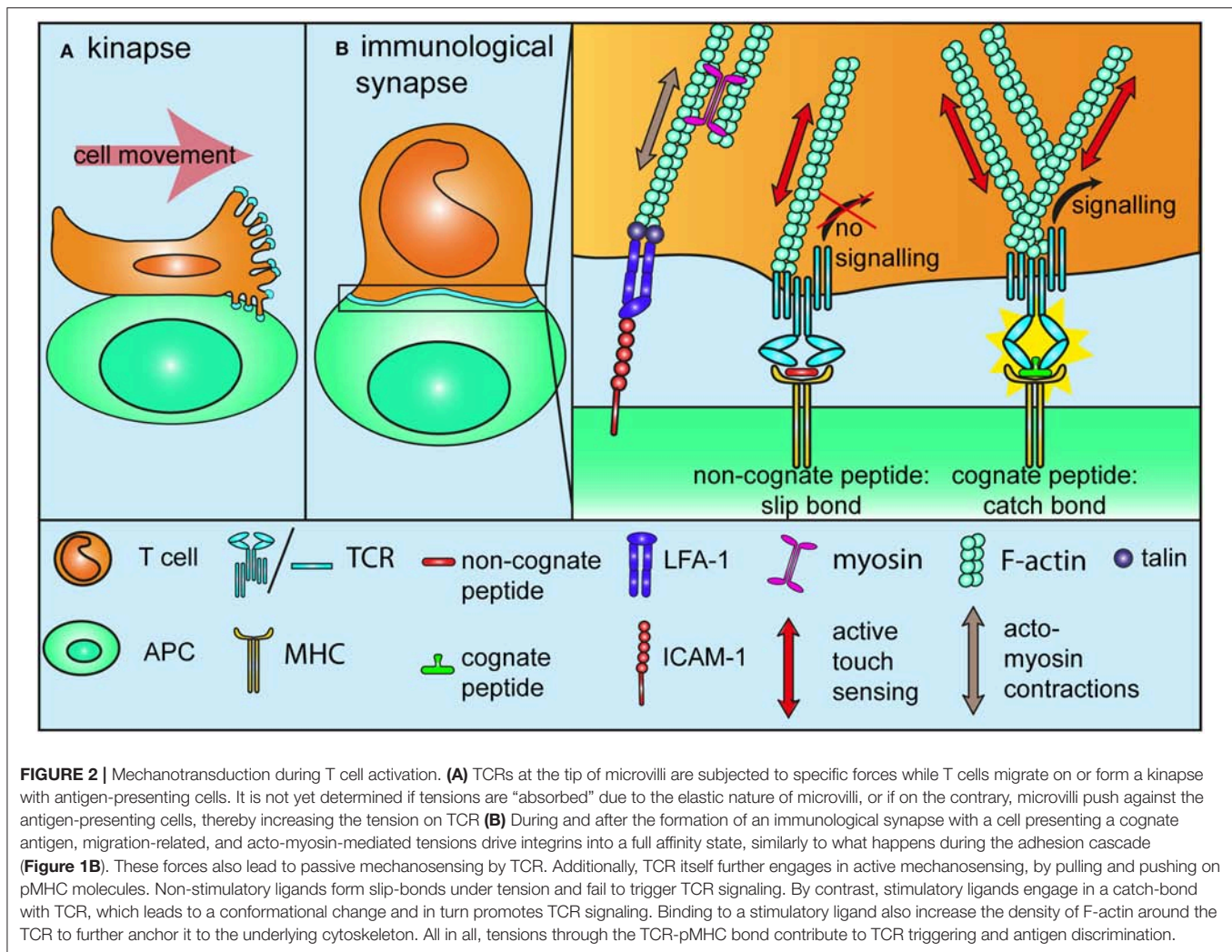
A direct consequence of the active touch sensing through TCR is that T cell activation is influenced by substrate stiffness. As a matter of fact, T cells pull more on stiffer substrates than on softer ones (37). CD4 T cells produce also more IL-2 on harder substrates up to 100 kPa (27, 43), but the stiffness contribution to T cell activation is somehow lost beyond 100 kPa (43, 44). More generally, every aspect of T cell activation is potentiated by stiffer surfaces up to 100 kPa (27). The effect of substrate stiffness on T cell activation could even be larger than reported in these studies, which all used functional antibodies against CD3 to activate T cells. It is indeed likely that differences in the rigidity of substrates might have a more pronounced effect on the binding of TCR to its natural ligand, a cognate peptide presented by major histocompatibility complex (MHC), than to an activating antibody.

The mechanism behind stiffness sensing in T cells is not identified yet, but talin might be involved. As part of the complex protein assembly between integrins and the actin cytoskeleton (45), talin is an essential element of the substrate stiffness sensing machinery and preventing talin to mechanically engage with integrin disrupts extracellular rigidity sensing (46). Interestingly, T cells lacking talin fail to stop migrating in response to TCR triggering (47). As mentioned above, talin is essential to integrin-mediated adhesion (17) and in particular to LFA-1 adhesiveness for ICAM-1 following TCR triggering (48). It is likely that the affinity of LFA-1 for ICAM-1 is increased during T cell arrest upon TCR activation through a similar mechanism than described above during the arrest on endothelial cells in the blood flow. One can indeed consider that during activation, the LFA-1–ICAM-1 bond is put under tension by acto-myosin contractions and actin retrograde flow in a similar fashion that it is stretched by extracellular forces resulting from shear flow during the adhesion cascade (**Figure 2B**). As a matter of fact

it has been shown that ICAM-1 is immobilized at the surface of antigen-presenting cells in order to promote T cell-antigen presenting cells conjugation and T cell activation (33). Hence talin mechanosensing properties could contribute to the stop signal that precedes the establishment of the immunological synapse and eventually to full T cell activation. However, a recent study somehow challenges the idea that the talin-LFA-1 axis supports the stop signal. Feigelson et al. reported that the integrin ligands on antigen-presenting cells, ICAM-1 and -2, are dispensable for these cells trigger arrest activation of T cells (49). Finally, intravital microscopy studies have shown that T cells do not necessarily stop when encountering a stimulatory antigen-presenting cells. Antigen recognition can happen during long-lasting contact, the immunological synapse, but also during shorter and more dynamic interactions, termed *kinapse* [(28, 50), **Figure 2A**]. While the functional difference between synapse and kinapse has not been fully established, the duration and nature of the antigen-presenting cell-T cell interaction contribute to shape the outcome of T cell activation (51). Therefore, it is likely that the mechanosensitive properties of integrin and TCR contribute to this process by leading to distinct signaling in the context of a synapse or of a kinapse.

Thus, T cells pull on activating substrates and they are more susceptible to be activated by stiffer substrates. Having this in mind, it does not take a bit leap to imagine that the active touch used by T cells is not only a mechanism to interrogate substrate stiffness. Indeed, a few recent studies indicate that putting TCR under tension is in fact an integral part of the activation process (**Figure 2B**). Presenting T cells with activating peptide-MHC complex (pMHC) on an AFM microscope showed that T cell activation requires both the binding of a cognate antigen and forces through TCR (52). An in depth analysis of the kinetics of TCR-pMHC interactions using a biomembrane force probe showed that TCR establishes catch bonds with cognate pMHC and slip bonds—molecular interactions whose dissociation rate increases with force—with non-agonistic pMHC, thereby making force applied through TCR a component of the antigen discrimination process (53). The formation of catch bond is even what distinguishes stimulatory from non-stimulatory ligands between peptides that bind TCR with similar affinity (54). These results are further confirmed by two studies from Lang and colleagues using optical tweezers and DNA tethers. They first identified an elongated structural element of the TCR β constant chain, the FG loop (55), as a key factor for the contribution of the force in antigen discrimination (56). More recently, they demonstrated that TCR needs non-physiological levels of pMHC molecules to be triggered in the absence of forces (57). Using DNA-based nanoparticle tension sensors Liu et al. further demonstrated that piconewton forces are transmitted through TCR-CD3 complexes a few seconds after activation and that these forces are required for antigen discrimination (58).

In summary, passive mechanosensing of the forces resulting from migration and activation, and active touch sensing through the TCR-CD3 complex probably act together to connect TCR triggering at the same time to the physical environment (speed of migration, stiffness of the presenting cells) the T cell evolves in and to ligand selectivity (8). This maybe brings us back to a model



described just 10 years ago, which proposed that the TCR-CD3 complex requires to be stretched in order to be activated (59). A postulate that is strengthened by the fact that TCR triggering involves a mechanical switch of its structure (60).

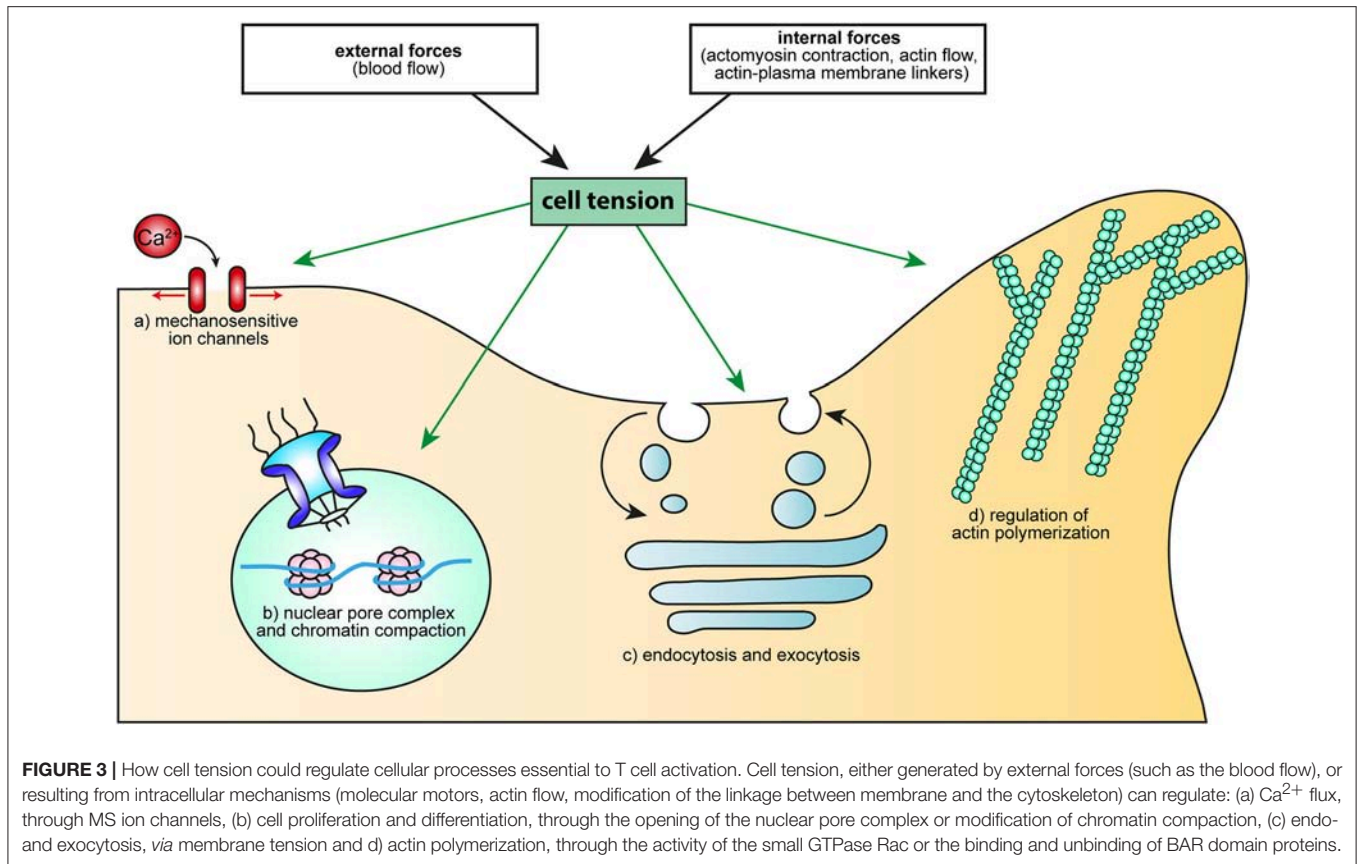
Forces that T cells generate upon activation do not relate only to signal intensity and specificity, but also contribute to the T cell response, notably in the context of killing. Cancer target cells that express a higher number of adhesion molecules facilitate the release of lytic granules by cytotoxic T lymphocytes (61). More strikingly, tension induced on target cells by cytotoxic T lymphocyte facilitates perforin pore formation in target cells and thereby increases the transfer of granzyme proteases and cytotoxicity (62).

TENSION IN T CELLS: FURTHER FACTS AND PERSPECTIVES

Cell tension is the result of a complex interplay between tension mediated through the cytoskeleton and membrane tension. The cortical actin—plasma membrane relationship plays a central role

in mechanobiology and is very well described in recent reviews (63, 64). In this regard, proteins that link the plasma membrane to the underlying cortical actin such as Ezrin/Radixin/Moesin (65) are likely to play a determining role in T cell mechanical properties and mechanotransduction. Ezrin, which directly regulates membrane tension (66) is deactivated upon T cell activation to promote cell relaxation and *in fine* conjugation to antigen-presenting cells (67). Similarly, constitutively active Ezrin increases membrane tension and impairs T cell migration *in vivo* (68). Hence, it appears that the ability of T cells to relax and deform their membrane is directly related to their ability to migrate and be activated. This is confirmed by the fact that naïve T cells are less deformable than T lymphoblasts, as assessed by a micropipette aspiration assay. The same study showed that depolymerization of the actin cytoskeleton makes naïve T cells and T lymphoblasts more deformable altogether (69).

Variations in membrane tension can influence T cell signaling in various ways. Mechanosensitive (MS) channels open up to mediate ion flux in response to membrane stretch (32, 70). First discovered in bacteria where they compensate for sudden changes in environmental osmolality, MS channels have



been shown to mediate intracellular Ca^{2+} rise in response to tension applied to focal adhesion or along actin fibers (71). T cells express a large variety of potential MS channels (72) and an electrophysiological study showed that one of them, TRPV2, opens and mediates Ca^{2+} entry in T cells subjected to mechanical stress (73). It has recently been shown that the most potent mechanosensitive ion channel identified to date, Piezo 1, is expressed in T cells, where it contributes to T cell activation through Ca^{2+} -influx, albeit the study did not actually investigate if this is through mechanical stress (74). In this regard, a study using AFM in synchronization with fluorescence imaging reported that mechanical stimulation alone, without TCR stimulation, is sufficient to elicit an increase in intracellular Ca^{2+} (75). This is in agreement with the expression of Piezo 1 in T cells, but somehow in contradiction with Hu and Butte, who reported that mechanical stimulation triggers Ca^{2+} flux only when coupled with TCR triggering (52). Further studies are still required to determine whether or not mechanical stimuli alone are sufficient to trigger Ca^{2+} flux through Piezo 1 in T cells.

Whether or not MS channels play a role in T cell migration also remains to be determined. It is however likely that membrane tension contributes to organize polarity during T cell migration, in light of what has been observed in neutrophils. Ten years after the inhibitory effect of cell tension on the small GTPase Rac had been shown (76), Houk et al used micropipette aspiration to show that cell tension acts as a

long-range inhibitor to prevent Rac-mediated actin protrusions elsewhere than at the leading edge of motile neutrophils (77). These results were extended to further demonstrate that cell tension limits actin assembly through a negative feedback pathway involving phospholipase D2 and the mammalian target of rapamycin complex 2 (mTORC2) (78). Membrane tension also impact on the distribution and dynamics of membrane-bending proteins, such as BAR domain proteins (79), and reciprocally (80). In this context, it is interesting to note that tension promotes membrane tensformation of the leading edge of COS-1 cells, through the recruitment of FBP17, a membrane-bending and curvature-sensing activator of WASP-dependent actin polymerization (81). Even though T cells and COS-1 cells have noticeably different mechanisms of migration, it seems likely that tension and actin polymerization could act in concert to install polarity in migrating and in activated T cells *via* similar mechanisms.

Carrying the speculation further, we could even imagine that the contribution of membrane tension to T cell activation or migration extends to the regulation of intracellular trafficking. As discussed in comprehensive reviews, the plasma membrane is largely inelastic and can increase in area only 2–3% before rupture occurs (63, 82, 83). Consequently, cells actively respond to membrane tension through regulation of intracellular trafficking, increased membrane tension favoring exocytosis (84–86) and reduced membrane tension leading to endocytosis (87).

This means that cell tension could act as a mechanical long-range messenger to directly influence and coordinate endocytic and exocytic events (82, 83, 88) taking place during T cell migration and activation. In fact, intracellular trafficking is a key factor in establishing functional polarity by spatially restricting membrane proteins at a specific localization in the cell, thereby confining signaling and interactions with other cells or with the extracellular matrix. Selective endocytosis of a given receptor can locally reduce its surface expression. Similarly, targeted recycling can increase the local concentration of a protein within the plasma membrane. Incidentally, T cells are highly polarized, both during migration (uropod vs. leading edge) and during activation (immunological synapse). It is thus possible that membrane tension contributes to the regulation of these processes through the organization of specific endocytic and exocytic events. For instance, endocytosis and recycling are essential to integrin polarization and activity in motile cells in general (89, 90) and in T cells in particular (91, 92). Similarly, targeted delivery of vesicles to the immunological synapse is required for full T cell activation (93, 94) and secretion of cytotoxic granules (95, 96). A good illustration of how this could happen can be found during phagocytosis by macrophages, a process that is in many ways similar to the formation of the immunological synapse and during which membrane tension coordinates the actin-driven formation of the phagocytic cup and exocytosis-fusion of vesicles (97).

Finally, cell tension does not stop at the plasma membrane or the cortical cytoskeleton. As well described in a recent review, forces are transferred from the cell surface to the nuclear envelope through the intermediate of the cytoskeleton or directly from the external environment (98). The structure and function of the nucleus are affected by these tensions, which allows it to function as a mechanosensor (99, 100). Accordingly, tensions can regulate gene expression by modifying the connection of heterochromatin to the nuclear lamina (101). Forces transferred to the nuclear envelope have also been reported to favor cell proliferation (98). Nuclear deformation has further been shown to directly lead to the import of specific transcription factors through the opening of nuclear pore complexes (102, 103). Because of its size and rigidity, the nucleus is the limiting factor during cell migration in a dense meshwork (104). Typically, dendritic cells use myosin II-driven contractions (105) and produce a dense actin network around the nucleus (106) to promote nucleus deformation and

in turn facilitate squeezing through constrictions. 3D migration of T cells in confined environments is thus very likely to lead to compression of the nucleus. Similarly, the pulling exerted by T cells on antigen-presenting cells is susceptible to lead to compression or even flattening of the nuclear envelope. Hence it is conceivable that tension resulting from prolonged migration in confined environment or from T cell binding to an antigen-presenting cell can lead to rearrangement of the chromatin structure or to the opening of nuclear pores and thereby influence the regulation of gene expression leading to T cell differentiation or proliferation.

CONCLUSION

T cells are subjected to ever-changing forces, either generated intracellularly or from their environment. They further interact tightly with cells displaying various levels of stiffness and with molecules whose anchorage to the underlying actin cytoskeleton varies. But more important than the multiplicity of these mechanical contexts, is the fact that they very often are associated with specific processes participating to T cell function. It is therefore very likely that distinct mechanical signals team up with biochemical signals to ensure that T cells do the right thing at the right place and time. The role of mechanotransduction in the adhesion cascade preceding extravasation and in T cell activation is now well-established, although there is still room to refine the model describing it. Now is maybe the time to investigate the importance of cell tension for T cells (Figure 3), using what we have learned from other cell types and taking advantage of ever-improving biophysical approaches.

AUTHOR CONTRIBUTIONS

JR and DL conceived and wrote the manuscript, JL draw the illustrations.

ACKNOWLEDGMENTS

This work was funded in part by grants from the Swiss National Science Foundation (SNSF 172969 to JR and 169936 to DL), the Thurgauische Stiftung für Wissenschaft und Forschung, and the State Secretariat for Education, Research and Innovation.

REFERENCES

- Gaud G, Lesourne R, Love PE. Regulatory mechanisms in T cell receptor signalling. *Nat Rev Immunol.* (2018) 18:485–97. doi: 10.1038/s41577-018-0020-8
- Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. *Immunity* (2014) 41:694–707. doi: 10.1016/j.immuni.2014.10.008
- Krummel MF, Bartumeus F, Gérard A. T cell migration, search strategies and mechanisms. *Nat Rev Immunol.* (2016) 16:193–201. doi: 10.1038/nri.2015.16
- Dupré L, Houmadi R, Tang C, Rey-Barroso J. T Lymphocyte migration: an action movie starring the actin and associated actors. *Front Immunol.* (2015) 6:586. doi: 10.3389/fimmu.2015.00586
- Gomez TS, Billadeau DD. T Cell activation and the cytoskeleton: You can't have one without the other. *Adv Immunol.* (2008) 97:1–64. doi: 10.1016/S0065-2776(08)00001-1
- Bufl N, Saitakis M, Dogniaux S, Buschinger O, Bohineust A, Richert A, et al. Human primary immune cells exhibit distinct mechanical properties that are modified by inflammation. *Biophys J.* (2015) 108:2181–90. doi: 10.1016/j.bpj.2015.03.047
- Comrie WA, Burkhardt JK. Action and traction: cytoskeletal control of receptor triggering at the immunological synapse. *Front Immunol.* (2016) 7:68. doi: 10.3389/fimmu.2016.00068
- Depoil D, Dustin ML. Force and affinity in ligand discrimination by the TCR. *Trends Immunol.* (2014) 35:597–603. doi: 10.1016/j.it.2014.10.007

9. Huse M. Mechanical forces in the immune system. *Nat Rev Immunol.* (2017) 17:679–90. doi: 10.1038/nri.2017.74
10. Brazin KN, Mallis RJ, Das DK, Feng Y, Hwang W, Wang J, et al. Structural features of the α TCR mechanotransduction apparatus that promote pMHC discrimination. *Front Immunol.* (2015) 6:441. doi: 10.3389/fimmu.2015.00441
11. Chen W, Zhu C. Mechanical regulation of T-cell functions. *Immunol Rev.* (2013) 256:160–76. doi: 10.1111/immr.12122
12. Hunter MC, Teixeira A, Halin C. T cell trafficking through lymphatic vessels. *Front Immunol.* (2016) 7:613. doi: 10.3389/fimmu.2016.00613
13. Alon R, Dustin ML. Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen-presenting cells. *Immunity* (2007) 26:17–27. doi: 10.1016/j.immuni.2007.01.002
14. Marshall BT, Long M, Piper JW, Yago T, McEver RP, Zhu C. Direct observation of catch bonds involving cell-adhesion molecules. *Nature* (2003) 423:190–3. doi: 10.1038/nature01605
15. Sarangapani KK, Qian J, Chen W, Zarnitsyna VI, Mehta P, Yago T, et al. Regulation of catch bonds by rate of force application. *J Biol Chem.* (2011) 286:32749–61. doi: 10.1074/jbc.M111.240044
16. Laufer JM, Legler DF. Beyond migration-chemokines in lymphocyte priming, differentiation, and modulating effector functions. *J Leukoc Biol.* (2018) 104:301–12. doi: 10.1002/JLB.2MR1217-494R
17. Alon R, Feigelson SW. Chemokine-triggered leukocyte arrest: force-regulated bi-directional integrin activation in quantal adhesive contacts. *Curr Opin Cell Biol.* (2012) 24:670–6. doi: 10.1016/j.ceb.2012.06.001
18. Sun Z, Guo SS, Fässler R. Integrin-mediated mechanotransduction. *J Cell Biol.* (2016) 215:445–56. doi: 10.1083/jcb.201609037
19. Woolf E, Grigorova I, Sagiv A, Grabovsky V, Feigelson SW, Shulman Z, et al. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat Immunol.* (2007) 8:1076–85. doi: 10.1038/ni1499
20. Bachir AI, Horwitz AR, Nelson WJ, Bianchini JM. Actin-based adhesion modules mediate cell interactions with the extracellular matrix and neighboring cells. *Cold Spring Harb Perspect Biol.* (2017) 9:a023234. doi: 10.1101/cshperspect.a023234
21. Lin H-H, Lin H-K, Lin I-H, Chiou Y-W, Chen H-W, Liu C-Y, et al. Mechanical phenotype of cancer cells: cell softening and loss of stiffness sensing. *Oncotarget* (2015) 6:20946–58. doi: 10.18632/oncotarget.4173
22. Wirtz D, Konstantopoulos K, Searson PC. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat Rev Cancer* (2011) 11:512–22. doi: 10.1038/nrc3080
23. Chin L, Xia Y, Discher DE, Jamney PA. Mechanotransduction in cancer. *Curr Opin Chem Eng.* (2016) 11:77–84. doi: 10.1016/j.coche.2016.01.011
24. Handorf AM, Zhou Y, Halanski MA, Li W-J. Tissue stiffness dictates development, homeostasis, and disease progression. *Organogenesis* (2015) 11:1–15. doi: 10.1080/15476278.2015.1019687
25. Oakes PW, Patel DC, Morin NA, Zitterbart DP, Fabry B, Reichner JS, et al. Neutrophil morphology and migration are affected by substrate elasticity. *Blood* (2009) 114:1387–95. doi: 10.1182/blood-2008-11-191445
26. Jannat RA, Dembo M, Hammer DA. Traction forces of neutrophils migrating on compliant substrates. *Biophys J.* (2011) 101:575–84. doi: 10.1016/j.bpj.2011.05.040
27. Saitakis M, Dogniaux S, Goudot C, Bufi N, Asnacios S, Maurin M, et al. Different TCR-induced T lymphocyte responses are potentiated by stiffness with variable sensitivity. *Elife* (2017) 6:e23190. doi: 10.7554/eLife.23190
28. Dustin ML. Hunter to gatherer and back: immunological synapses and kinapses as variations on the theme of amoeboid locomotion. *Annu Rev Cell Dev Biol.* (2008) 24:577–96. doi: 10.1146/annurev.cellbio.24.110707.175226
29. Mescher MF. Surface contact requirements for activation of cytotoxic T lymphocytes. *J Immunol.* (1992) 149:2402–5.
30. Kim ST, Takeuchi K, Sun Z-YJ, Touma M, Castro CE, Fahmy A, et al. The alphabeta T cell receptor is an anisotropic mechanosensor. *J Biol Chem.* (2009) 284:31028–37. doi: 10.1074/jbc.M109.052712
31. Kim ST, Shin Y, Brazin K, Mallis RJ, Sun Z-YJ, Wagner G, et al. TCR mechanobiology: torques and tunable structures linked to early T cell signaling. *Front Immunol.* (2012) 3:76. doi: 10.3389/fimmu.2012.00076
32. Kobayashi T, Sokabe M. Sensing substrate rigidity by mechanosensitive ion channels with stress fibers and focal adhesions. *Curr Opin Cell Biol.* (2010) 22:669–76. doi: 10.1016/j.ceb.2010.08.023
33. Comrie WA, Li S, Boyle S, Burkhardt JK. The dendritic cell cytoskeleton promotes T cell adhesion and activation by constraining ICAM-1 mobility. *J Cell Biol.* (2015) 208:457–73. doi: 10.1083/jcb.201406120
34. Ilani T, Vasiliver-Shamis G, Vardhana S, Bretscher A, Dustin ML. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. *Nat Immunol.* (2009) 10:531–9. doi: 10.1038/ni.1723
35. Tabdanov E, Gondarenko S, Kumari S, Liapis A, Dustin ML, Sheetz MP, et al. Micropatterning of TCR and LFA-1 ligands reveals complementary effects on cytoskeleton mechanics in T cells. *Integr Biol.* (2015) 7:1272–84. doi: 10.1039/c5ib00032g
36. Husson J, Chemin K, Bohineust A, Hivroz C, Henry N. Force generation upon T cell receptor engagement. *PLoS ONE* (2011) 6:e19680. doi: 10.1371/journal.pone.0019680
37. Hui KL, Balagopal L, Samelson LE, Upadhyaya A. Cytoskeletal forces during signaling activation in Jurkat T cells. *Mol Biol Cell.* (2014) 26:685–95. doi: 10.1091/mbc.E14-03-0830
38. Bashour KT, Gondarenko A, Chen H, Shen K, Liu X, Huse M, et al. CD28 and CD3 have complementary roles in T-cell traction forces. *Proc Natl Acad Sci USA.* (2014) 111:2241–6. doi: 10.1073/pnas.1315606111
39. du Roure O, Saez A, Buguin A, Austin RH, Chavrier P, Silberzan P, et al. Force mapping in epithelial cell migration. *Proc Natl Acad Sci USA.* (2005) 102:2390–5. doi: 10.1073/pnas.0408482102
40. Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) 356:eaa13118. doi: 10.1126/science.aal3118
41. Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) 113:E5916–24. doi: 10.1073/pnas.1605399113
42. von Andrian UH, Hasslen SR, Nelson RD, Erlandsen SL, Butcher EC. A central role for microvillous receptor presentation in leukocyte adhesion under flow. *Cell* (1995) 82:989–99.
43. Judokusumo E, Tabdanov E, Kumari S, Dustin ML, Kam LC. Mechanosensing in T lymphocyte activation. *Biophys J.* (2012) 102:L5–7. doi: 10.1016/j.bpj.2011.12.011
44. O'Connor RS, Hao X, Shen K, Bashour K, Akimova T, Hancock WW, et al. Substrate rigidity regulates human T cell activation and proliferation. *J Immunol.* (2012) 189:1330–9. doi: 10.4049/jimmunol.1102757
45. Kanchanawong P, Shtengel G, Pasapera AM, Ramko EB, Davidson MW, Hess HF, et al. Nanoscale architecture of integrin-based cell adhesions. *Nature* (2010) 468:580–4. doi: 10.1038/nature09621
46. Austen K, Ringer P, Mehlich A, Chrostek-Grashoff A, Kluger C, Klingner C, et al. Extracellular rigidity sensing by talin isoform-specific mechanical linkages. *Nat Cell Biol.* (2015) 17:1597–606. doi: 10.1038/ncb3268
47. Wernimont SA, Wiemer AJ, Bennin DA, Monkley SJ, Ludwig T, Critchley DR, et al. Contact-dependent T cell activation and T cell stopping require talin1. *J Immunol.* (2011) 187:6256–67. doi: 10.4049/jimmunol.1102028
48. Simonson WTN, Franco SJ, Huttenlocher A. Talin1 regulates TCR-mediated LFA-1 function. *J Immunol.* (2006) 177:7707–14. doi: 10.4049/jimmunol.177.11.7707
49. Feigelson SW, Solomon A, Biram A, Hatzav M, Lichtenstein M, Regev O, et al. ICAMs are not obligatory for functional immune synapses between naive CD4 T cells and lymph node DCs. *Cell Rep.* (2018) 22:849–59. doi: 10.1016/j.celrep.2017.12.103
50. Azar GA, Lemaître F, Robey EA, Bousso P. Subcellular dynamics of T cell immunological synapses and kinapses in lymph nodes. *Proc Natl Acad Sci USA.* (2010) 107:3675–80. doi: 10.1073/pnas.0905901107
51. Stein J V. T Cell motility as modulator of interactions with dendritic cells. *Front Immunol.* (2015) 6:559. doi: 10.3389/fimmu.2015.00559
52. Hu KH, Butte MJ. T cell activation requires force generation. *J Cell Biol.* (2016) 213:535–42. doi: 10.1083/jcb.201511053
53. Liu B, Chen W, Evavold BD, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* (2014) 157:357–68. doi: 10.1016/j.cell.2014.02.053

54. Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell* (2018) 174:672–87.e27. doi: 10.1016/j.cell.2018.06.017.
55. Touma M, Chang H-C, Sasada T, Handley M, Clayton LK, Reinherz EL. The TCR C β FG loop regulates $\alpha\beta$ T cell development. *J Immunol.* (2006) 176:6812–23. doi: 10.4049/jimmunol.176.11.6812
56. Das DK, Feng Y, Mallis RJ, Li X, Keskin DB, Hussey RE, et al. Force-dependent transition in the T-cell receptor β -subunit allosterically regulates peptide discrimination and pMHC bond lifetime. *Proc Natl Acad Sci USA.* (2015) 112:1517–22. doi: 10.1073/pnas.1424829112
57. Feng Y, Brazin KN, Kobayashi E, Mallis RJ, Reinherz EL, Lang MJ. Mechanosensing drives acuity of $\alpha\beta$ T-cell recognition. *Proc Natl Acad Sci USA.* (2017) 114:E8204–13. doi: 10.1073/pnas.1703559114
58. Liu Y, Blanchfield L, Ma VP-Y, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci USA.* (2016) 113:5610–5. doi: 10.1073/pnas.1600163113
59. Ma Z, Janmey PA, Finkel TH. The receptor deformation model of TCR triggering. *FASEB J.* (2008) 22:1002–8. doi: 10.1096/fj.07-9331hyp
60. Lee MS, Glassman CR, Deshpande NR, Badgandi HB, Parrish HL, Uttamapinant C, et al. A mechanical switch couples T cell receptor triggering to the cytoplasmic juxtamembrane regions of CD3 ζ . *Immunity* (2015) 43:227–39. doi: 10.1016/j.immuni.2015.06.018
61. Steblyanko M, Anikeeva N, Campbell KS, Keen JH, Sykulev Y. Integrins influence the size and dynamics of signaling microclusters in a Pyk2-dependent manner. *J Biol Chem.* (2015) 290:11833–42. doi: 10.1074/jbc.M114.614719
62. Basu R, Whitlock BM, Husson J, Le Floch A, Jin W, Oyer-Yaniv A, et al. Cytotoxic T cells use mechanical force to potentiate target cell killing. *Cell* (2016) 165:100–10. doi: 10.1016/j.cell.2016.01.021
63. Sens P, Plastino J. Membrane tension and cytoskeleton organization in cell motility. *J Phys Condens Matter.* (2015) 27:273103. doi: 10.1088/0953-8984/27/27/273103
64. Köster D V, Mayor S. Cortical actin and the plasma membrane: inextricably intertwined. *Curr Opin Cell Biol.* (2016) 38:81–9. doi: 10.1016/j.ccb.2016.02.021
65. Niggli V, Rossy J. Ezrin/radixin/moesin: Versatile controllers of signaling molecules and of the cortical cytoskeleton. *Int J Biochem Cell Biol.* (2008) 40:344–9 doi: 10.1016/j.biocel.2007.02.012
66. Rouven Brückner B, Pietuch A, Nehls S, Rother J, Janshoff A. Ezrin is a major regulator of membrane tension in epithelial cells. *Sci Rep.* (2015) 5:14700. doi: 10.1038/srep14700
67. Faure S, Salazar-Fontana LI, Semichon M, Tybulewicz VLJ, Bismuth G, Trautmann A, et al. ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. *Nat Immunol.* (2004) 5:272–9. doi: 10.1038/ni1039
68. Liu Y, Belkina NV, Park C, Nambiar R, Loughhead SM, Patino-Lopez G, et al. Constitutively active ezrin increases membrane tension, slows migration, and impedes endothelial transmigration of lymphocytes *in vivo* in mice. *Blood* (2012) 119:445–53. doi: 10.1182/blood-2011-07-368860
69. Guillou L, Babataheri A, Saitakis M, Bohineust A, Dogniaux S, Hivroz C, Barakat AI. T-lymphocyte passive deformation is controlled by unfolding of membrane surface reservoirs. *Mol Biol Cell* (2016) 27:3574–82. doi: 10.1091/mbc.E16-06-0414
70. Martinac B. The ion channels to cytoskeleton connection as potential mechanism of mechanosensitivity. *Biochim Biophys Acta* (2014) 1838:682–91. doi: 10.1016/j.bbame.2013.07.015
71. Kiyoshima D, Kawakami K, Hayakawa K, Tatsumi H, Sokabe M. Force- and Ca²⁺-dependent internalization of integrins in cultured endothelial cells. *J Cell Sci.* (2011) 124:3859–70. doi: 10.1242/jcs.088559
72. Wenning AS, Neblung K, Strauss B, Wolfs M-J, Sappok A, Hoth M, Schwarz EC. TRP expression pattern and the functional importance of TRPC3 in primary human T-cells. *Biochim Biophys Acta* (2011) 1813:412–23. doi: 10.1016/j.bbame.2010.12.022
73. Pottosin I, Delgado-Enciso I, Bonales-Alatorre E, Nieto-Pescador MG, Moreno-Galindo EG, Dobrovinskaya O. Mechanosensitive Ca²⁺-permeable channels in human leukemic cells: pharmacological and molecular evidence for TRPV2. *Biochim Biophys Acta* (2015) 1848:51–9. doi: 10.1016/j.bbame.2014.09.008
74. Liu CSC, Raychaudhuri D, Paul B, Chakrabarty Y, Ghosh AR, Rahaman O, et al. Cutting edge: piezo1 mechanosensors optimize human T cell activation. *J Immunol.* (2018) 200:1255–60. doi: 10.4049/jimmunol.1701118
75. Cazaux S, Sadoun A, Biarnes-Pelicot M, Martinez M, Obeid S, Bongrand P, et al. Synchronizing atomic force microscopy force mode and fluorescence microscopy in real time for immune cell stimulation and activation studies. *Ultramicroscopy* (2016) 160:168–81. doi: 10.1016/j.ultramicro.2015.10.014
76. Katsumi A, Milanini J, Kiosses WB, del Pozo MA, Kaunas R, Chien S, et al. Effects of cell tension on the small GTPase Rac. *J Cell Biol.* (2002) 158:153–64. doi: 10.1083/jcb.200201105
77. Houk AR, Jilkine A, Mejean CO, Boltyskiy R, Dufresne ER, Angenent SB, et al. Membrane tension maintains cell polarity by confining signals to the leading edge during neutrophil migration. *Cell* (2012) 148:175–88. doi: 10.1016/j.cell.2011.10.050
78. Diz-Muñoz A, Thurley K, Chintamen S, Altschuler SJ, Wu LF, Fletcher DA, et al. Membrane tension acts through PLD2 and mTORC2 to limit actin network assembly during neutrophil migration. *PLoS Biol.* (2016) 14:e1002474. doi: 10.1371/journal.pbio.1002474
79. Simunovic M, Voth GA. Membrane tension controls the assembly of curvature-generating proteins. *Nat Commun.* (2015) 6:7219. doi: 10.1038/ncomms8219
80. Shi Z, Baumgart T. Membrane tension and peripheral protein density mediate membrane shape transitions. *Nat Commun.* (2015) 6:5974. doi: 10.1038/ncomms6974
81. Tsujita K, Takenawa T, Itoh T. Feedback regulation between plasma membrane tension and membrane-bending proteins organizes cell polarity during leading edge formation. *Nat Cell Biol.* (2015) 17:749–58. doi: 10.1038/ncb3162
82. Apodaca G. Modulation of membrane traffic by mechanical stimuli. *Am J Physiol Renal Physiol.* (2002) 282:F179–90. doi: 10.1152/ajrenal.2002.282.2.F179
83. Gauthier NC, Masters TA, Sheetz MP. Mechanical feedback between membrane tension and dynamics. *Trends Cell Biol.* (2012) 22:527–35. doi: 10.1016/j.tcb.2012.07.005
84. Bretou M, Jouannot O, Fanget I, Pierobon P, Larochette N, Gestraud P, et al. Cdc42 controls the dilation of the exocytotic fusion pore by regulating membrane tension. *Mol Biol Cell.* (2014) 25:3195–209. doi: 10.1091/mbc.E14-07-1229
85. Wen PJ, Grenklo S, Arpino G, Tan X, Liao H-S, Heureaux J, et al. Actin dynamics provides membrane tension to merge fusing vesicles into the plasma membrane. *Nat Commun.* (2016) 7:12604. doi: 10.1038/ncomms12604
86. Gauthier NC, Fardin MA, Roca-Cusachs P, Sheetz MP. Temporary increase in plasma membrane tension coordinates the activation of exocytosis and contraction during cell spreading. *Proc Natl Acad Sci USA.* (2011) 108:14467–72. doi: 10.1073/pnas.1105845108
87. Raucher D, Sheetz MP. Membrane expansion increases endocytosis rate during mitosis. *J Cell Biol.* (1999) 144:497–506.
88. Abu Shah E, Keren K. Mechanical forces and feedbacks in cell motility. *Curr Opin Cell Biol.* (2013) 25:550–7. doi: 10.1016/j.ccb.2013.06.009
89. Paul NR, Jacquemet G, Caswell PT. Endocytic trafficking of integrins in cell migration. *Curr Biol.* (2015) 25:R1092–105. doi: 10.1016/j.cub.2015.09.049
90. Maritzen T, Schachtner H, Legler DF. On the move: endocytic trafficking in cell migration. *Cell Mol Life Sci.* (2015) 72:2119–34. doi: 10.1007/s00018-015-1855-9
91. Nishikimi A, Ishihara S, Ozawa M, Etoh K, Fukuda M, Kinashi T, et al. Rab13 acts downstream of the kinase Mst1 to deliver the integrin LFA-1 to the cell surface for lymphocyte trafficking. *Sci Signal.* (2014) 7:ra72. doi: 10.1126/scisignal.2005199
92. Stanley P, Tooze S, Hogg N. A role for Rap2 in recycling the extended conformation of LFA-1 during T cell migration. *Biol Open* (2012) 1:1161–68. doi: 10.1242/bio.20122824
93. Onnis A, Finetti F, Baldari CT. Vesicular trafficking to the immune synapse: how to assemble receptor-tailored pathways from a basic building set. *Front Immunol.* (2016) 7:50. doi: 10.3389/fimmu.2016.00050

94. Compeer EB, Kraus F, Ecker M, Redpath G, Amiezer M, Rother N, et al. A mobile endocytic network connects clathrin-independent receptor endocytosis to recycling and promotes T cell activation. *Nat Commun.* (2018) 9:1597. doi: 10.1038/s41467-018-04088-w
95. Lou J, Rossy J, Deng Q, Pagon S V., Gaus K. New Insights into How trafficking regulates T cell receptor signaling. *Front Cell Dev Biol.* (2016) 4:77. doi: 10.3389/fcell.2016.00077
96. Griffiths GM, Tsun A, Stinchcombe JC. The immunological synapse: a focal point for endocytosis and exocytosis. *J Cell Biol.* (2010) 189:399–406. doi: 10.1083/jcb.201002027
97. Masters TA, Pontes B, Viasnoff V, Li Y, Gauthier NC. Plasma membrane tension orchestrates membrane trafficking, cytoskeletal remodeling, and biochemical signaling during phagocytosis. *Proc Natl Acad Sci USA.* (2013) 110:11875–80. doi: 10.1073/pnas.1301766110
98. Aureille J, Belaadi N, Guilluy C. Mechanotransduction via the nuclear envelope: a distant reflection of the cell surface. *Curr Opin Cell Biol.* (2017) 44:59–67. doi: 10.1016/j.ceb.2016.10.003
99. Fedorchak GR, Kaminski A, Lammerding J. Cellular mechanosensing: Getting to the nucleus of it all. *Prog Biophys Mol Biol.* (2014) 115:76–92. doi: 10.1016/j.PBIOMOLBIO.2014.06.009
100. Guilluy C, Osborne LD, Van Landeghem L, Sharek L, Superfine R, Garcia-Mata R, et al. Isolated nuclei adapt to force and reveal a mechanotransduction pathway in the nucleus. *Nat Cell Biol.* (2014) 16:376–81. doi: 10.1038/ncb2927
101. Le HQ, Ghatak S, Yeung C-YC, Tellkamp F, Günschmann C, Dieterich C, et al. Mechanical regulation of transcription controls Polycomb-mediated gene silencing during lineage commitment. *Nat Cell Biol.* (2016) 18:864–75. doi: 10.1038/ncb3387
102. Elosegui-Artola A, Andreu I, Beedle AEM, Lezamiz A, Uroz M, Kosmalska AJ, et al. Force triggers YAP nuclear entry by regulating transport across nuclear pores. *Cell* (2017) 171:1397–410.e14. doi: 10.1016/j.CELL.2017.10.008
103. Lomakin A, Nader G, Piel M. Forcing entry into the nucleus. *Dev Cell* (2017) 43:547–548. doi: 10.1016/j.devcel.2017.11.015
104. McGregor AL, Hsia C-R, Lammerding J. Squish and squeeze — the nucleus as a physical barrier during migration in confined environments. *Curr Opin Cell Biol.* (2016) 40:32–40. doi: 10.1016/j.ceb.2016.01.011
105. Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R, Hirsch K, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature* (2008) 453:51–55. doi: 10.1038/nature06887
106. Thiam H-R, Vargas P, Carpi N, Crespo CL, Raab M, Terriac E, et al. Perinuclear Arp2/3-driven actin polymerization enables nuclear deformation to facilitate cell migration through complex environments. *Nat Commun.* (2016) 7:10997. doi: 10.1038/ncomms10997

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with the authors DL and JL.

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GLOSSARY

Shear stress (or shear force)	Shear stress is the tangential force applied by a flowing fluid on the surface of an object
Catch bond/Slip bond	A catch bond is a bond that becomes stronger (increased lifetime) when a pulling force is applied to it. By contrast, a slip bond becomes weaker (decreased lifetime) with applied force
Mechanosensing	Mechanosensing is the process through which cells or proteins detect and respond to variations of forces and mechanical properties of their environment
Mechanosensor	A mechanosensor is a molecule/protein that mediates mechanosensing
Passive mechanosensing	During passive mechanosensing, a mechanosensor detects applied mechanical stimuli (without applying force or tension itself)
Active touch sensing	Active touch sensing is the process through which cells actively probe the mechanical properties of their environment (for instance substrate stiffness)
Mechanotransduction	Mechanotransduction is the process during which mechanosensors translate mechanical inputs into intracellular signaling events
Stiffness (or elastic modulus)	Stiffness is a measure of the ability of an object or a substance to resist deformation upon an applied force
Durotaxis	Migrating cells can sense stiffness of the substrate they migrate in or on, typically <i>via</i> active touch sensing. Durotaxis is the ability of cells to move up rigidity gradients.



Immunophysical Evaluation of the Initiating Step in the Formation of the Membrane Attack Complex

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OPEN ACCESS

Edited by:

Andrzej Stasiak,
Université de Lausanne, Switzerland

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Specialty section:

This article was submitted to
Biophysics,
a section of the journal
Frontiers in Physics

Received: 11 August 2018

Accepted: 26 October 2018

Published: 20 November 2018

Citation:

Zewde N, Mohan RR and Morikis D
(2018) Immunophysical Evaluation of
the Initiating Step in the Formation of
the Membrane Attack Complex.
Front. Phys. 6:130.
doi: 10.3389/fphy.2018.00130

The complex between complement system proteins C5b and C6 is the cornerstone for the assembly of the membrane attack complex (MAC, also known as C5b6789_n). MAC is the terminal product of three converging pathways of the complement system and functions as a pore forming complex on cell surfaces, as a response of the immune system in fighting pathogens. However, when proper regulation of the complement system is compromised, MAC also attacks host tissues and contributes to several complement-mediated autoimmune diseases. We performed a molecular dynamics and electrostatics study to elucidate the mechanism of interaction between C5b and C6 and the formation of the C5b6 complex. The C5b6 interface consists of three binding sites stabilized predominantly by van der Waals interactions, and several critical salt bridges and hydrogen bonds. We discuss differences between domains C5d and C3d that lead to mono-functionality of C5d in acting as the scaffold for MAC formation, as opposed to dual functionality of C3d in acting as an opsonin for phagocytosis and as a link between innate and adaptive immunity, based on a comparative sequence, structural, and physicochemical analysis. We also extended our analysis to pathway dynamics to demonstrate the significance of consumption-production rates of C5b, C6, and C5b6 that lead toward MAC formation. Finally, we propose that C5d is a target for drug discovery, aiming to the inhibition of the MAC formation in autoimmune diseases originating from MAC-mediated host cell lysis.

Keywords: complement system, complement protein C5, complement protein C6, C5b6 complex, membrane attack complex, molecular dynamics, electrostatics

INTRODUCTION

As part of the innate immunity, the complement system orchestrates a cascade of biochemical reactions that result in pathogen elimination and in activation of the adaptive immune response [1, 2]. The versatile response of the complement system emerges from its three pathways known as alternative, classical, and lectin, that are either constitutively active in the fluid phase (alternative and classical pathway [3–5]) or initiate upon sensing danger-associated molecular patterns on pathogens (classical and lectin pathways). Activation of all three pathways converges on the cleavage of complement protein C3 into C3b and C3a [6]. Subsequently, continued propagation leads to the terminal cascade by cleavage of complement C5 to form C5b and C5a. Complement C6 then binds to C5b to form the complex C5b6 [7]. This soluble complex then associates with C7 to form C5b67, which later anchors to a nearby surface. Subsequently, the surface bound C5b67

binds to C8 to form C5b678 [8]. This complex, unlike the anchored C5b67, forms a pore of 0.9-nm diameter, which expands later in time to a 3-nm pore [9–11]. Finally, surface bound C5b678 recruits multiple C9s, to a maximum of 18, to form the membrane attack complex (MAC or C5b678_n, where $n = 1-18$) [9, 12]. Although the proteins that make up the MAC pores are the same (C5b, C6, C7, C8, and C9), there is oligomeric heterogeneity in the assembly process of MACs. For instance, oligomerization of two to four C5b678 complexes can bind to a variable number of C9s to form a joined MAC pore [9]. In any case, structures of a single MAC pore, comprised of C5b678 in complex with a polymerized C9, are cylindrical in shape, contain a single stalk protrusion, and have an inner lumen diameter of 10- to 11.5-nm [9, 12]. MACs evolved as the only direct killing mechanism deployed by the complement system to fight against pathogens; indeed MAC deficiency has been associated with an increased risk of recurrent meningitis [1]. In addition to eliminating pathogens, MAC instigates numerous signaling pathways that directly affect cell cycles. For instance, sublytic MACs affect cell proliferation and apoptosis by enhancing or inhibiting the processes [13]. In addition, MACs can directly mediate cytokine production and platelet activation [13].

The instigation of an immune response via C5b6 is also detrimental to host-cells unless complement is properly regulated at the terminal stage [14]. To ensure tissue homeostasis, multiple checkpoints are present in fluid and surface phases that target complement propagators such as C3/C5 convertases. Furthermore, membrane-bound complement regulator, CD59, is present on host-cells to directly inhibit the assembly of the MAC and mitigate its deadly effects. However, despite the regulatory checkpoints, disease-related mutations over-activate the complement system and disrupt tissue homeostasis by generating elevated levels of MAC. This level of impairment propels complement in becoming one of the key drivers for diseases like hemolytic uremic syndrome (aHUS), age-related macular degeneration (AMD), and paroxysmal nocturnal hemoglobinuria (PNH) [15–20].

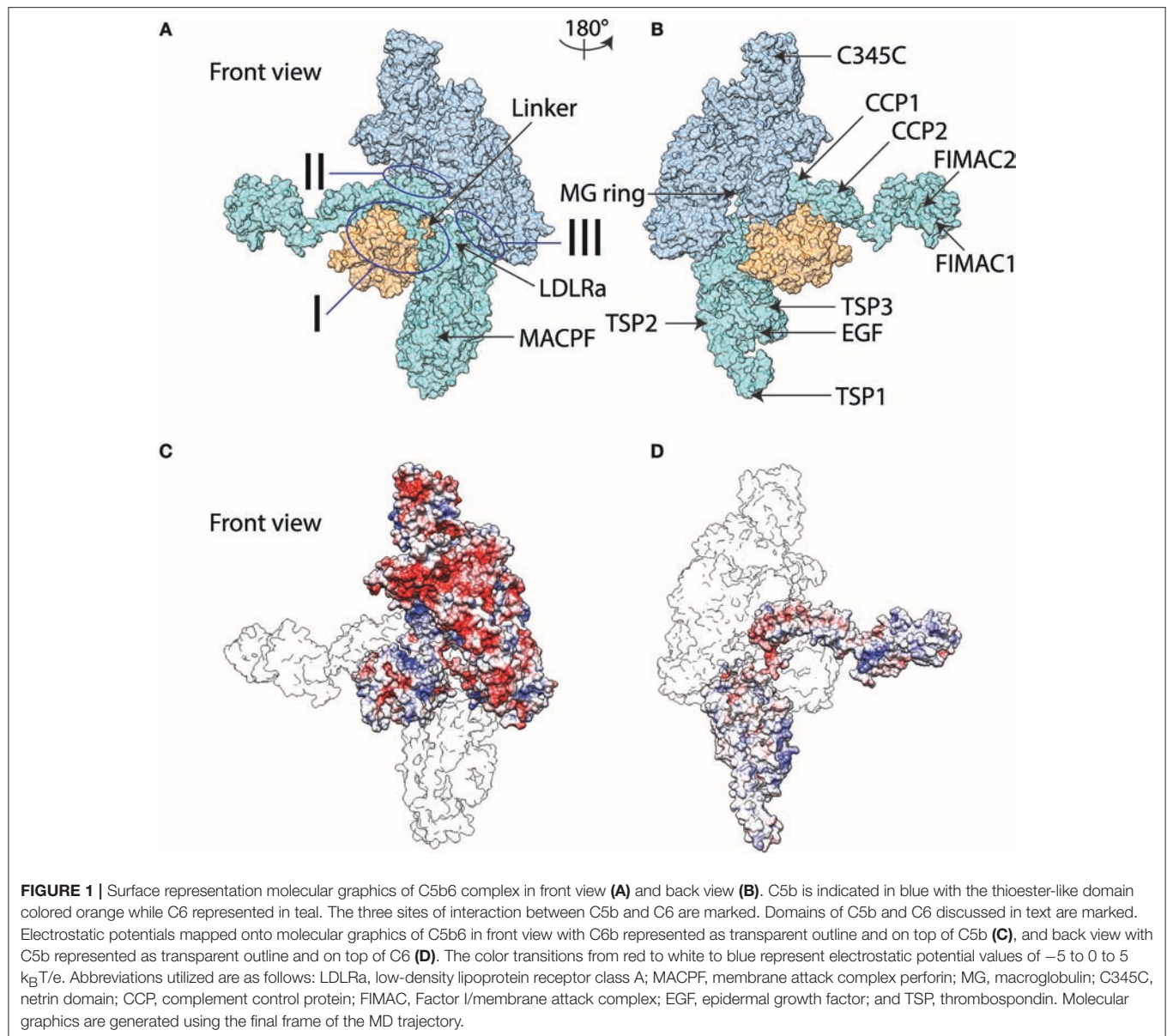
The formation of C5b6 complex sets the stage for a cascade of reactions that go beyond just the elimination of pathogens. C5b6 provides the junction at which the early- and late-stage complement pathway propagation converge to instigate signaling cues that are vital for cell survival [13]. Thus, understanding the governing mechanism behind C5b6 formation provides the basis for the first step in the assembly of terminal MAC complex that initiates a range of events, from immune defense to development of autoimmune diseases.

Complement proteins C3, C4, and C5 are structurally homologous [21–23] but only C3 and C4 have an internal thioester bond moiety that is capable of undergoing hydrolysis, followed by covalent attachment to cell surfaces [24, 25]. After cleavage of C3 and C4 by convertases to form fragments C3a/C3b and C4a/C4b, the C3b and C4b fragments are opsonins that attach to cells surfaces through their thioester domains (TED), also known as C3d and C4d when they become stand-alone proteins after additional cleavage steps. The cell-bound C3b is recognizable by phagocytes for elimination of the C3b-tagged cells, and also C3b and C4b become part of the convertase

complexes that are responsible for C3 and C5 cleavage. On the other hand, C5b is missing an internal thioester bond, but it contains a TED-like domain that is structurally homologous to the TEDs of C3 and C4. For simplicity we will call hereafter C3d, C4d, and C5d the TED domains of C3 and C4, and the TED-like domain of C5, respectively.

Crystal structures of C3b in complex with structurally homologous modular regulators, Factor H (FH), complement receptor 1 (CR1) membrane cofactor protein (MCP), decay accelerating factor (DAF), and smallpox inhibitor of complement enzymes (SPICE), are available [26, 27]. These regulators are composed of repeated complement control protein (CCP) modules and have shown a shared binding mode along the structure of C3b, comprising modules CCP1-4 (FH, MCP, SPICE), CCP2-4 (DAF), and CCP15-17 (CR1). All regulators show contact of one module at the C3d domain of C3b. The viral vaccinia control protein (VCP), that is structurally and functionally homologous to SPICE, is also expected to have a similar binding mode to C3b. In addition, the stand-alone C3d domain, is known to interact with modules CCP1-2 of complement receptor 2 (CR2) [28], modules CCP19-20 of FH [29], in addition to modules CCP1-4 (mentioned above as interacting along C3b), and *S. aureus* proteins Efb-C, Ecb, and Sbi [30, 31]. These structural observations make the C3d domain multifunctional in interacting with complement natural and viral regulators, when C3d is part of C3b, and in attracting CR2, FH (CCP19-20), and bacterial regulators when C3d is stand-alone. On the other hand, C5b is not known to possess similar properties as C3b, and C5d is not known to exist in a stand-alone form. Instead, C5b acts as the first block of a scaffold that initiates the membrane attack complex, interacting first with C6, and subsequently with C7, C8, and several C9s, mentioned above. The crystal structures of C5b6 [32, 33] reveal that an elongated C6 surrounds half of the C5d domain and has three sites of contacts with C5b (**Figures 1A,B**). In addition, the crystal structure reveals the presence of charged patches on the surfaces of C5b and C6, and at the binding interface, which are expected to contribute to structural stability of the complex through the formation of ionic and hydrogen bonding contacts (**Figures 1C,D**).

In this study, first we examine the physicochemical mechanism of the interaction between C5b and C6. Given that the C5d domain contributes to the interaction of C5b and C6, and the multifunctionality of C3d with several sites of interaction with native regulators and receptors and bacterial and viral regulators, we present a comparative sequence, structural, and physicochemical analysis between C5d and C3d. For completion, we also include C4d in the comparative analysis. Our goal is to contribute toward understanding mechanisms of function of C3d, C4d, and C5d at the structural and physicochemical property level. Finally, we present a systems-biology approach to understand the pathway dynamics of the terminal complement cascade that starts at C5b6 complex and ends at MAC [25]. We performed molecular dynamics (MD) simulations to relax the crystallographic structure from crystal packing effects, and to obtain insight on the dynamic character of the structure and the persistence of the intermolecular contacts at the amino acid



side chain level. Guided by the findings of our MD analysis, and by our previous work that has shown that electrostatics plays a fundamental role on the regulation and function of C3b [34–40] and C3d [28–31, 41, 42], we performed electrostatic calculations using conformational states extracted from the MD data.

RESULTS

Molecular Dynamics Analysis

Our goal is to identify the stabilizing interactions that lead to formation of the C5b6 complex, the initial scaffold for the assembly of MAC. The crystal structure [32] shows three major sites of interaction between C5b and C6, named I, II, III (**Figures 1A,B**). Interactions between C6 and the thioester domain of C5b fall under Site I, while interactions of C6 with the macroglobulin (MG) ring of C5b fall under Sites II and III.

The crystal structure also shows the presence of charged patches on the surfaces of the two proteins, C5b and C6 (**Figures 1C,D**), suggesting that charges may be contributing factors to binding. We performed an explicit solvent MD simulation, using a crystal structure as initial conformation, to optimize local geometries and chemistry and to delineate distinct conformational states visited throughout the simulation. Analysis of the MD trajectory, using the molecular mechanics-Poisson-Boltzmann surface area (MM-PBSA) method, showed an overall favorable binding energy for the solvated complex, dominated by van der Waals interactions (**Table 1**). Given the observation of many charged patches on the surfaces of C5b and C6 (**Figures 1C,D**), but overall unfavorable polar contribution to binding (**Table 1**), we analyzed the frequency of occurrence of intermolecular pairwise polar interactions to obtain a closer look into the nature of polar contributions. Specific intermolecular salt bridges often

TABLE 1 | Calculated MM-PBSA energies from the MD trajectory.

Energy component	Mean $\pm$ SD (kJ/mol)
MM van der Waals (vdW)	-983.7 ± 107.5
MM electrostatic	795.4 ± 390.8
Poisson-Boltzmann (PB) solvation	-450.2 ± 386.6
Nonpolar solvation	-140.2 ± 11.7
$\Delta G(\text{total})$	-778.6 ± 94.1

stabilize protein complexes, an effect that has been termed ionic tethering, and so do intermolecular hydrogen bonds, even if the overall energetic contribution is dominated by van der Waals interactions of hydrophobic chemical groups [43].

We characterized the significance of intermolecular polar interactions by evaluating the number of salt bridges and hydrogen bonds that occur across the C5b6 binding interface at a frequency of at least 20% during the MD trajectory. **Figures 2A,B** shows occupancy (frequency of occurrence during the MD trajectory) maps for intermolecular salt bridges and hydrogen bonds, respectively. The C5b6 complex has 13 intermolecular salt bridges with distance cutoff of 5 Å, and 11 intermolecular hydrogen bonds, demonstrating varying levels of persistence throughout the trajectory.

The 13 intermolecular salt bridges (and protein/domain) in decreasing order of persistence (81–20%) are: Lys1117 (C5b/C5d)-Glu2217(C6/CCP1), Lys884(C5b/CUB)-Asp2226 (C6/CCP1), Glu646(C5b/MG Ring)-Arg1704(C6/LDLRa), Asp1076(C5b/C5d)-Arg2279(C6/CCP2), Lys1139(C5b/C5d)-Glu2187(C6/CCP1), Asp643(C5b/MG Ring)-Lys1693(C6/TSP2), Lys1139(C5b/C5d)-Asp2186(C6/CCP1), Asp648(C5b/MG Ring)-Arg1704(C6/LDLRa), Glu414(C5b/MG Ring)-Arg1734 (C6/LDLRa), Glu149(C5b/MG Ring)-Arg2244(C6/CCP1), Asp648(C5b/MG Ring)-Lys1702(C6/LDLRa), Arg435 (C5b/MG Ring)-Glu1716(C6/LDLRa), and Lys1133(C5b/C5d)-Glu2187(C6/CCP1) (**Figure 2A**). All intermolecular salt bridges (cutoff distance 5 Å) at all occupancy levels are listed in **Supplementary Data Sheet 1**, and all intermolecular Coulombic interactions (cutoff distance 8 Å, including salt bridges with cutoff distance of 5 Å) at all occupancy levels are listed in **Supplementary Data Sheet 2**.

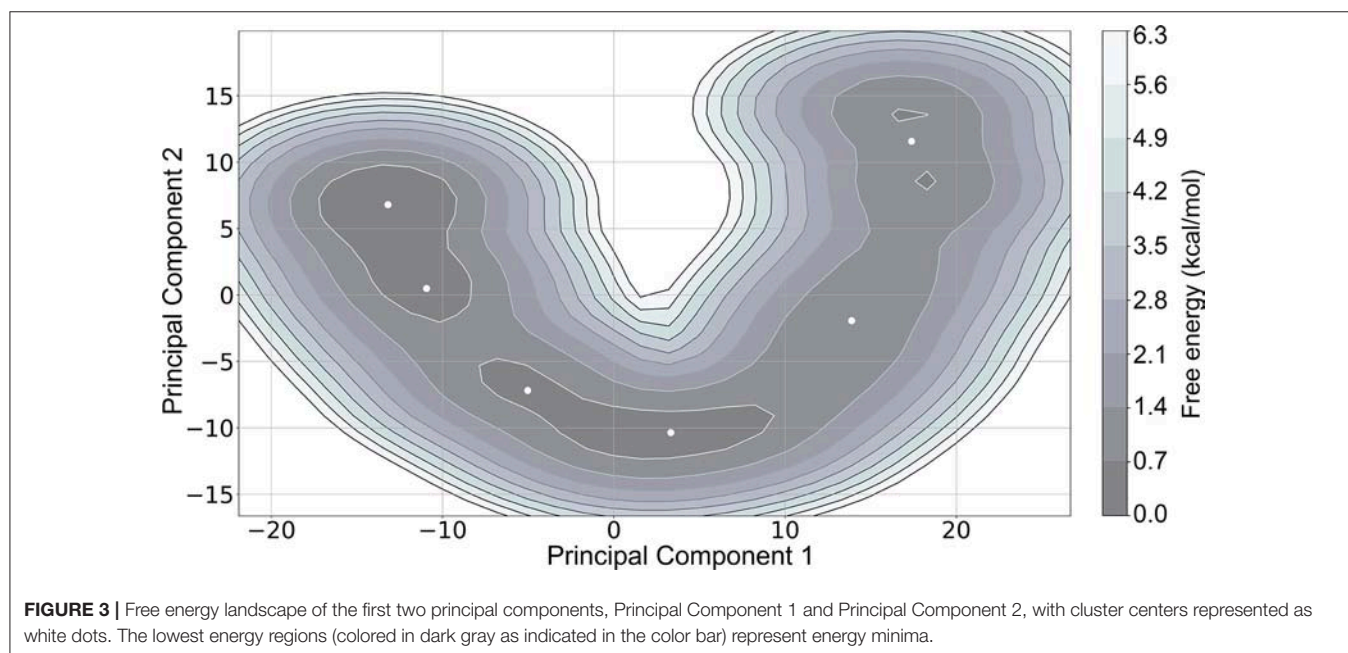
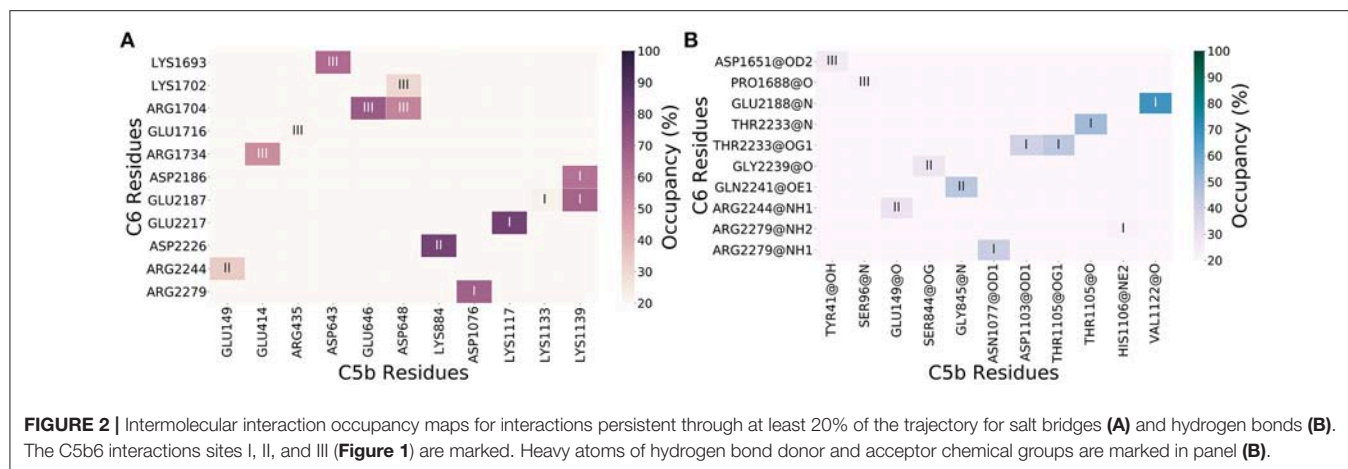
Unlike the persistence of intermolecular salt bridges, most of the intermolecular hydrogen bonds formed between C5b and C6 appear less than half of the time in the trajectory. The 11 intermolecular hydrogen bonds (and protein/domain) in decreasing order of persistence (68–20%) are: Val1122(C5b/C5d)-Glu2188(C6/CCP1), Thr1105(C5b/C5d)-Thr2233(C6/CCP1) (backbone-backbone), Gly845(C5b/CUB)-Gln2241(C6/CCP1), Thr1105(C5b/C5d)-Thr2233(C6/CCP1) (side chain-side chain), Asn1077(C5b/C5d)-Arg2279(C6/CCP2), Asp1103(C5b/C5d)-Thr2233(C6/CCP1), Glu149(C5b/MG Ring)-Arg2244(C6/CCP1), Ser844(C5b/CUB)-Gly2239(C6/CCP1), Tyr41(C5b/MG Ring)-Asp1651(C6/TSP2), Ser96(C5b/MG Ring)-Pro1688(C6/TSP2), and His1106(C5b/C5d)-Arg2279(C6/CCP2) (**Figure 2B**). All intermolecular hydrogen bonds at all occupancy levels are listed in **Supplementary Data Sheet 3**.

Six salt bridges occur in Site III, followed by five in Site I, and two in Site II (**Figures 1A, 2A**). On the other hand, six hydrogen bonds occur in Site I, followed by three hydrogen bonds occurring in Site II and two hydrogen bonds occurring in site III (**Figures 1A, 2B**). Three bifurcated salt bridges are observed. These are Site I salt bridge Lys1139(C5b) with Asp2186(C6) and Glu2187(C6), Site III salt bridge Arg1704(C6) with Glu646(C5b) and Asp648(C5b), and Site III salt bridge Asp648(C5b) with Lys1702(C6) and Arg1704(C6) (**Figures 1A, 2A**). Two residues of C6 are hydrogen bonded with more than one partner, both in Site I. These are Thr2233(C6) side chain with Asp1103(C5b) side chain and Thr1105(C5b) side chain, and Arg2279(C6) side chain with Asn1077(C5b) backbone and His1106(C5b) side chain (**Figures 1A, 2B**). Finally, two residues of C5b and three of C6 participate in both salt bridge side chain-side chain interactions and hydrogen bonding side chain-backbone interactions. These are Glu149 and Asp648 of C5b, and Arg1704, Arg2244, and Arg2279 of C6. Among them are Arg1704(C6)-Asp648(C5b) salt bridge and hydrogen bond, involving Arg1704(C6) which is also part of a bifurcated salt bridge with Glu646(C5b) (Site III), Arg2244(C6)-Glu149(C5b) salt bridge and hydrogen bond (Site II), and Arg2279(C6) salt bridge with Asp1070(C6b), and hydrogen bond Asn1077(C5b) (Site I) (**Figures 1A, 2**).

Unfavorable charge-charge interactions within 5 Å of each other were not observed at occupancies greater than 10% (**Supplementary Data Sheet 4**). Thirteen unfavorable charge-charge interactions were observed within 8 Å of each other using the 20% occupancy threshold, as follows: four at Site I, three at Site II, and six at Site III. At Site I, the unfavorable charge-charge interactions are between Lys1139(C5b) and Lys1889(C6), Asp1140(C5b) and Glu2187(C6), Asp1167(C5b) and Glu2217(C6) and, Glu1181(C5b) and Asp2185(C6). At Site II, the unfavorable charge-charge interactions are between Arg177(C5b) and Arg2244(C6), Arg840(C5b) and Arg2252(C6) and, Lys884(C5b) and Arg2244(C6). At Site III, the unfavorable charge-charge interactions are between Asp43(C5b) and Asp1651(C6), Arg98(C5b) and Lys1690(C6), Arg435(C5b) and Arg1734(C6), Asp643(C5b) and Asp1706(C6), Glu646(C5b) and Glu1695(C6) and, Lys652(C5b) and Lys1702(C6). Of these, the only interactions with frequency above 50% are between Asp1167(C5b) and Glu2217(C6) at Site I, and Lys884(C5b) and Arg2244(C6) at Site II. All unfavorable Coulombic interactions with cutoff distances 5 Å and 8 Å at all occupancy levels are listed in **Supplementary Data Sheets 4, 5**, respectively. Overall, unfavorable charge-charge interactions are weak.

We further analyzed the MD trajectory to capture the dynamic changes in electrostatic contributions, by evaluating distinct conformational states. We identified six representative structures from the MD trajectory through principal component analysis (PCA) decomposition on phi and psi angles, followed by k-means clustering of the PCA components. The cluster centers (**Figure 3**) were extracted as representative structures. Structural representation of clustering conformational states is shown in **Supplementary Figure 1**.

We performed MM-PBSA calculations for the 20 most representative structures of each cluster to evaluate possible differences in the binding free energies among the



six clusters. Although we observe differences among the clusters, the overall trends in their free energies are similar (Table 2).

Electrostatic Analysis

We used the alanine scan method of our AESOP computational framework [44] to explore in detail the significance of the many pairwise charge-charge interactions in contributing to the stability of the C5b6 complex. We performed computational alanine scan by mutating every ionizable amino acid residue to alanine, one at a time, to generate families of single mutants for C5b and C6, and their C5b6 complex. Subsequently, we performed Poisson-Boltzmann electrostatic calculations to obtain electrostatic contributions to the free energies of binding for the binding reaction of each mutant. We present the results in reference to the parent structure, as differences between the calculated electrostatic free energies of binding of mutant

structures minus the electrostatic free energy of the parent structure (see Methods). We performed the calculations for the representative structures of all six conformational clusters, and the results for those residues that participate in salt bridges (shown in Figure 2A) are presented in Figure 4. Loss of binding upon mutation is denoted by an electrostatic free energy of binding with a positive value, whereas gain of binding is denoted by a negative value. Loss of binding for the mutant indicates that the mutated residue is a contributor to binding of the parent protein by forming favorable charge-charge interactions. Likewise, gain of binding for the mutant indicates that the mutated residue opposes binding of the parent protein by contributing to unfavorable charge-charge interactions.

A few mutants show perturbations greater than thermal energy of $2k_B T$ at room temperature, most notably Lys1139A of C5b, followed by Lys884A, Asp1076A, Lys1117A, and Lys1133A

TABLE 2 | Calculated MM-PBSA energies of six conformational states, extracted from the MD trajectory.

Energy	Mean ± SD (kJ/mol)					
	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
MM vdW	−869.9 ± 36.4	−1115.9 ± 34.3	−876.5 ± 46.9	−901.2 ± 31.0	−985.3 ± 47.7	−887.4 ± 35.6
MM electrostatic	786.2 ± 171.1	511.7 ± 119.2	931.4 ± 100.0	1190.8 ± 349.8	555.2 ± 172.8	−1290.3 ± 184.1
PB solvation	−523.0 ± 151.9	−125.5 ± 109.2	−664.0 ± 106.7	−819.2 ± 332.2	−224.7 ± 173.2	−954.4 ± 168.6
Non-polar solvation	−133.1 ± 3.8	−154.4 ± 3.3	−130.5 ± 2.9	−124.3 ± 4.6	−137.7 ± 2.9	−131.0 ± 3.8
ΔG(total)	−736.4 ± 35.6	−884.1 ± 40.6	−739.7 ± 33.5	−654.0 ± 47.7	−791.6 ± 32.2	−682.8 ± 37.2

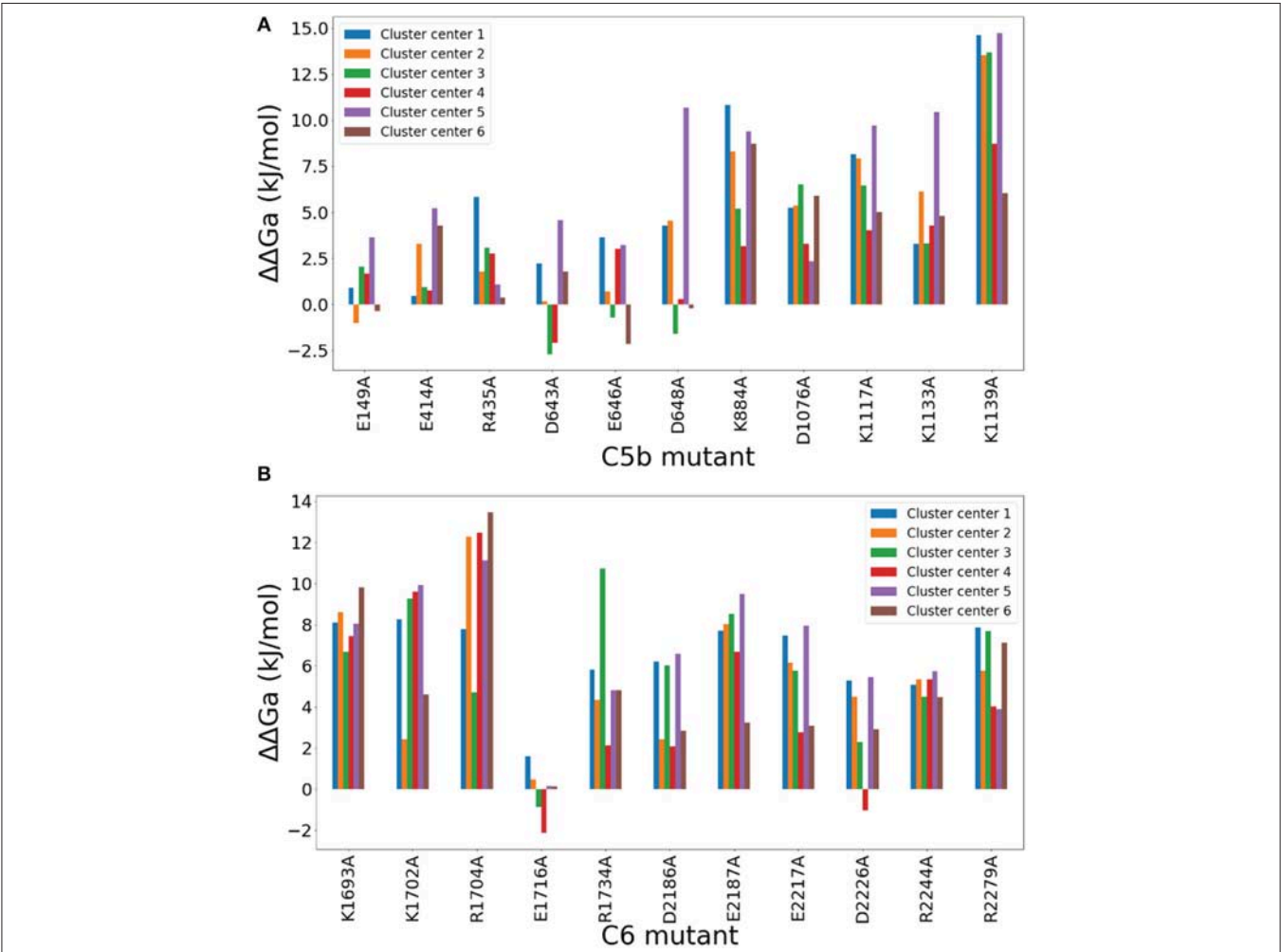


FIGURE 4 | Computational Alanine Scan results of C5b (A) and C6 (B) mutants that participate in salt bridges. The vertical axis represents differences in the calculated electrostatic free energies of binding between the mutant and the parent structure for the mutants shown in the horizontal axis. Each mutant is represented by six free energy values, corresponding to the six representative (cluster center) structures of the conformational clusters of the MD simulation. The mutant notation denotes the residue number surrounded by the type of replaced residue on the left and the replacing residue (alanine, A) on the right. A positive value denotes that the mutation causes loss of binding, indicating that the mutated residue favors binding in the parent structure. A negative value denotes that the mutation causes gain of binding, indicating that the mutated residue disfavors binding in the parent structure. The one-letter amino acid code is used, instead if the three-letter code used in text, to simplify the figure.

(Figure 4A), and nearly every mutant of C6, most notably Arg1704, with the exception of Glu1716A (Figure 4B). These significant charge interactions depicted by the mutations of Figure 4 have been identified in the salt bridge occupancy maps of Figure 2A. AESOP data for all ionizable amino acid replacements within 8 Å of the C5b6 complex interface are listed in Supplementary Data Sheet 6. The most notable charge interactions, involving Lys1139(C5b) and Arg1704(C6)

as depicted by the data of **Figure 4**, form strong bifurcated salt bridges in Sites I and II, respectively (see above). Another charge interaction, but of lower strength, involving Asp648(C5b) also forms a bifurcated salt bridge in Site III (see above).

Sequence and Electrostatic Potential Comparison of Thioester Domains of C3d, C4d, and C5d

The complement system contains thioester domains, referred herein as C3d and C4d, that can covalently attach to different surfaces and tag the cellular species with complement complexes and fragments. However, the C5d thioester-like domain of C5 is distinct from C3d and C4d because it does not have the ability to covalently tag a surface, or to exist as a standalone domain, but has gained the molecular capability to instigate MAC formation [32, 33]. Despite all three thioester/thioester-like domains, C3d, C4d, and C5d, having similar structures, C3d is a molecular hub for reactions that mediate inflammatory processes such as opsonophagocytosis and B-cell activation, whereas C5d participates in the binding of C6 and acts as the cornerstone for the MAC assembly. Thus, to elucidate on how C3d and C5d achieve their versatile functions, we performed a comparative analysis of the physicochemical properties of C3d and C5d, also including a comparative analysis of C3d and C4d for completion. Earlier studies have identified the residues of C3d that interact with FH [29] and CR2 [28]. FH is a potent regulator of C3b, and C3b's convertase complexes, that inhibits opsonophagocytosis on host cells. The CR2-C3d complex is formed as part of the B cell receptor-coreceptor complex and is a link between innate and adaptive immunity.

First, we performed a sequence alignment of all three domains, C3d, C4d, and C5d, to compare the conservation of their structurally and functionally important residues (**Figure 5**). The percent identity between C3d and C5d is 29.2%, between C3d and C4d is 35.7%, and between C4d and C5d is 28.8%. The contact sites with FH and CR2 are spread across the sequence of C3d. Of the total 310 C3d residues, 87 residues are involved in contacts with FH and CR2 combined (union of red and blue horizontal boxes in **Figure 5**). On the other hand, only nine C5d residues are involved in contacts with C6 (green horizontal boxes in **Figure 5**), and from those nine only one is a common site with C3d, that of Asp1104(C5d) and Gly1179(C3b). From the 87 residues that are involved in C3d-CR2/FH interactions, only 19 are homologous to C5d residues (intersection of red/blue horizontal boxes and identical C5d and C3d residues, denoted by vertical blue blocks, in **Figure 5**).

Lastly, of the nine residues of C5d that are involved in binding to C6, four are charged residues (Asp1076, Lys1117, Lys1133, and Lys1139) that interact by forming salt bridges with residues in the CCP1 and CCP2 domains of C6 (Site I, **Figures 1, 2A**). Contact map analysis showed that Lys1137 forms strong bifurcated salt bridges (**Figure 2A**). Also, the AESOP analysis showed that out of these four charged residues, alanine perturbation of Lys1139, produces the highest loss of binding mutation, hence making it one of the most important contact sites (**Figure 4**). In addition, Val1122 of C5d also makes a strong a hydrogen bond with

Glu2188 in the CCP1 domain of C6, with a 68% frequency of contact from the total trajectory (**Figure 2**). Thus, from the nine residues of C5d that are involved in contacts with C6, Lys1139, and Val1122 play the most important role by participating in salt bridges and a hydrogen bond.

We also performed comparative binding/conservation analysis for C3d and C4d. There are 19 conserved C4d residues with C3d that are also found in the binding interfaces of C3d with FH and CR2 (union of red and blue horizontal boxes in **Figure 5**). The majority of the conserved C4d residues (17 out of 19) overlap with the FH contact sites of C3d (red-only horizontal boxes in **Figure 5**), with one of them overlapping with FH and CR2 contact site and two additional ones overlapping with CR2 only (blue-only horizontal boxes in **Figure 5**). The side chains of the two C4d/C3d conserved residues that overlap with CR2 contact sites are negatively charged. On the other hand, 11 of the 17 C4d/C3d conserved residues that overlap with FH contact sites have hydrophobic side chains, including the residue that overlaps with both FH and CR2 contact sites.

We also performed a comparative analysis of the electrostatic properties of C3d, C4d, and C5d. Earlier works have studied the properties of a highly negatively charged concave surface on C3d that is involved in recognition and binding of complement regulators [29] and receptors [28, 41] as well as bacterial inhibitors [30, 31]. **Figure 6A** shows the C3d concave surface with the negatively charged patch, surrounded by a neutral rim. C4d also has a negatively charged patch but spans mostly the cavity's periphery (**Figure 6C**). C5d on the other hand, contains a slightly shallower but more extended cavity, with a sparsely negatively charged patch on its center and distinct positively charged patches at the edges of the surface (**Figure 6E**). The size and charge density of the concave cavity in C3d may be the determining factor for binding to CR2, a property that is not shared by C4d and C5d. We also examined the electrostatic properties of the internal thioester bond face (**Figures 6B,D,F**), focusing on the small region that is responsible for covalently tagging pathogen surfaces after hydrolysis of the thioester bond. Both, C3d and C4d, contain positively charged patches, but the patch on C3d is more extended (**Figures 6B,D**). Unlike C3d and C4d, the positively charged patch of the thioester bond moiety is lost in C5d, which has slightly negatively charged character. In addition, this region forms a crevice, which is absent in C3d and C4d, to assist packing of three hydrophobic residues of Phe2172, Ile2174, and Met2175 (part of C6's FSIM sequence motif), as stated in [33]. C6 contacts C5d through an elongated linker that contains the predominantly hydrophobic FSIM sequence motif at one end, and a polar/negatively charged DDEE sequence motif, proposed here, toward the other end, before it loops out and returns to form additional contacts with C5d. The occupancy of intermolecular contacts of FSIM residues throughout the MD trajectory is as follows (>20% for 5 Å between heavy atoms): Phe2172-Gln898, 85.7%; Phe2172-Glu899, 62.6%; Phe2172-Asn902, 40.9%; Phe2172-Phe938, 39.2%; Ser2173-Gln898, 36.3%; Ile2174-Ala894, 25.0%; Ile2174-Gln898, 77.6%. The DDEE sequence motif comprises residues Asp2185, Asp2186, Glu2187, and Glu2188. Asp2186 and Glu2187 participate in the high-occupancy bifurcated salt bridge

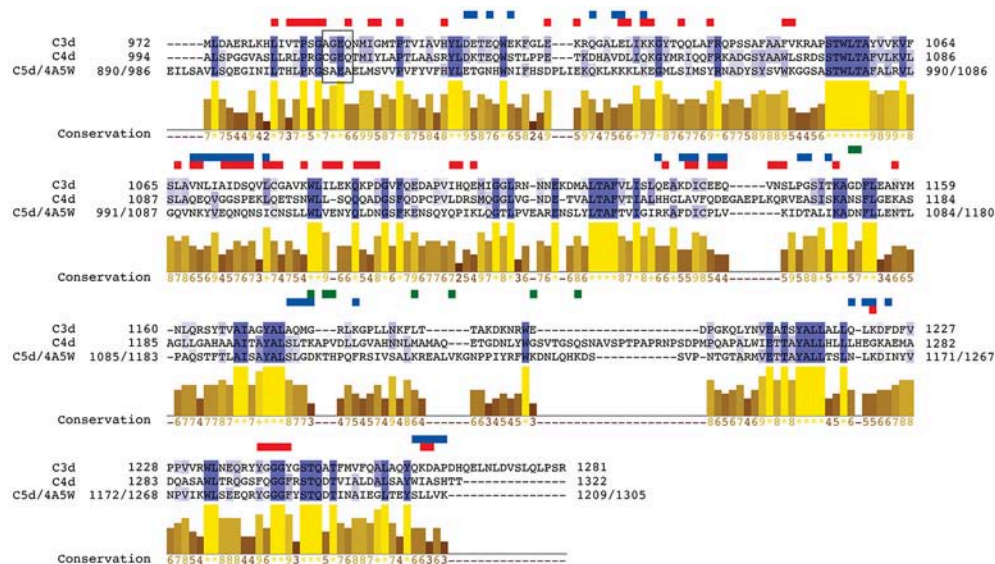


FIGURE 5 | Sequence alignment of C3d, C4d, and C5d. Regions of intermolecular polar contacts between C3d and FH, C3d and CR2, and C5d and C6 are designated with horizontal rectangles above the sequences, colored in red, blue, and green respectively. Polar contacts: ionic interactions (salt bridges and long-range Coulombic) and hydrogen bonds (including side chain and backbone donor/acceptor groups). The vertical blocks in dark blue color denote identity in all three sequences, and those in light blue color denote identity in two out of three sequences. The bar plots denote similarity in conservation across all three sequences with numbers in yellow indicating the level of conservation where a larger number corresponds to higher conservation ("*" and "+" correspond to scores of 11 and 10 respectively). Residue numbering corresponds to the numbering of the crystal structures used for C3d and C4d. For C5d the residue numbering used in our study is shown, followed by the numbering of the crystal structure (PDB code 4A5W, see Methods). The thioester moiety for all three sequences is represented within a black box. Note that the cysteine that participates in the thioester bond is mutated to alanine, as was the case in the crystal structure used (PDB code 3OED).

with C5b's Lys1139; Glu2187 also participates in bifurcated salt bridge with C5b's Lys1133 and Lys 1139; and Glu2188 participates in hydrogen bonding with C5b's Val1122, which is the highest occupancy hydrogen bond of the complex (Figure 2). Both, FSIM and DDEE side chains interact with part of Site I that is depicted by the ellipse of Figure 6F.

Pathway Modeling of Complement System Terminal Cascade

Formation of the MAC pore is propagated through the cleavage of complement C5 that forms C5b and C5a. The smaller fragment, C5a, is an anaphylatoxin that mediates inflammation through activation of immune cells. The larger fragment, C5b, interacts with C6 to form C5b6. The C5b6 complex interacts with C7 and C8 to form the C5b78 complex. Subsequently, multiple C9 molecules combine with C5b78 to form the transmembrane pore C5b6789₁₈ (MAC). We have recently developed quantitative models to study the dynamics of the alternative [24] and the combined alternative and classical pathways [25] of the complement system. These models are based on describing the biochemical reactions of the complement pathways using ordinary differential equations and are parameterized using experimental kinetic data. We used our latest model that encompasses the alternative and classical pathways to gain a systems level understanding of the terminal cascade of the complement system, starting at fluid phase C5 and ending in MAC pore formation on cell membranes [25]. Figure 7 shows the concentration-time profiles of C5, C5b, C6, C5b6, and

MAC pores. As C5 is consumed (Figure 7A), MAC is produced (Figure 7B). C5 is minorly consumed to form C5b (Figure 7C), and so does C6 (Figure 7D) when it combines with C5b to form the C5b6 complex (Figure 7E). C5b and C5b6 show similar time responses, but with different concentration magnitudes. The time response of MAC pore formation shows a short lag phase, followed by an accelerated production phase. Unlike complement products C5b and C5b6 that are mostly consumed in ~40 min, the profile of MAC pores shows a continuous production after the 90th min mark.

Our results present the prompt activation and propagation kinetics of the complement system. In spite of the terminal pathway taking root later in the complement cascade (after the cleavage of C3), our results show MACs can be generated in less than 10 min. Furthermore, our results show despite the presence of complement regulators acting on the terminal cascade and numerous biochemical steps from C5 to C5b, C5b6, C5b67, C5b678, C5b6789, and the polymerization of C9 to form MACs (C5b6789₁₋₁₈), the rates of C5 or C6 consumption may be good predictors for the rate of MAC formation (Figure 7). This is highlighted where an accelerated rate of consumption for C5/C6 are present within the first 30 min, and this rate subsequently is reduced after the 30th min. Similarly, but inversely related to the concentration-time profiles of C5/C6, the MAC production exhibits an accelerated phase within the first 30 min that also subsequently diminishes after 30 min. However, it should be pointed out that the rate of production in MACs is still higher compared to the rate of C5/C6 consumption after the 30th min.

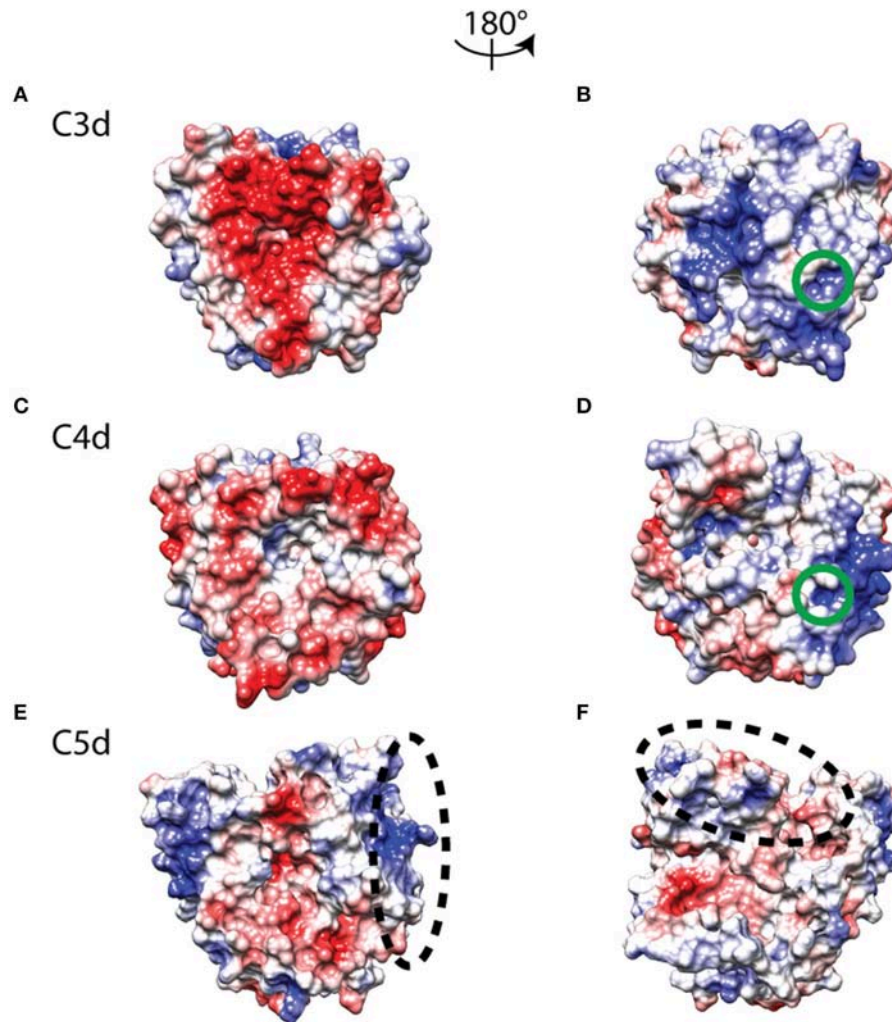


FIGURE 6 | Electrostatic potentials mapped onto molecular surface representations of C3d (**A,B**), C4d (**C,D**), and C5d (**E,F**), showing the CR2/FH binding face on left and the thioester face on right. The color transitions from red to white to blue represent electrostatic potential values of -3 to 0 to $+3$ kBT/e. The green circles mark the internal thioester bond region of C3d (**B**) and C4d (**D**), and the two black dashed ellipses indicate distinct C6 binding regions in Site I of C5d (**E,F**).

In summary, kinetics of C5 or C6 consumption may be sufficient to study the kinetics of MAC formation despite the presence of numerous biochemical steps from C5/C6 to MACs.

DISCUSSION

Activation and propagation of the complement system through the alternative, classical, and lectin pathways leads to the cleavage of C5 to form C5b and C5a. Presence of C5b sets the stage for recruitment of complement protein C6 through C9 to form the MAC pore. Pathogens such as *Neisseria* species (causing meningitis) are susceptible to MAC-induced killing, and deficiency in the terminal proteins leads to recurrent meningitis [45]. On the other hand, dysregulation of the complement system leads to excessive propagation of the terminal step that plays a critical role in diseases such as AMD, aHUS, and PNH [15–20]. In addition to diseases, sublytic MAC of

the terminal cascade can induce multiple signaling pathways associated with proliferation, apoptosis, and protein synthesis [13, 46]. Overall, formation of C5b6 initiates a cascade of reactions that affects numerous events from pathogen killing, to complement-mediated diseases, and signaling pathways. Here, we performed biophysical analysis on C5b6 complex to identify the key components that are involved in the mechanism of C5b and C6 binding.

We initiated our study to quantify the physicochemical origins of C5b6 binding, with focus on electrostatic interactions, guided by the large number of charged patches on the surfaces of C5b and C6 and in their binding interface. Our analysis is based on the crystal structure and MD trajectory of the C5b6 complex. MM-PBSA calculations throughout the MD trajectory showed an overall unfavorable electrostatic energy component in the free energy of binding, which is dominated by favorable van der Waals interactions. However, analysis of the binding

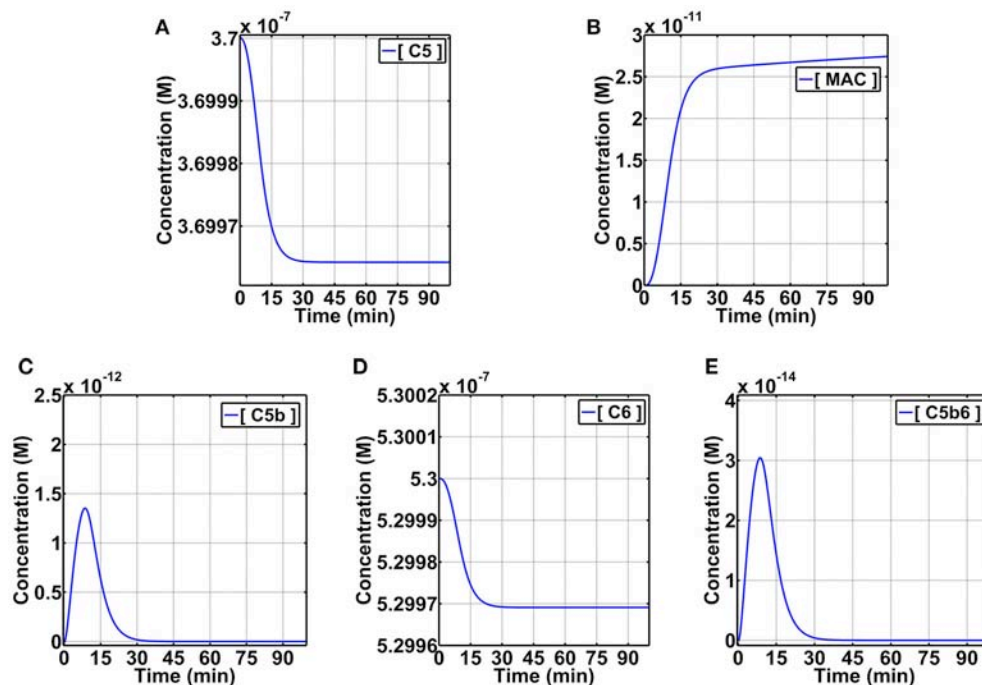


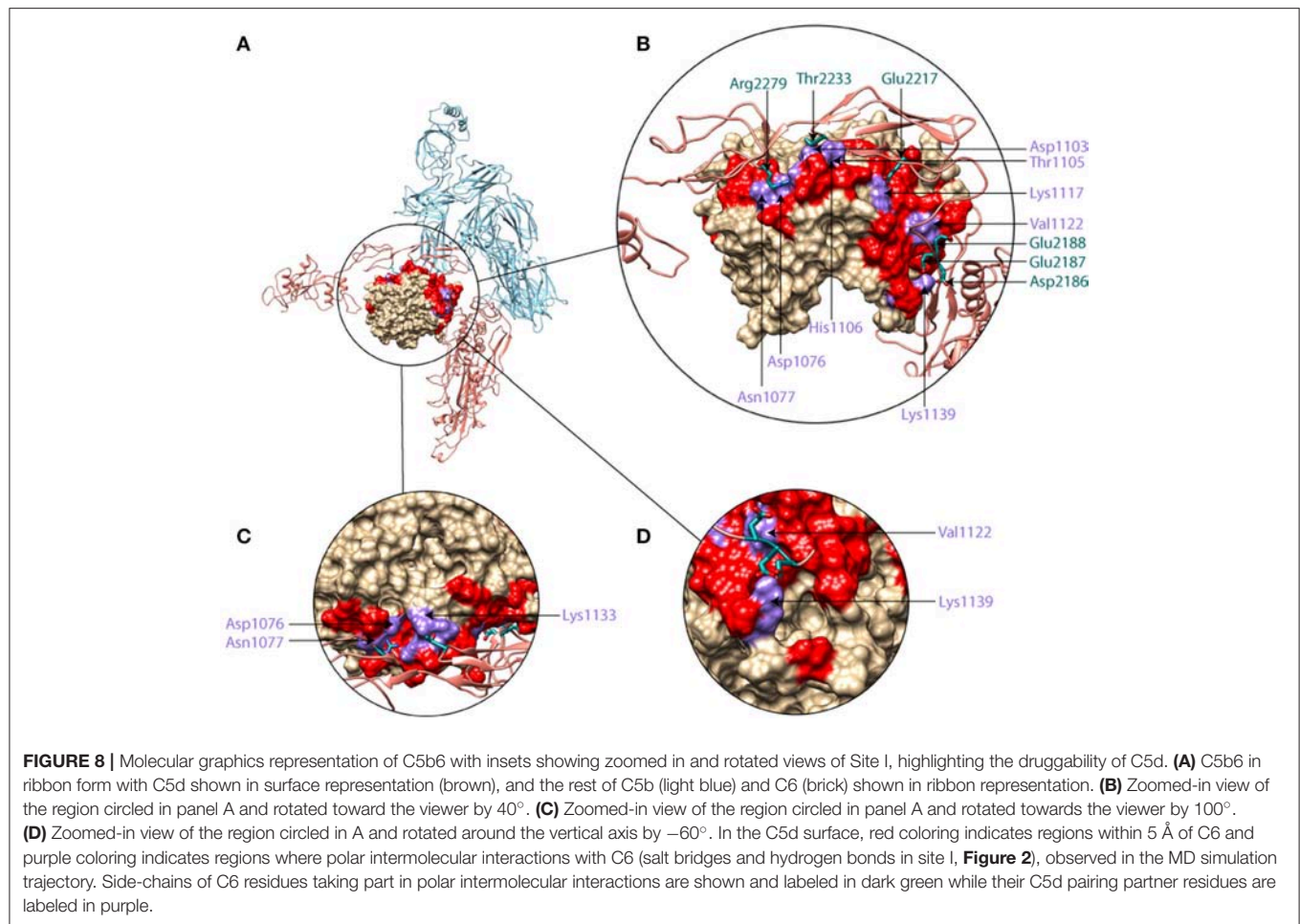
FIGURE 7 | Dynamic pathway modeling of the complement terminal cascade. Rates of consumption of C5 (A) and production of MAC (B) are shown. Intermediate steps of production and consumption of C5b (C), consumption of C6 (D), and production and consumption of C5b6 (E) are also shown. C5, C5b, C6, and C5b6 are in fluid phase, whereas MAC is cell membrane bound.

interface throughout the MD trajectory revealed the presence of 13 intermolecular salt bridges and 11 intermolecular hydrogen bonds, which is a large number of electrostatic interactions for a complex of approximately $3200 \text{ \AA}^2$ buried solvent accessible surface area (Supplementary Figure 2). Additionally, AESOP analysis using six representative structures from MD conformational clusters, identified several charged residues with strong contributions to the electrostatic free energy of binding. Therefore, we conclude that the stability of the C5b6 complex is dominated by van der Waals interactions and by the presence of several distinct pairwise electrostatic interactions in the form of hydrogen bonds and salt bridges. Disrupting the electrostatic interactions of residues identified herein as being important for structural stability of the C5b6 complex, may be a good strategy for future drug discovery aiming to inhibit MAC assembly.

The complement system contains two thioester domains, C3d and C4d, and one thioester-like domain, C5d, which function as initiators and propagators of the cascade of reactions that leads to the elimination of pathogens or apoptotic/damaged cells [1]. C3d and C4d function as parts of C3b and C4b, respectively, and unlike C5d both C3b and C4b can covalently attach to host/pathogen cell surfaces and tag the cellular species for inflammatory response and opsonophagocytosis. C3d and C4d also exist as standalone proteins, after degradation cleavage of C3b by complement regulators, and remain covalently linked to the surface of cells long after complement activation. On the other hand, the thioester-like domain of C5d cannot covalently

attach to surfaces of pathogens/apoptotic cells; however, C5d makes extensive contacts with C6 to form the complex C5b6 [32, 33]. The assembly of C5b6 presents the junction between the different phases of complement activation that initiates the formation of MAC. But unlike C5d (and also C4d), complement fragment C3d has numerous binding partners ranging from complement components to pathogenic proteins [41, 47–49]. For instance, C3d can mediate adhesion by interacting with complement receptors, bind the domain of *Staphylococcus aureus* protein Sbi, and also form the link between innate and adaptive immunity by interacting with CR2 on B-cells [50–54]. Although all three thioester domains are structurally similar, they have distinct electrostatic potential projections on their surfaces (Figure 6). We identified C3d has 87 of its 310 residues that are involved in binding to complement proteins FH and CR2. Furthermore, 19 of the 87 C3d residues are conserved on both C4d and C5d (Figure 5). These differences indicate that C3d has evolved to have multiple binding partners by having more binding residues present its thioester domain.

A major contributing factor to the multifaceted functionality of C3d, has been associated with its dual electrostatic nature, through two distinct faces [41]. The thioester face is positively charged and functions by tagging cells. The concave negatively charged face functions for recruiting (or as an aid to) adaptive immunity and inducing immune response. These charged regions contain functional sites that serve to accelerate protein association and stabilize protein complexes [55, 56].



Furthermore, our results also show complement C4d contains a negatively charged ring surrounding the concave face, rather than entirely covering it, and differences in the distribution of positive charge in its thioester face (**Figure 6**). C5d also shows electrostatic differences on both the concave face and the thioester-like face compared to C3d and C4d (**Figure 6**). Another difference in C5d is the presence of a positive region that accommodates the C6 domain for the formation of C5b6 complex (**Figure 6**). This C6-binding site of C5d contains a deep groove, which facilitates the binding of conserved C6 linker residues of the $^{2172}\text{FSIM}^{2175}$ sequence motif. In addition, the linker contains the $^{2185}\text{DDEE}^{2188}$ sequence motif of acidic charged residues Asp2186 and Glu2187, which form salt bridges with basic residues of Lys1139 (Asp2186 and Glu2187) and Lys1133 (Glu2187) of C5d, respectively (**Figure 2A**). The perturbation Lys1139Ala most notably showed the strongest destabilizing effect on the interaction of C5b6 in AESOP analysis (**Figure 4**). Similar to C6, complement protein C7 also contains an analogous linker. However, as stated in [33], C5b may discriminate toward C6 because C7 lacks the FSIM motif and has a shorter linker that makes less extensive contacts with C5d. Overall, these results show complement proteins C3d and C4d that perform similar functions by covalently attaching to

different surfaces for propagation of complement cascades, also have similar electrostatic profiles. However, C5d shows distinct charge properties, with lower densities of negative and positive charges in its concave and thioester-like surfaces. Instead, C5d has a positive groove that facilitates C6 binding and instigates the terminal step of complement propagation.

A distinct structural feature of C3 activation product, C3b, is that its C3d domain packs at the bottom of macroglobulin domain MG1. In contrast, the C5d domain of C5b is located 50 Å from the base of MG1 when in complex with C6 [32, 33]. Although C3 and C5 are structurally similar, the distinct locations of their C3d/C5d domains in their active products C3b/C5b highlight not only how their active products are topologically different, but also on how each of them propagates its distinct function on a surface of pathogen/host cells. For instance, C3b uses its C3d domain to covalently attach to nearby pathogens and interact with FB to form C3bB. This complex subsequently is activated by FD to form the C3/C5 convertase, C3bBb [57, 58]. C3bBb then propagates a cascade of reactions on the surface by cleaving C3 to form more C3b and C3a, and to promote pathogen elimination through enhanced opsonophagocytosis. The placement of C3d close to base of MG1, in conjunction with the active thioester moiety and the

positively charged thioester face, makes the covalent attachment of C3b possible. On other hand, C5b does not covalently attach to surface using its C5d domain, but promotes a complementary mechanism of pathogen elimination through the formation of the pore-forming MAC. Recent cryo-electron microscopy and tomography studies of MACs proposed that the role of C5b is to assist priming C6 to initiate the pore formation [9, 12]. Close examination of MAC showed the cholesterol-dependent cytolysin/MAC perforin (CDC/MACPF) domain in C6, C7, C8, and C9 are responsible for pore formation, and this domain is absent in C5b. Furthermore, these studies showed that the structural arrangement of C5b6 is also maintained in the MAC pore assembly. The conformation of C5b6, where C5d remains half-way elevated from the base of the MG1 domain, ensures the absence of steric clashes with complement C9, which is situated below the thioester-like domain of C5b in the MAC assembly. Subsequently, the maximum number of C9s (18 polymerization copies) can fit to form the MAC's cylindrical shape. Furthermore, downstream reactions of C5b6 to form C5b678 also affects the arc-like structure formed by C9 polymerization. Recent cryo-electron microscopy studies show polymerized C9 (poly-C9) in the absence of C5b678, assembles into a closed symmetric ring with 22 C9 components [59, 60]. This contrasts the recent MAC structures where MACs are shown to be asymmetric with a maximum number of 18 C9s [9, 12]. Superposition of poly-C9 ring with that of a physiological MAC pore shows dimensional differences in height and spacing of C9 molecules [61]. These data highlight the presence of C5b678 as one of the key factors in affecting the molecular assembly of C9s to form an asymmetric pore. All in all, formation of the C5b6 complex results in priming C6 for MAC assembly and aiding in the polymerization of C9. These functions are brought together by the stabilization of C5d's position by C6 (in the complex C5b6), and the downstream reactions that form C5b678 complex.

Through the course of our analysis of the structural and physicochemical properties underlying the mechanism of formation and stability of the C5b6 complex, we identified druggable C5d regions with the potential to disrupt C5b6 interface. In **Figure 8**, the C5d intermolecular polar contacts contributing to the stability of the C5b6 complex are emphasized, with surrounding residues that are in close proximity to C6 highlighted as well. The number of polar intermolecular interactions at Site I are restricted to a few narrow regions due to the shape and size of C6. Deriving short peptides from C6 and applying modifications to improve the interfacial SASA and specific intermolecular interactions could result in stronger binders to C5d in search of inhibitory ligands against C6 binding. We also observe that several of the polar intermolecular contacts are adjacent to pockets that could be leveraged in the development of drug-like or peptidic inhibitors (**Figures 8C,D**).

In contrast to the potential therapeutic sites on C5d, C5 inhibitors eculizumab and SKY59 interact with domains MG7 and MG1, respectively [62, 63]. Unlike C5d, MG7 and MG1 do not contain deep pockets but have charged residues that are critical for interactions with eculizumab or SKY59. For instance, MG1 domain has three charged residues, Glu48, Asp51, and

Lys109 (within 3.5 Å from SKY59 antigen-binding fragment, Fab) that mediate binding by forming critical salt bridges and numerous hydrogen bonds [62]. Mutating any of these charged residues on C5 to alanine had severe effects on the binding affinity of SKY59 [62]. And hence, the binding interface between C5 and SKY59 is highly mediated by the charged residues positioned in MG1. Similarly, the MG7 domain of C5 (targeted by eculizumab) does not contain deep pockets as observed for the thioester domains of C3d/C4d or that of C5d (TED-like). Similarly the eculizumab epitope on MG7 is also highly charged, containing six charged residues comprised of one glutamic acid (Glu915) and five positively charged residues of four lysine (Lys858, Lys882, Lys887, Lys920) and one arginine (Arg885) [63]. Furthermore, Schatz-Jakobsen et al. showed out of the 66 single-point mutants on Fab residues that interact with C5, three residues in the heavy chain (Trp107, Phe101, Trp33) and one residue in light chain (Ala32) severely impaired hemolysis inhibition when mutated to histidine [63]. Using PDBsum [64] on the binding interface between C5 and the Fab fragment, we observed half of these key mutants (Phe101 and Trp33) make extensive contacts with the positively charged residue Arg885 on the MG7 domain of C5. Interestingly, a small number of PNH patients that are resistant to eculizumab carry a common single nucleotide polymorphism where Arg885 is replaced by histidine [65]. These results show mutations on C5 that affect charged residues in drug sites have severe consequences on the functionality of the complement inhibitors. And hence, accounting for the electrostatic nature of the C5 epitopes may significantly improve binding affinities and subsequently enhance complement inhibition.

Although most of our study is structure/dynamics-based at molecular level, we expanded our efforts to understand the role of C5b and C6 at pathway dynamics level. There is small consumption of C5 and C6 from their initial blood plasma concentrations, and this is reflected in the production of C5b and C5b6, and eventually in MAC production. C5b and C6 show an initial accelerated production phase, followed by a consumption phase as they are converted to MAC. The production of MAC shows a lag phase, corresponding to the production phases of C5b and C5b6, followed by an accelerated production phase, corresponding to the consumption phases of C5b and C6. Despite this level of production, the concentration of MAC pores in 90 min is 27 pM, about four orders of magnitude lower than the initial concentrations of C5 and C6. Given that the calculation has performed with full complement regulation in place, representing homeostasis, such MAC pore concentration is not expected to have any significant effects on host cells. However, in pathogen cells, where negative complement regulation is absent, a larger amount of MAC deposition is observed [24]. Substantial increase in MAC pore formation is also expected in host diseases such as AMD, where there is severe complement dysregulation.

Overall, our molecular dynamics and electrostatics study revealed that the large and multi-site C5b6 interface is stabilized predominantly by van der Waals interactions, but also contains an unusually large number of stabilizing salt bridges and hydrogen bonds. We identified critical salt bridges and hydrogen

bonds for the stability of the C5b6 complex. Furthermore, the C5d domain of C5b contains a sparsely negatively charged patch enclosed with positively charged patches. C5d does not contain a C3d-like cavity which in C3d is an electrostatic hotspot primed for interaction with CR2 for the formation of a link between innate and adaptive immunity. This suggests mono-functionality for C5b for the formation of the MAC assembly. We propose that C5d is a target for drug discovery, by designing inhibitors capable of disrupting the critical salt bridge and hydrogen bonding interactions at the C5d-C6 interface. We also showed the presence of small cavities neighboring the critical electrostatic contacts that can be leveraged in the development of drug-like or peptidic inhibitors. Lastly, we extended our study from molecular level dynamics to pathway dynamics to demonstrate the specifics in consumption-production rates of C5, C5b, C6, C5b6, toward MAC formation. Inhibition of the C5b6 interaction may be an efficient way to block MAC formation for diseases such as PNH, where MAC is responsible for hemolytic activity of red blood cells.

METHODS

Structure Preparations

The three-dimensional cocrystal structure of C5b6 with code 4A5W [32] was obtained from the Protein Data Bank (PDB). C5b is represented as chain A, whereas C6 is represented as chain B. All missing residues of C5b and C6 were added with MODELLER [66]. The crystal structure of C3d with code 3OED [35] was obtained from the PDB. The structures of C4d and C5d were extracted from the crystal structures of C4b and C5b6, with PDB codes 5JTW [36] and 4A5W, respectively. Missing residues for C4d were modeled using SWISS-MODEL [67]. Structural visualization and comparisons were performed using Chimera [68]. Molecular graphics were generated using Chimera. Protonation states of histidine were assigned using PDB2PQR [69]. The following transformations are needed to convert residue numbering used in 4A5W [32] to residue numbering used in our study: for C5b residues 19-765, subtract 18 from the 4A5W residue numbers; for C5b residues 766-1676, subtract 96 from the 4A5W residue numbers; for C6 residues 22-934, add 1559 to the 4A5W residue numbers.

Sequence Alignment and Analysis

Sequences of C3d, C4d, and C5d were extracted from the PDB entries 3OED [70], 5JTW [71], and 4A5W [32], respectively. Sequence alignments of C3d, C4d, and C5d were performed using Clustal Omega [72] and visualized with Jalview [73]. Identification of C3d residues involved in binding with FH and CR2 were acquired from MD simulation analysis performed in previous studies [28, 29]. Identification of C5b6 intermolecular interactions was performed as outlined in the MD trajectory analysis section, below.

Molecular Dynamics

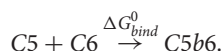
Initial minimization of structure in the absence of water was performed using NAMD and the CHARMM36 force field [74, 75]. Subsequently, the structure was solvated in a cubic TIP3P water box leaving a minimum margin of 12 Å between any protein atom and the cube boundary. Sodium and chloride counterions were added to the system to achieve 150 mM ionic strength and neutralize protein charges. Adding water and counterions increased the total system size to 747,620 atoms. The solvated structure was energy minimized by undergoing 50,000 steps of conjugate gradient energy minimization before heating from 0 to 310 K with all protein atoms harmonically constrained to their positions after minimization. Next, five equilibration steps were performed in which the first five steps for a total time of 7 ns. During the four stages of equilibration, all protein atoms were constrained at a force constant of 10, 5, 2, and 1 kcal/mol/Å, respectively. The final equilibration step was concluded by only constraining backbone atoms with a force constant of 1 kcal/mol/Å. Following equilibration, AMBER16 [76, 77] was used for the production run for 100 ns with the following conditions: periodic boundary conditions, Langevin temperature control, a non-bonded interaction cutoff of 12 Å, with SHAKE algorithm used for constraining hydrogen bonds, and an integration time step of 2 fs.

Molecular Dynamics Trajectory Analysis

Characterization and visualization of intermolecular interactions was performed using CPPTRAJ, pandas, and seaborn [78–80]. Analysis of buried solvent accessible surface area upon binding and visualization were performed with MDTraj [81] and matplotlib [82], respectively over all 2,000 frames in the trajectory. MSMBuilder [83] and MSMExplorer [84] were used for clustering, visualization and extracting representative structures from the trajectory for electrostatic analysis. Salt bridges between C5b and C6 residues was calculated with custom R scripts in conjunction with Bio3D package [85]. A distance cutoff of 5 Å was used. CPPTRAJ was used to analyze hydrogen bonds formed between C5b and C6 over the course of the trajectory. For hydrogen bonds, the default distance cutoff of 3 Å was used between acceptor to donor heavy atom, and an angle cutoff of 135°. To extract representative structures, PCA decomposition was performed on the phi and psi angles observed throughout the trajectory to reduce to four principal components using MSMBuilder. The MiniBatchKMeans method in MSMBuilder was utilized to cluster the four principal components to six distinct clusters and cluster centers were extracted as representative structures.

MM-PBSA calculations were performed using a thermodynamic cycle that decomposes the calculation of the free energy of binding into molecular mechanics (MM) force field calculations in a state of low dielectric coefficient ($\epsilon = 2$) and solvation calculations by transferring the proteins from the low dielectric coefficient environment to a high dielectric environment ($\epsilon = 80$). A frame interval of 4 was chosen for the MM-PBSA calculations, and hence a total of 500 frames from a

total of 2,000 were processed. In our case, the MM calculations include van der Waals and electrostatic free energies, but not covalent geometry energy (bonds, angles, torsions) or entropic effects. We use the one-trajectory approximation, according to which we separate the structures of the components of the complex from the structure of the complex without additional minimization and without performing separate MD simulations of the complex components. Given that we used the one-trajectory approximation, covalent geometry energies and entropic effects are expected to cancel out in the binding scheme



The solvation free energy calculations include electrostatic contribution according to Poisson-Boltzmann electrostatic calculations, and nonpolar contribution (cavity solvation) described by an empirical term based on loss of solvent accessible surface area upon binding [86]. The following equations describe the MM-PBSA calculations.

$$\Delta G_{bind}^0 = G_{C5b6}^0 - (G_{C5b}^0 + G_{C6}^0) \quad (1)$$

Where the Binding Free Energy According to MM Force Field Calculations is Given by

$$\Delta E_{bind,MM}^0 = \Delta E_{MM,vdW}^0 + \Delta E_{MM,electro}^0, \quad (2)$$

and electrostatic contributions of individual components, C5b and C6, and complex, C5b6, to solvation free energy are given by Poisson-Boltzmann (PB) free energy differences

$$\Delta G_{solv,polar}^0 = G_{PB,\epsilon=80}^0 - G_{PB,\epsilon=2}^0. \quad (3)$$

The overall solvation contributions to binding, including polar and nonpolar effects, and are given by

$$\begin{aligned} \Delta \Delta G_{solv}^0 &= \Delta G_{solv,C5b6}^0 - (\Delta G_{solv,C5b}^0 + \Delta G_{solv,C6}^0) \\ &+ \Delta G_{nonpolar}^0, \end{aligned} \quad (4)$$

and the MM-PBSA free energy of binding is given by

$$\Delta G_{bind,solv}^0 = \Delta E_{MM,vdW}^0 + \Delta E_{MM,electro}^0 + \Delta \Delta G_{solv}^0 \quad (5)$$

Electrostatic Analysis

The Alanine scan method in the AESOP (Analysis of Electrostatic Structures Of Proteins) python package [44] was utilized to perform a computational alanine scan on ionizable residues at the C5b6 interface, and to evaluate their electrostatic contributions to binding. Alanine scans were performed for each of the six representative structures of the MD-derived conformational states.

AESOP utilizes the Adaptive Poisson-Boltzmann Solver (APBS) [87] to calculate grid-based electrostatic potentials, which are converted to electrostatic free energies. The program PDB2PQR is used to pre-assign charges and atomic radii for

each atom according to the PARSE force field [88, 89], as well as to convert the PDB format to PQR format used by APBS. The selection of parameters for AESOP calculations has been described before [90].

Electrostatic free energies of binding were calculated according to a thermodynamic cycle that is similar to the one used for the MM-PBSA calculations, described above, except that binding at the reference state is evaluated using Coulomb's equation instead of the molecular mechanics method. The following equations are used, as described previously [44] and in recent applications of AESOP on C3d and its ligands [28–30]:

$$\begin{aligned} \Delta G_{bind,solv}^0 &= \Delta E_{bind,Coulomb}^0 - \Delta G_{solv,C5b6}^0 \\ &- (\Delta G_{solv,C5b}^0 + \Delta G_{solv,C6}^0) \end{aligned} \quad (6)$$

where,

$$\Delta G_{solv}^0 = \Delta G_{electro,\epsilon=80}^0 - \Delta G_{electro,\epsilon=20}^0 \quad (7)$$

Electrostatic free energies of binding for the family of alanine scan mutants are generated as deviations from the electrostatic free energy of binding of the parent protein, according to:

$$\Delta G_{bind}^0 = \Delta G_{bind,solv,mutant}^0 - \Delta G_{bind,solv,parent}^0. \quad (8)$$

APBS is used to calculate electrostatic potentials for the solvation steps, and the program COULOMB (part the APBS suite) is used to calculate binding at the reference state. For the solvated state, dielectric coefficients of 78.54 and 20 were used for solvent and protein interior, respectively, while for the reference state a dielectric coefficient of 20 was used to resemble that of the protein interior [90]. The ionic strength of the solvated state corresponded to monovalent counterions of 150 mM concentration (physiological ionic strength), whereas the reference state had zero ionic strength. For the electrostatic analysis of the representative structures of C5b6 the number of grid points and mesh dimensions were set to $321 \times 257 \times 257$ and $282 \text{ \AA} \times 245 \text{ \AA} \times 239 \text{ \AA}$, respectively. For the electrostatic analysis of C3d, C4d, and C5d, the number of grid points and mesh dimensions were set to $84 \times 98 \times 96$ and $129 \text{ \AA} \times 129 \text{ \AA} \times 97 \text{ \AA}$, respectively.

Pathway Dynamics of the Terminal Cascade

The dynamics of the terminal cascade of complement system activation were modeled using a previously developed mathematical model that describes the biochemical reactions of the alternative and classical pathways [25]. The output of the model is reaction rates in the form of concentration-time profiles for all complement system proteins, enzymatic cleavage fragments, and association complexes. The model consists of a system of 290 ordinary differential equations (ODEs) and 142 kinetic parameters. Equations, initial concentrations, and kinetic parameters can be found in

the **Supplementary Information** of [25]. The system of ODEs was solved using the ode15s solver of MATLAB (Mathworks, Natick, MA).

AUTHOR CONTRIBUTIONS

NZ, RM, and DM: designed the study, interpreted the data, and wrote the manuscript; NZ and RM: performed calculations and data analysis; DM: conceived and directed the study. All authors approved the submitted manuscript.

REFERENCES

- Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol.* (2015) 6:262. doi: 10.3389/fimmu.2015.00262
- Schatz-Jakobsen JA, Pedersen DV, Andersen GR. Structural insight into proteolytic activation and regulation of the complement system. *Immunol Rev.* (2016) 274:59–73. doi: 10.1111/imr.12465
- Tseng Y, Poon PH, Zavodszky P, Schumaker VN. Spontaneous activation of serum C1 *in vitro*. Role of C1 inhibitor. *J Immunol.* (1991) 147:1884–90.
- Bianchino AC, Poon PH, Schumaker VN. A mechanism for the spontaneous activation of the first component of complement, C1, and its regulation by C1-inhibitor. *J Immunol.* (1988) 141:3930–6.
- Ziccardi RJ. Spontaneous activation of the first component of human complement (C1) by an intramolecular autocatalytic mechanism. *J Immunol.* (1982) 128:2500–4.
- Ricklin D, Reis ES, Mastellos DC, Gros P, Lambris JD. Complement component C3-the “Swiss Army Knife” of innate immunity and host defense. *Immunol Rev.* (2016) 274:33–58. doi: 10.1111/imr.12500
- Bayly-Jones C, Bubeck D, Dunstone MA. The mystery behind membrane insertion: a review of the complement membrane attack complex. *Philos Trans R Soc Lond B Biol Sci.* (2017) 372:20160221. doi: 10.1098/rstb.2016.0221
- Morgan BP, Boyd C, Bubeck D. Molecular cell biology of complement membrane attack. *Semin Cell Dev Biol.* (2017) 72:124–32. doi: 10.1016/j.semcdb.2017.06.009
- Sharp TH, Koster AJ, Gros P. Heterogeneous MAC initiator and pore structures in a lipid bilayer by phase-plate cryo-electron tomography. *Cell Rep.* (2016) 15:1–8. doi: 10.1016/j.celrep.2016.03.002
- Ramm LE, Whitlow MB, Mayer MM. Size of the transmembrane channels produced by complement proteins C5b-8. *J Immunol.* (1982) 129:1143–6.
- Zalman LS, Müller-Eberhard HJ. Comparison of channels formed by poly C9, C5b-8 and the membrane attack complex of complement. *Mol Immunol.* (1990) 27:533–7. doi: 10.1016/0161-5890(90)90072-8
- Serna M, Giles JL, Morgan BP, Bubeck D. Structural basis of complement membrane attack complex formation. *Nat Commun.* (2016) 7:ncomms10587. doi: 10.1038/ncomms10587
- Morgan BP. The membrane attack complex as an inflammatory trigger. *Immunobiology* (2016) 221:747–51. doi: 10.1016/j.imbio.2015.04.006
- Schmidt CQ, Lambris JD, Ricklin D. Protection of host cells by complement regulators. *Immunol Rev.* (2016) 274:152–71. doi: 10.1111/imr.12475
- Liszewski MK, Java A, Schramm EC, Atkinson JP. Complement dysregulation and disease: insights from contemporary genetics. *Annu Rev Pathol.* (2017) 12:25–52. doi: 10.1146/annurev-pathol-012615-044145
- De Vriese AS, Sethi S, Van Praet J, Nath KA, Fervenza FC. Kidney disease caused by dysregulation of the complement alternative pathway: an etiologic approach. *J Am Soc Nephrol.* (2015) 26:2917–29. doi: 10.1681/ASN.2015020184
- Angioi A, Fervenza FC, Sethi S, Zhang Y, Smith RJ, Murray D, et al. Diagnosis of complement alternative pathway disorders. *Kidney Int.* (2016) 89:278–88. doi: 10.1016/j.kint.2015.12.003
- Clark SJ, Bishop PN. The eye as a complement dysregulation hotspot. *Semin Immunopathol.* (2018) 40:65–74. doi: 10.1007/s00281-017-0649-6
- Ricklin D, Reis ES, Lambris JD. Complement in disease: a defence system turning offensive. *Nat Rev Nephrol.* (2016) 12:383–401. doi: 10.1038/nrneph.2016.70
- Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. *Front Immunol.* (2015) 6:257. doi: 10.3389/fimmu.2015.00257
- Janssen BJC, Huizinga EG, Raaijmakers HCA, Roos A, Daha MR, Nilsson-Ekdahl K, et al. Structures of complement component C3 provide insights into the function and evolution of immunity. *Nature* (2005) 437:505–11. doi: 10.1038/nature04005
- Kidmose RT, Laursen NS, Dobó J, Kjaer TR, Sirotkina S, Yatime L, et al. Structural basis for activation of the complement system by component C4 cleavage. *Proc Natl Acad Sci USA.* (2012) 109:15425–30. doi: 10.1073/pnas.1208031109
- Laursen NS, Andersen KR, Braren I, Spillner E, Sottrup-Jensen L, Andersen GR. Substrate recognition by complement convertases revealed in the C5-cobra venom factor complex. *EMBO J.* (2011) 30:606–16. doi: 10.1038/emboj.2010.341
- Zewde N, Gorham RD Jr, Dorado A, Morikis D. Quantitative modeling of the alternative pathway of the complement system. *PLOS ONE* (2016) 11:e0152337. doi: 10.1371/journal.pone.0152337
- Zewde N, Morikis D. A computational model for the evaluation of complement system regulation under homeostasis, disease, and drug intervention. *PLOS ONE* (2018) 13:e0198644. doi: 10.1371/journal.pone.0198644
- Wu J, Wu Y-Q, Ricklin D, Janssen BJC, Lambris JD, Gros P. Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat Immunol.* (2009) 10:728–33. doi: 10.1038/ni.1755
- Forneris F, Wu J, Xue X, Ricklin D, Lin Z, Sfyrera G, et al. Regulators of complement activity mediate inhibitory mechanisms through a common C3b-binding mode. *EMBO J.* (2016) 35:1133–49. doi: 10.15252/emboj.201593673
- Mohan RR, Gorham RD, Morikis D. A theoretical view of the C3d:CR2 binding controversy. *Mol Immunol.* (2015) 64:112–22. doi: 10.1016/j.molimm.2014.11.006
- ES Harrison R, Gorham RD, Morikis D. Energetic evaluation of binding modes in the C3d and Factor H (CCP 19–20) complex. *Protein Sci Publ Protein Soc.* (2015) 24:789–802. doi: 10.1002/pro.2650
- Gorham RD, Rodriguez W, Morikis D. Molecular Analysis of the Interaction between Staphylococcal Virulence Factor Sbi-IV and Complement C3d. *Biophys J.* (2014) 106:1164–73. doi: 10.1016/j.bpj.2014.01.033
- Gorham RD, Kieslich CA, Morikis D. Complement Inhibition by *Staphylococcus aureus*: electrostatics of C3d-EfbC and C3d-Ehp association. *Cell Mol Bioeng.* (2012) 5:32–43. doi: 10.1007/s12195-011-0195-6
- Hadders MA, Bubeck D, Roversi P, Hakobyan S, Forneris F, Morgan BP, et al. Assembly and regulation of the membrane attack complex based on structures of C5b6 and sC5b9. *Cell Rep.* (2012) 1:200–7. doi: 10.1016/j.celrep.2012.02.003
- Aleshin AE, DiScipio RG, Stec B, Liddington RC. Crystal structure of C5b-6 suggests structural basis for priming assembly of the membrane attack complex. *J Biol Chem.* (2012) 287:19642–52. doi: 10.1074/jbc.M112.361121
- Ojha H, Panwar HS, Gorham RD, Morikis D, Sahu A. Viral regulators of complement activation: structure, function and evolution. *Mol Immunol.* (2014) 61:89–99. doi: 10.1016/j.molimm.2014.06.004

FUNDING

This work was partially supported by NIH grant R01 EY027440.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphy.2018.00130/full#supplementary-material>

35. Kieslich CA, Tamamis P, Gorham RD Jr, de Victoria AL, Sausman NU, Morikis GA et al. Exploring protein-protein and protein-ligand interactions in the immune system using molecular dynamics and continuum electrostatics. *Curr Phys Chem.* (2012) 2:324–43. doi: 10.2174/1877946811202040324
36. Gorham RD, Kieslich CA, Morikis D. Electrostatic clustering and free energy calculations provide a foundation for protein design and optimization. *Ann Biomed Eng.* (2011) 39:1252–63. doi: 10.1007/s10439-010-0226-9
37. Kieslich CA, Vazquez H, Goodman GN, López de Victoria A, Morikis D. The effect of electrostatics on factor H function and related pathologies. *J Mol Graph Model.* (2011) 29:1047–55. doi: 10.1016/j.jmgm.2011.04.010
38. Pyaram K, Kieslich CA, Yadav VN, Morikis D, Sahu A. Influence of electrostatics on the complement regulatory functions of Kaposic, the complement inhibitor of Kaposi's sarcoma-associated herpesvirus. *J Immunol.* (2010) 184:1956–67.
39. Zhang L, Morikis D. Immunophysical properties and prediction of activities for vaccinia virus complement control protein and smallpox inhibitor of complement enzymes using molecular dynamics and electrostatics. *Biophys J.* (2006) 90:3106–19. doi: 10.1529/biophysj.105.068130
40. Sfyrera G, Katragadda M, Morikis D, Isaacs SN, Lambris JD. Electrostatic modeling predicts the activities of orthopoxvirus complement control proteins. *J Immunol.* (2005) 174:2143–51. doi: 10.4049/jimmunol.174.4.2143
41. Kieslich CA, Morikis D. The two sides of complement C3d: evolution of electrostatics in a link between innate and adaptive immunity. *PLOS Comput Biol.* (2012) 8:e1002840. doi: 10.1371/journal.pcbi.1002840
42. Mohan RR, Huber GA, Morikis D. Electrostatic steering accelerates C3d:CR2 association. *J Phys Chem.* (2016) 120:8416–23. doi: 10.1021/acs.jpcc.6b02095
43. Wade RC, Gabdoulline RR, Lüdemann SK, Lounnas V. Electrostatic steering and ionic tethering in enzyme-ligand binding: insights from simulations. *Proc Natl Acad Sci USA.* (1998) 95:5942–9. doi: 10.1073/pnas.95.11.5942
44. Harrison RES, Mohan RR, Gorham RD, Kieslich CA, Morikis D. AESOP: a python library for investigating electrostatics in protein interactions. *Biophys J.* (2017) 112:1761–6. doi: 10.1016/j.bpj.2017.04.005
45. Lewis LA, Ram S. Meningococcal disease and the complement system. *Virulence* (2014) 5:98–126. doi: 10.4161/viru.26515
46. Morgan BP, Dankert JR, Esser AF. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *J Immunol.* (1987) 138:246–53.
47. Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. *Nat Rev Microbiol.* (2008) 6:132–42. doi: 10.1038/nrmicro1824
48. Serruto D, Rappuoli R, Scarselli M, Gros P, van Strijp JAG. Molecular mechanisms of complement evasion: learning from staphylococci and meningococci. *Nat Rev Microbiol.* (2010) 8:393–9. doi: 10.1038/nrmicro2366
49. Clark EA, Crennell S, Upadhyay A, Zozulya AV, Mackay JD, Svergun DI, et al. A structural basis for Staphylococcal complement subversion: X-ray structure of the complement-binding domain of *Staphylococcus aureus* protein Sbi in complex with ligand C3d. *Mol Immunol.* (2011) 48:452–62. doi: 10.1016/j.molimm.2010.09.017
50. Shaw CD, Storek MJ, Young KA, Kovacs JM, Thurman JM, Holers VM, et al. Delineation of the complement receptor type 2–C3d complex by site-directed mutagenesis and molecular docking. *J Mol Biol.* (2010) 404:697–710. doi: 10.1016/j.jmb.2010.10.005
51. Kovacs JM, Hannan JP, Eisenmesser EZ, Holers VM. Mapping of the C3d ligand binding site on complement receptor 2 (CR2/CD21) using nuclear magnetic resonance and chemical shift analysis. *J Biol Chem.* (2009) 284:9513–20. doi: 10.1074/jbc.M808404200
52. Li K, Okemefuna AI, Gor J, Hannan JP, Asokan R, Holers VM, et al. Solution structure of the complex formed between human complement C3d and full-length complement receptor type 2. *J Mol Biol.* (2008) 384:137–50. doi: 10.1016/j.jmb.2008.08.084
53. Clemenza L, Isenman DE. Structure-guided identification of C3d residues essential for its binding to complement receptor 2 (CD21). *J Immunol.* (2000) 165:3839–48. doi: 10.4049/jimmunol.165.7.3839
54. Nagar B, Jones RG, Diefenbach RJ, Isenman DE, Rini JM. X-ray crystal structure of C3d: a C3 fragment and ligand for complement receptor 2. *Science* (1998) 280:1277–81. doi: 10.1126/science.280.5367.1277
55. Honig B, Nicholls A. Classical electrostatics in biology and chemistry. *Science* (1995) 268:1144–9. doi: 10.1126/science.7761829
56. Sheinerman FB, Norel R, Honig B. Electrostatic aspects of protein-protein interactions. *Curr Opin Struct Biol.* (2000) 10:153–9. doi: 10.1016/S0959-440X(00)00065-8
57. Pangburn MK, Müller-Eberhard HJ. The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase. *Biochem J.* (1986) 235:723–30. doi: 10.1042/bj2350723
58. Rawal N, Pangburn MK. C5 convertase of the alternative pathway of complement. Kinetic analysis of the free and surface-bound forms of the enzyme. *J Biol Chem.* (1998) 273:16828–35. doi: 10.1074/jbc.273.27.16828
59. Spicer BA, Law RHP, Caradoc-Davies TT, Ekkel SM, Bayly-Jones C, Pang S-S, et al. The first transmembrane region of complement component-9 acts as a brake on its self-assembly. *Nat Commun.* (2018) 9:3266. doi: 10.1038/s41467-018-05717-0
60. Dudkina NV, Spicer BA, Reboul CF, Conroy PJ, Lukyanova N, Elmlund H, et al. Structure of the poly-C9 component of the complement membrane attack complex. *Nat Commun.* (2016) 7:10588. doi: 10.1038/ncomms10588
61. Morgan BP, Walters D, Serna M, Bubeck D. Terminal complexes of the complement system: new structural insights and their relevance to function. *Immunol Rev.* (2016) 274:141–51. doi: 10.1111/imr.12461
62. Fukuzawa T, Sampei Z, Haraya K, Ruike Y, Shida-Kawazoe M, Shimizu Y, et al. Long lasting neutralization of C5 by SKY59, a novel recycling antibody, is a potential therapy for complement-mediated diseases. *Sci Rep.* (2017) 7:1080. doi: 10.1038/s41598-017-01087-7
63. Schatz-Jakobsen JA, Zhang Y, Johnson K, Neill A, Sheridan D, Andersen GR. Structural basis for eculizumab-mediated inhibition of the complement terminal pathway. *J Immunol.* (2016) 197:337–44. doi: 10.4049/jimmunol.1600280
64. Laskowski RA. PDBsum new things. *Nucleic Acids Res.* (2009) 37:D355–9. doi: 10.1093/nar/gkn860
65. Nishimura J, Yamamoto M, Hayashi S, Ohyashiki K, Ando K, Brodsky AL, et al. Genetic variants in C5 and poor response to eculizumab. *N Engl J Med.* (2014) 370:632–9. doi: 10.1056/NEJMoa1311084
66. Webb B, Sali A. Comparative protein structure modeling using MODELLER. *Curr Protoc Bioinformatics* (2016) 54:5.6.1–5.6.37. doi: 10.1002/0471250953.bi0506s15
67. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* (2018) 46:W296–303. doi: 10.1093/nar/gky427
68. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem.* (2004) 25:1605–12. doi: 10.1002/jcc.20084
69. Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* (2004) 32:W665–7. doi: 10.1093/nar/gkh381
70. van den Elsen JMH, Isenman DE. A crystal structure of the complex between human complement receptor 2 and its ligand C3d. *Science* (2011) 332:608–11. doi: 10.1126/science.1201954
71. Croll TI, Andersen GR. Re-evaluation of low-resolution crystal structures via interactive molecular-dynamics flexible fitting (iMDFF): a case study in complement C4. *Acta Crystallogr Sect Struct Biol.* (2016) 72:1006–16. doi: 10.1107/S2059798316012201
72. Sievers F, Higgins DG. Clustal Omega, accurate alignment of very large numbers of sequences. *Methods Mol Biol.* (2014) 1079:105–16. doi: 10.1007/978-1-62703-646-7_6
73. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* (2009) 25:1189–91. doi: 10.1093/bioinformatics/btp033
74. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kalé L, Schulten K. Scalable molecular dynamics with NAMD. *J Comput Chem.* (2005) 26:1781–802. doi: 10.1002/jcc.20289
75. MacKerell AD, Bashford D, Bellott M, Dunbrack RL, Evanseck JD, Field MJ, et al. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J Phys Chem.* (1998) 102:3586–616. doi: 10.1021/jp973084f
76. Case DA, Cheatham TE, Darden T, Gohlke H, Luo R, Merz KM, et al. The Amber biomolecular simulation programs. *J Comput Chem.* (2005) 26:1668–88. doi: 10.1002/jcc.20290

77. Pearlman DA, Case DA, Caldwell JW, Ross WS, Cheatham TE, DeBolt S, et al. AMBER, a package of computer programs for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculations to simulate the structural and energetic properties of molecules. *Comput Phys Commun.* (1995) **91**:1–41. doi: 10.1016/0010-4655(95)00041-D
78. Roe DR, Cheatham TE. PTRAJ and CPPTRAJ: software for processing and analysis of molecular dynamics trajectory data. *J Chem Theory Comput.* (2013) **9**:3084–95. doi: 10.1021/ct400341p
79. McKinney W. Data structures for statistical computing in python. In: van der Walt S, Millman J, editors. *Proceedings of the 9th Python in Science Conference*. Austin, TX (2010). p. 51–6.
80. Waskom M, Botvinnik O, drewokane, Hobson P, David, Halchenko Y, et al. *seaborn: v0.7.1* (June 2016). (2016).
81. McGibbon RT, Beauchamp KA, Harrigan MP, Klein C, Swails JM, Hernández CX, et al. MDTraj: a modern open library for the analysis of molecular dynamics trajectories. *Biophys J.* (2015) **109**:1528–32. doi: 10.1016/j.bpj.2015.08.015
82. Hunter JD. Matplotlib: A 2D graphics environment. *Comput Sci Eng.* (2007) **9**:90–5. doi: 10.1109/MCSE.2007.55
83. Beauchamp KA, Bowman GR, Lane TJ, Maibaum L, Haque IS, Pande VS. MSMBuilder2: modeling conformational dynamics at the picosecond to millisecond scale. *J Chem Theory Comput.* (2011) **7**:3412–9. doi: 10.1021/ct200463m
84. Hernández CX, Harrigan MP, Sultan MM, Pande VS. MSMExplorer: data visualizations for biomolecular dynamics. *J Open Source Softw.* (2017) **2**:1–2. doi: 10.21105/joss.00188
85. Grant BJ, Rodrigues APC, ElSawy KM, McCammon JA, Caves LSD. Bio3d: an R package for the comparative analysis of protein structures. *Bioinformatics* (2006) **22**:2695–6. doi: 10.1093/bioinformatics/btl461
86. Kollman PA, Massova I, Reyes C, Kuhn B, Huo S, Chong L, et al. Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Acc Chem Res.* (2000) **33**:889–7. doi: 10.1021/ar000033j
87. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci USA.* (2001) **98**:10037–41. doi: 10.1073/pnas.181342398
88. Tang CL, Alexov E, Pyle AM, Honig B. Calculation of pKas in RNA: on the structural origins and functional roles of protonated nucleotides. *J Mol Biol.* (2007) **366**:1475–96. doi: 10.1016/j.jmb.2006.12.001
89. Sitkoff D, Sharp KA, Honig B. Accurate calculation of hydration free energies using macroscopic solvent models. *J Phys Chem.* (1994) **98**:1978–88. doi: 10.1021/j100058a043
90. Gorham RD, Kieslich CA, Nichols A, Sausman NU, Foronda M, Morikis D. An evaluation of Poisson-Boltzmann electrostatic free energy calculations through comparison with experimental mutagenesis data. *Biopolymers* (2011) **95**:746–54. doi: 10.1002/bip.21644

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Injectable Biomimetic Hydrogels as Tools for Efficient T Cell Expansion and Delivery

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 30 May 2018

Accepted: 13 November 2018

Published: 28 November 2018

Citation:

Weiden J, Voerman D, Dölen Y,
Das RK, van Duffelen A, Hammink R,
Eggermont LJ, Rowan AE, Tel J and
Figdor CG (2018) Injectable
Biomimetic Hydrogels as Tools for
Efficient T Cell Expansion and Delivery.
Front. Immunol. 9:2798.
doi: 10.3389/fimmu.2018.02798

Biomaterial-based scaffolds are promising tools for controlled immunomodulation. They can be applied as three dimensional (3D) culture systems *in vitro*, whereas *in vivo* they may be used to dictate cellular localization and exert spatiotemporal control over cues presented to the immune system. As such, scaffolds can be exploited to enhance the efficacy of cancer immunotherapies such as adoptive T cell transfer, in which localization and persistence of tumor-specific T cells dictates treatment outcome. Biomimetic polyisocyanopeptide (PIC) hydrogels are polymeric scaffolds with beneficial characteristics as they display reversible thermally-induced gelation at temperatures above 16°C, which allows for their minimally invasive delivery via injection. Moreover, incorporation of azide-terminated monomers introduces functional handles that can be exploited to include immune cell-modulating cues. Here, we explore the potential of synthetic PIC hydrogels to promote the *in vitro* expansion and *in vivo* local delivery of pre-activated T cells. We found that PIC hydrogels support the survival and vigorous expansion of pre-stimulated T cells *in vitro* even at high cell densities, highlighting their potential as 3D culture systems for efficient expansion of T cells for their adoptive transfer. In particular, the reversible thermo-sensitive behavior of the PIC scaffolds favors straightforward recovery of cells. PIC hydrogels that were injected subcutaneously gelled instantly *in vivo*, after which a confined 3D structure was formed that remained localized for at least 4 weeks. Importantly, we noticed no signs of inflammation, indicating that PIC hydrogels are non-immunogenic. Cells co-delivered with PIC polymers were encapsulated within the scaffold *in vivo*. Cells egressed gradually from the PIC gel and migrated into distant organs. This confirms that PIC hydrogels can be used to locally deliver cells within a supportive environment. These results demonstrate that PIC hydrogels are highly promising for both the *in vitro* expansion and *in vivo* delivery of pre-activated T cells. Covalent attachment of biomolecules onto azide-functionalized PIC polymers provides the opportunity to steer the phenotype, survival or functional response of the adoptively transferred cells. As such, PIC hydrogels can be used as valuable tools to improve current adoptive T cell therapy strategies.

Keywords: adoptive T cell transfer, biomaterial-based scaffold, polyisocyanopeptide hydrogel, 3D culture, injectable, T cells

INTRODUCTION

Scaffolds produced from a variety of biomaterials are now widely applied as engineered microenvironments or delivery vehicles in biomedical applications. These biomaterial-based scaffolds can be used as three-dimensional (3D) culture systems *in vitro* to more faithfully recapitulate the complex set of cues that cells receive in the body (1). Alternatively, biomaterial-based scaffolds can be applied *in vivo* as delivery vehicles of bioactive molecules or cells, since they can exert spatiotemporal control over the release of bioactive molecules (2, 3) and dictate cellular localization (4, 5). Precisely these characteristics can be highly valuable for the field of immunoengineering to benefit cancer immunotherapy, as scaffolds can be applied as tools to induce strong and durable anti-cancer immune responses (6, 7).

Biomaterial-based scaffolds are able to overcome several limitations associated with current cancer immunotherapeutic strategies and thereby enhance efficacy and reduce treatment-related toxicity. For instance, scaffolds have been used for efficient cancer vaccination by recruiting dendritic cells (DCs) toward a depot of tumor antigens and adjuvants in the context of a local 3D environment in the body, which obviates the need for time-intensive *ex vivo* DC culturing protocols (8–10). Alternatively, toxicity associated with systemic immune checkpoint blockade can be reduced by the local and sustained release of anti-programmed death ligand 1 (PD-L1) and chemotherapy from scaffolds (11). By acting as molecular and cellular delivery vehicles with high spatiotemporal resolution, biomaterial-based scaffolds can have a clear additive value to current cancer immunotherapeutic strategies.

The ability to control the 3D environment and direct cellular localization can be especially beneficial to enhance the efficacy of cellular cancer immunotherapies such as adoptive T cell transfer (ACT). Adoptive transfer of T lymphocytes is aimed at eliminating tumor cells by infusing cancer patients with high numbers of autologous tumor-reactive tumor infiltrating lymphocytes (TILs). This potent strategy exploits the natural capacity of cytotoxic T cells to recognize and kill cancerous cells, and encouraging results have been reported for various solid cancer types (12–15). However, systemic injection of expanded tumor-reactive T cells results in insufficient localization of infused lymphocytes to the tumor site and a lack of *in vivo* persistence (16, 17), even though high cell quantities (typically 10^{10} cells) are administered. Moreover, for many cancer patients it is not feasible to generate these large amounts of TILs, which is one of the factors that hampers widespread application of ACT across different solid cancers types (18). Lymphodepleting conditioning of the host and co-infusion of high dose bolus IL-2 are applied to enhance the accumulation and survival of adoptively transferred cells (19), but both cause significant wide-spread toxicity (18). Thus, poor T cell persistence and functionality hamper the clinical efficacy of ACT for solid tumors (20–24), particularly since the degree of persistence of the administered lymphocytes is associated with outcome (25, 26). There is a great medical need to develop more efficient and rapid approaches for the *ex vivo* expansion of TILs and to improve the delivery and persistence of T lymphocytes. These hurdles

can be overcome by making use of biomaterial-based scaffolds as efficient 3D culture systems and by dictating cellular localization by exploiting scaffolds as cellular delivery vehicles.

In this study, we explore the potential of an injectable scaffold to harbor and support the expansion of pre-activated T cells *ex vivo* and we studied the feasibility of injecting these gels *in vivo* for localized T cell delivery. We present a scaffold that consists of a polymeric hydrogel that is based on fully-synthetic tri-ethylene glycol-substituted polyisocyanopeptides (PIC). Hydrogels generally provide excellent biocompatibility due to their high water-content which facilitates rapid diffusion of nutrients and chemical cues. The PIC hydrogels are composed of a bundled network of synthetic PIC polymers (27), which have the advantage that they are well-defined and have a tunable composition. These polymers allow for incorporation of azide click-handles, which can be used to functionalize the gels with biomolecules such as integrin-binding motifs (28, 29). In particular, a unique feature of PIC hydrogels is their biomimetic strain-stiffening behavior that resembles the mechanical behavior found in natural polymers, where upon strain the stiffness of the material increases (27, 30). The PIC polymers display thermally-induced gelation at temperatures above 16°C, upon which they form into a transparent hydrogel (27, 30). This enables straightforward encapsulation of cells and importantly enables delivery of these gels *in vivo* via needle-mediated injection. The PIC gels can therefore be administered in a minimally invasive manner, which diminishes risks of complications that are associated with implantation of scaffolds.

Here, we demonstrate that PIC hydrogels support the survival and expansion of *ex vivo* cultured T lymphocytes. We furthermore describe the *in vivo* gelation of PIC polymers after injection and study its biocompatibility. Collectively, we provide evidence that PIC hydrogels are highly promising 3D scaffolds for the *in vitro* expansion and *in vivo* delivery of pre-activated T cells which puts PIC hydrogels forward as valuable tools to improve current adoptive T cell therapy strategies.

MATERIALS AND METHODS

Preparation of Polyisocyanopeptide Polymers and Conjugation of GRGDS Peptide

Non-functionalized and azide-functionalized tri-ethylene glycol-substituted polyisocyanopeptide (PIC) polymers were synthesized as previously described (28, 29). Briefly, non-functionalized isocyano-(D)-alanyl-(L)-alanine monomer and azide-terminated monomer mixed at a molar ratio of 1:30 were dissolved in toluene. After adding nickel percholate ($\text{Ni}(\text{ClO}_4)_2$) as a catalyst (catalyst-to-monomer molar ratio of 1:1,000), the reaction mixture was stirred at room temperature for 72 h. The reaction product was precipitated three times from dichloromethane in di-isopropyl ether. To produce GRGDS-functionalized PIC polymers (RGD-PIC), a solution of DBCO-NHS in DMSO was mixed with GRGDS peptide dissolved in borate buffer (pH 8.4) at 2 mg/mL and stirred for 3 h at room temperature. Mass spectrometry was performed to

confirm the formation of DBCO-GRGDS conjugates. DBCO-GRGDS peptide was conjugated to azide-functionalized PIC polymers via strain-promoted azide-alkyne cycloaddition at a ratio of one DBCO-GRGDS per 100 monomers (28). DBCO-sulfo-Cy5 (Jena Bioscience) at a ratio of one DBCO-sulfo-Cy5 per 5,000 monomers was conjugated to the azide-functionalized PIC polymers in a similar manner to generate fluorescent PIC polymers.

Rheological Analysis and Characterization of PIC Polymers and Hydrogels

RGD-PIC and PIC polymers were dissolved at 3 mg/mL in *X-VIVO*-15 medium (Lonza) supplemented with 2% human serum (HS) by rotation at 4°C for 24–36 h and dissolved polymers were stored at –20°C. Bulk stiffness measurements on gels was done by rheology analysis at 37°C were performed as described (28) using temperature sweep rheology followed by time sweep experiments. Peripheral blood leukocytes or pan T cells were incorporated in the gels at a concentration of 0.5 or 1×10^6 cells/mL at gel concentrations of 0.75 and 1.5 mg/mL. PIC polymers were furthermore characterized by circular dichroism at 0.1 mg/mL in PBS on a Jasco 815 CD spectrophotometer. Atomic force microscopy was performed to confirm appropriate length and molecular weight (28). In all functional experiments rheological analysis was performed to validate proper gel formation and gel bulk stiffness.

Preparation of Collagen Gels

Three-dimensional collagen matrices were prepared at 1.7 mg/mL by mixing 55.5% (v/v) of pepsinized bovine type 1 collagen (3.1 mg/mL, PureCol, Advanced Biomatrix), 3.7% (v/v) 0.75% sodium bicarbonate solution (Life Technologies), 7.4% (v/v) minimum essential Eagle's medium (Sigma-Aldrich) and 33.3% (v/v) *X-VIVO*-15 medium with 2% HS containing cells at a final concentration of at a concentration of 0.5 or 1×10^6 cells/mL. The matrices (final pH 7.4) were polymerized at 37°C for 30–45 min.

Cell Isolation, Cell Culture, and Reagents

Human dendritic cells, pan T cells and natural killer (NK) cells were isolated from buffy coats obtained from healthy volunteers. This study was carried out in accordance with the recommendations of institutional guidelines. All subjects gave written informed consent in accordance with the Declaration of Helsinki. CD1c⁺ mDCs were isolated from peripheral blood mononuclear cells (PBMCs) using the CD1c (BDCA-1) DC isolation kit. Pan T cells and NK were isolated from peripheral blood leukocytes using the Pan T cell isolation kit and the NK cell isolation kit, respectively, according to manufacturer's prescription (all Miltenyi Biotec). Cell phenotype was determined using flowcytometry staining: CD11c (BD Biosciences)/CD1c (Miltenyi Biotec) for CD1c⁺ mDCs (purity >85%), CD3 (eBioscience) for pan T cells (purity >98%), CD69 and CD25 (both BD Pharmingen) for T cell activation, CD56 (BD Biosciences) for NK cells (purity >92%). Human DCs, T cells and NK cells were cultured in *X-VIVO*-15 medium supplemented

with 2% HS. For NK cell culture an additional 100 IU/mL of IL-2 (Proleukin, Novartis) was added.

Embedding Cells Within PIC Gels and Cell Viability

Human pan T cells were activated overnight using plate-immobilized α CD3 monoclonal antibodies (clone OKT3, 1 μ g/mL, BioXcell) and α CD28 monoclonal antibodies (clone 9.3, 5 μ g/mL, BioXcell). Non activated pan T cells, activated pan T cells, immature DCs or NK cells were incorporated within (RGD-) PIC hydrogels by mixing cold (RGD-) PIC polymers with the cells on ice at a final gel concentration of 0.75 or 1.5 mg/mL and a cell concentration of $0.5\text{--}1 \times 10^6$ cells/ml unless indicated otherwise. Instantaneous gelation of the RGD-PIC gel when placed at 37°C ensured embedding of cells throughout the 3D matrix. Pan T cells were labeled with PKH-26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma Aldrich) and imaged on Olympus FV1000 Confocal Laser Scanning Microscope to test distribution in the gel, and a z-stack reconstruction was made using FIJI software. For cell viability experiments, cells were simultaneously encapsulated in collagen scaffolds. After 4, 24, 48, and 72 h, cells were recovered from (RGD-) PIC gels by incubating the gels at 4°C for 15–30 min, addition of ice cold PBS and collecting the cell pellet from the fluid after centrifugation. Cells were retrieved from the collagen scaffolds by enzymatic digestion for 45 min at 37°C using collagenase A (Roche). Cell viability was assessed using Annexin V (BD Pharmingen) and 7AAD staining (eBioscience). Flow cytometric analysis was performed on a FACS Calibur (BD Biosciences) or FACS Verse (BD Biosciences) and all data was analyzed using FlowJo software (Version X 10.0 Tree Star).

Time-Lapse Microscopy and Quantification of Cell Migration

Migration of individual cells (activated T cells or immature DCs) encapsulated within (RGD-) PIC hydrogels or collagen matrices was monitored by digital time-lapse, bright-field inverse microscopy in a humidified environmental chamber (37°C and 5% CO₂). Images were collected for 4 h at 2.5 min time intervals with a digital CCD camera (Nikon Diaphot 300 with Hamamatsu C8484-05G CCD Camera, okolab 2D time lapse software). Migration was quantified by tracking 30 randomly selected cells for 3 h with manual tracking FIJI software, beginning >30 min after the start of imaging and using the xy coordinates of cell paths. Cell velocity per cell was calculated as the length of each cell path divided by time, excluding stop phases. The xy trajectories were converted into the mean square displacement (MSD) as previously reported (31). Cells were classified as motile when they show a MSD of >200 μm^2 in the first 2 h of cell tracking. Chemotaxis and Migration Tool software (version 1.01, Ibbidi) was used to plot migration trajectories.

T Cell Proliferation in 3D Matrices

Human pan T cells were stained with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) to track cell proliferation by flowcytometry. To pre-activate T cells, pan T cells were stimulated overnight with α CD3/ α CD28 Dynabeads (Gibco).

The following day, T cells were harvested and re-plated into two-dimensional (2D) medium, 3D collagen matrices or 3D RGD-PIC hydrogels at varying cell densities as indicated. Alternatively, unstimulated T cells (1×10^6 cells/ml) were mixed with α CD3/ α CD28 Dynabeads (Gibco) or with IL-2 (90–100 IU/mL), PHA (phytohemagglutinin, 1 μ g/mL, Sigma Aldrich) and IL-6 (15 ng/mL, Cell Genix). Subsequently, cells were embedded within the RGD-PIC hydrogels or collagen matrices at varying densities to test *in situ* activation. Cells were recovered from the RGD-PIC gels and collagen gels and proliferation by CFSE dilution and activation were assessed by flow cytometry on a FACS Verse (BD Biosciences). Cell numbers were quantified using a MACSQuant Analyzer 10 (Miltenyi Biotec). Fixable Viability Dye eFluor[®] 780 (eBioscience) was used to exclude dead cells. Intracellular staining for interferon gamma (IFN γ) was done using anti-IFN γ (BD Biosciences) and the BD Cytotfix/Cytoperm Fixation/Permeabilization.

In vivo PIC Gel Stability and Adoptive Transfer of Pre-Stimulated T Cells

Mice were housed at the Central Animal Laboratory (Nijmegen, the Netherlands) where food and water were provided *ad libitum*. This study was carried out in accordance with European legislation. The protocol was approved by the local authorities (CCD, The Hague, the Netherlands) for the care and use of animals with related codes of practice. Animals were randomly allocated to groups. To pre-activate T cells *ex vivo* before adoptive transfer, mouse pan T cells [mouse pan T cell isolation kit (Miltenyi Biotec)] were isolated from the spleens and inguinal lymph nodes of wild-type female C57BL/6J mice (age 5–8 weeks, Charles River) congenic for the CD45.1 marker. T cells were stained with CellTrace[™] CFSE and activated for 16 h with immobilized α CD3 monoclonal antibodies (1 μ g/mL, clone 17A2, Cell Genix) and α CD28 monoclonal antibodies (2 μ g/mL, clone 37.51, Cell Genix). Cell phenotype was determined using flow cytometry staining: CD3 (purity typically >98%), CD69 and CD25 (all Biolegend). Azide-functionalized PIC polymers were dissolved in phenol-red free RPMI medium and were confirmed to be endotoxin free using the LAL test (Lonza). Rheology was performed to confirm adequate gel stiffness. PIC polymers were labeled for 2 h with DCBO-sulfo-Cy5 (Jena Bioscience) and for some experiments with 250 IU/mL IL-2 for 4 h at 4°C. Next, pre-activated CFSE-labeled CD45.1⁺ T cells were mixed with the PIC polymers or with phenol-red free RPMI medium (control), at a final concentration of 1.5×10^6 cells/100 μ L. Rheology confirmed that this number of cells did not significantly affect PIC gel stiffness. When IL-2 was attached onto PIC polymers, 250 IU/mL IL-2 was added to the control as well. The PIC polymers were kept on ice and injected s.c. under anesthesia into the dorsal flank of female C57BL/6J mice congenic for the CD45.2 marker (age 5–8 weeks; Charles River, housed at 28°C) in order to discriminate the adoptively transferred T cells from host T cells by flow cytometry. Fluorescent images were collected with the IVIS Lumina imaging system (Cy5 signal: 640 nm, Cy5.5 filter, CFSE signal: 465 nm, GFP filter, Perkin Elmer) after 2 h, 1, 3, 7, 14, 21, or 28 days to investigate gel localization and cellular

localization. At designated time points, gels were excised from the dorsal flank and the remaining PIC gel, blood, spleen, draining and non-draining lymph nodes (LNs) were collected. Single cells suspension were made from the excised gels, spleen and LNs by digestion with DNase (20 μ g/mL, Roche) /collagenase type III (1 mg/mL, Worthington) and from the PIC gels by cooling and DNase/collagenase treatment. The percentage, proliferation and phenotype of CFSE-labeled CD45.1⁺ T cells were quantified by flow cytometry. All flow cytometric analysis was performed on a FACS Verse. A TNF- α ELISA (eBioscience) was performed on serum collected on day 1.

Immunohistochemistry

On various time points after s.c. injection of Cy5-labeled PIC gels with T cells, PIC gels were resected from the dorsal flank of the mice. The tissues were fixed overnight in 4% PFA at RT. Tissues were embedded in paraffin and 10 μ m FFPE sections were cut. Antigen retrieval was performed using citrate pH 6.0 (Scytek). Slides were stained with primary anti-CD3 (1:300, clone CD3-12, #MCA 12477, Serotec) and secondary rabbit-anti rat HRP (1:100, Jackson ImmunoResearch). Tyramide signal amplification visualization was performed with the Opal seven-color IHC Kit according to protocol (PerkinElmer) containing fluorophores DAPI and Opal 540. Slides were mounted using Fluoromount without DAPI (SouthernBiotech) and scanned using the PerkinElmer Vectra (Vectra 3.0.5; PerkinElmer). Multispectral images were unmixed using spectral libraries using the inForm Advanced Image Analysis software (inForm 2.2.1; PerkinElmer) and analysis was performed by applying an inForm software algorithm (tissue segmentation, cell segmentation, phenotyping tool, and positivity score) based on training with on a selection of 10 representative original multispectral images.

Statistical Analysis

Statistical analyses were performed in GraphPad Prism 5 software using the appropriate testing methods, as indicated in the figure legends. Statistical significance was defined as a two-sided significance level of <0.05. ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

RESULTS

Characterization of Thermoresponsive Polyisocyanopeptide Hydrogels

We produced thermoresponsive hydrogels from tri-ethylene glycol-substituted polyisocyanopeptides (PIC). Methoxy-terminated and azide-terminated isocyanide monomers were co-polymerized with a nickel perchlorate (Ni(ClO₄)₂) catalyst in a molar ratio of 1:30 to create azide-containing PIC polymers (Figure 1A). These fully synthetic PIC polymers have an average of one azide functionality every ~3 nm of the polymer chain (28). In order to construct cell-adhesive polymers that would promote interaction of cellular integrin receptors with the matrix, RGD peptide ligands were grafted onto the azide-functionalized PIC polymers via strain-promoted cycloaddition at a ratio of one DBCO-GRGDS

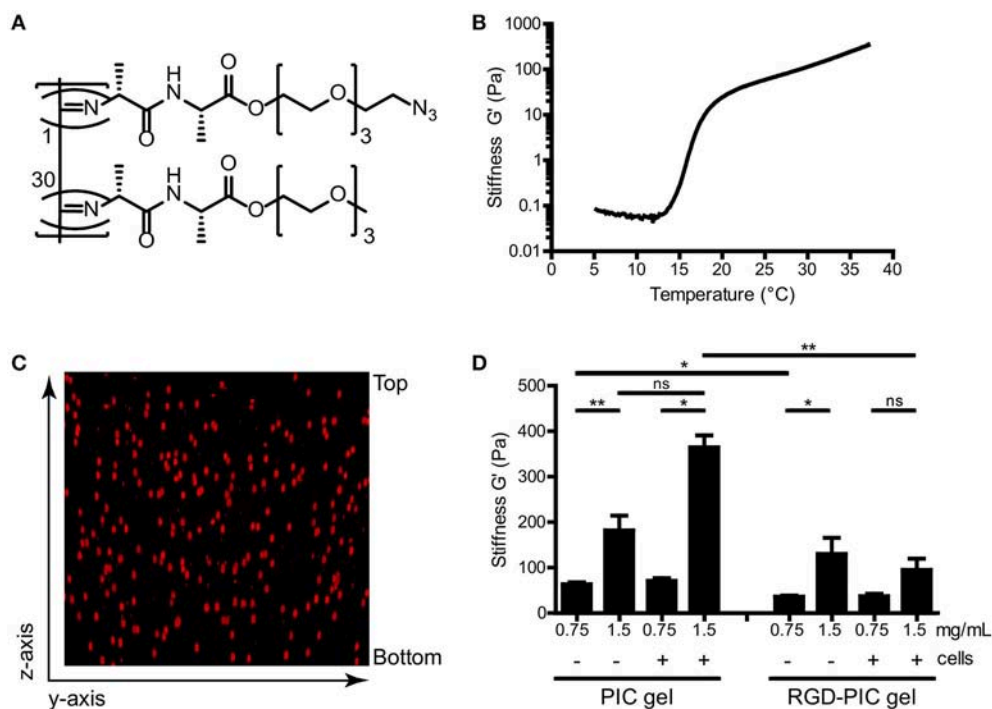


FIGURE 1 | Tri(ethylene glycol)-substituted polyisocyanopeptides (PIC) form soft thermoresponsive gels that can be functionalized via the azide-terminated tail. **(A)** Molecular structure of polyisocyanopeptides substituted with tri(ethylene glycols) and azide functionalization. Azide-functionalized monomer (top) and unfunctionalized monomer (bottom) are co-polymerized in a 1:30 ratio. **(B)** Representative rheological analysis of PIC polymers at 1.5 mg/mL without cells, where G' as a measure of bulk stiffness is set out against temperature. The material gels at the LCST of 16°C. **(C)** Z-stack reconstruction of confocal imaging of PKH-26 labeled T cells within PIC hydrogel at 1.5 mg/mL. **(D)** G' value as a measure of bulk stiffness of PIC hydrogel and RGD-PIC gels, determined with rheological analysis at 37°C. Between 0.5×10^6 and 1×10^6 cells/mL were incorporated into the gels. Values represent mean and standard error of the mean (SEM). Data were analyzed with the Kruskal Wallis test and the Dunns post-test. $n = 3-10$ in at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

per 100 monomers. We analyzed PIC polymers and GRGDS-functionalized PIC polymers (RGD-PIC) by rheology to investigate their bulk stiffness. The polymers demonstrated instantaneous temperature-dependent gelation with an average lower critical solution temperature (LCST) of 16°C (Figure 1B), resulting in the formation of transparent hydrogels at temperatures above the LCST. These thermoresponsive characteristics and gelation temperature are key to warrant straightforward cell encapsulation and injectability of the material.

Next, we mixed primary T cells with cold PIC and RGD-PIC polymer solutions at 4°C and warmed them to 37°C to encapsulate cells during gel formation. As gelation of the PIC polymers occurs virtually instantaneous (within 1 min) upon warming of the polymer solution, cells were encapsulated homogeneously in a genuine 3D fashion within the nanoporous network of bundles (Figure 1C). We performed time sweep rheological analysis to measure bulk stiffness of different batches of PIC gel and RGD-PIC polymers, either empty or after encapsulating cells, to investigate the effect of encapsulating adhesion motifs and cells on the material properties. As anticipated, rheology measurements indicated that PIC and RGD-PIC hydrogels exhibit a concentration-dependent stiffness and form soft gels with a stiffness ranging between 0.03 and

0.4 kPa (Figure 1D). The presence of RGD within the hydrogels reduced the average bulk stiffness of the gels, especially for PIC gels with higher stiffness. The incorporation of cells did not affect stiffness significantly at a density of $0.5-1 \times 10^6$ cells/mL of gel.

Polyisocyanopeptide Hydrogels Are Biocompatible and Sequester T Cells

To establish whether PIC and RGD-PIC hydrogels are biocompatible and can promote the survival of primary T cells, we embedded pre-stimulated primary human T cells within (RGD-) PIC gels and cultured them at 37°C. Due to the thermoreversible behavior of the hydrogels, cells could be easily retrieved from the 3D matrices for phenotypical analysis by incubating the gels at 4°C. Primary T cells remained highly viable within PIC and RGD-PIC hydrogels for at least 3 days at levels comparable to that in medium and collagen type I hydrogels (Figure 2A). The wide applicability of PIC hydrogels is further supported by the notion that primary human dendritic cells (DCs) and primary natural killer (NK) cells are also viable within the PIC hydrogels at a similar level as cells cultured in medium or collagen (Supplementary Figures 1A,B), demonstrating that PIC

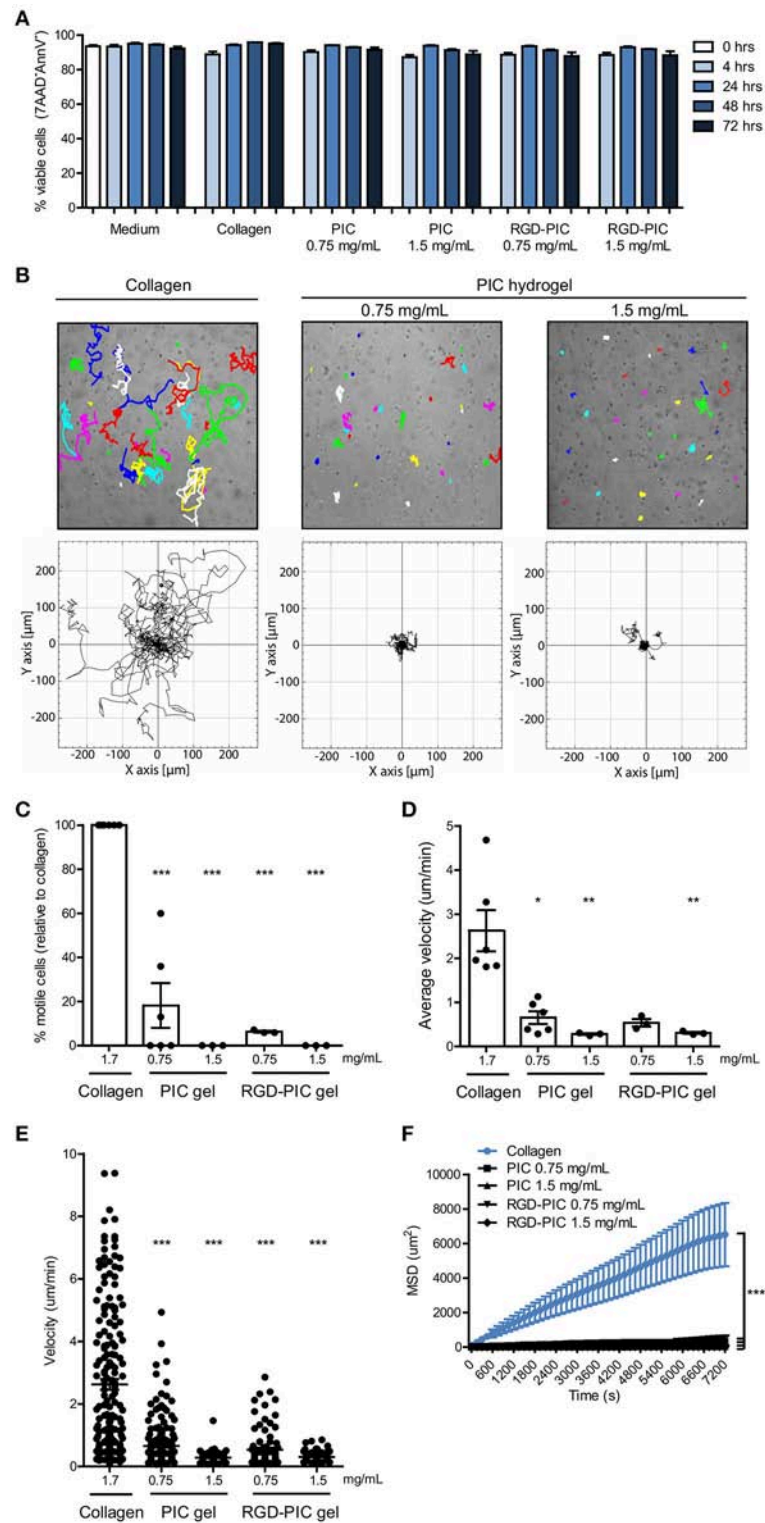


FIGURE 2 | PIC hydrogels support T cell survival and restrict T cell migration within the matrix. **(A)** Viability of α CD3/ α CD28 dynabead-activated T cells in medium, collagen gels, and (RGD-) PIC hydrogels, defined as the percentage of 7AAD⁻Annexin V⁻ cells. $n = 3$ in one experiment. **(B)** Top: Representative images of 3 h cell tracks of individual activated T cells in collagen gels and PIC hydrogels. Each dot and/or track represents one randomly chosen cell out of 30. Bottom: Trajectories of 30 individual activated T cells from one representative experiment. **(C)** The percentage of motile cells relative to the number of motile cells in collagen. Cells are

(Continued)

FIGURE 2 | classified as motile when they show a mean squared displacement (MSD) of $>200 \mu\text{m}^2$ in the first 2 h of cell tracking. **(D,E)** The average velocity depicted for the average of all cells **(D)** or per single cell **(E)** based on tracking of 30 randomly chosen cells for 3 h in collagen gels and (RGD-) PIC hydrogels. **(F)** The mean squared displacement of 30 cell tracks in collagen and (RGD-) PIC hydrogels that were tracked for 2 h. **(A,C–F)** Values represent mean and SEM. **(C–E)** Data were analyzed with the Kruskal Wallis test and the Dunns post-test. **(F)** A two-way ANOVA with a Bonferroni post-test was performed to test for statistical significance. **(C–F)** $n = 6$ for collagen and PIC gel 0.75 mg/mL and $n = 3$ for others in at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

hydrogels support 3D cell culture of a wide variety of primary immune cells.

The 3D matrix that is formed by the bundles of the PIC polymers consist of a tight nanoporous network with an estimated average pore size of 200 nm, which is physically crosslinked by bundling of polymer chains at temperatures above the LCST (27). We aimed to characterize the migratory behavior of immune cells within PIC hydrogels to understand how immune cells behave in this 3D culture system. We tracked non-directional migration of pre-activated T cells in PIC and RGD-PIC hydrogels within 4 h after their encapsulation, and compared it to random migration in collagen type I hydrogels with pore diameters of 2–6 μm as a mimic of a natural 3D matrix that supports immune cell migration (32). Time-lapse microscopy revealed that primary T cells hardly migrate through PIC hydrogels, in contrary to T cells encapsulated in collagen gels (**Figures 2B–F**). The ability to migrate was directly dependent on gel concentration as increasing PIC gel concentration to 1.5 mg/mL completely arrested cell migration, possibly as a result of enhanced gel stiffness (**Figure 1D**). A minor decrease in pore size could contribute to this as well as pore size correlates inversely with polymer concentration (27). Next, we investigated whether functionalizing PIC gels with RGD integrin-binding motifs would promote T cell migration. RGD peptide ligands interact with $\alpha 4\beta 1$ integrins on T cells (33) and are frequently incorporated into synthetic biomaterials in order to promote cellular adhesion and enhance interaction of cells with biomaterials (34). T cells did not display increased migration through gels bearing RGD adhesion-motifs compared to non-functionalized gels, even though RGD-PIC hydrogels generally have a lower bulk stiffness than PIC gels (**Figures 2B–F**). This could suggest that the compact bundled network of the PIC hydrogels is the main determining factor of cellular migration. Alternatively, it could also be a result predominantly integrin-independent amoeboid migratory behavior that T cells utilize (35), which is therefore not enhanced by the presence of integrin-binding motifs. Tracking random migration of immature myeloid DCs demonstrated that they displayed migratory behavior similar to that of T lymphocytes within PIC gels, as they were restricted in their migration through the tight network with small pores (**Supplementary Figures 1C–F**). However, DCs retained higher levels of migration compared to T cells in 0.75 mg/mL PIC hydrogels, probably because of differences in their ability to deform their nucleus as this is one of the determining factors when physical restrictions are imposed on migration (32). The finding that DCs preserve some of their migratory capacity within the PIC matrix implies that the polymer network does not inhibit the intrinsic migratory function of cells but instead poses a physical barrier. These observations together with

the notion that immune cells are viable within (RGD-) PIC gels (**Figure 2A**, **Supplementary Figures 1A,B**) suggest that cell migration is restricted because of physical restraints imposed by the PIC polymer bundles which sequesters cells within the 3D matrix.

Next, we studied whether the absence of immune cell migration in PIC gels affects their inherent capacity to become activated by stimulatory cues. Activation and proliferation of primary T cells embedded in RGD-PIC hydrogels could be induced by introducing T cell-activating $\alpha\text{CD}3/\alpha\text{CD}28$ dynabeads, but only when relatively high numbers of cells and high numbers of beads were mixed together which statistically enhances the chance of a T cell-activating bead and a non-migratory T cell to interact (**Supplementary Figures 2A–C**). This shows that sequestered T cells still have the potential to become activated and expand. Moreover, these T cells can also be activated using soluble T cell activators such as PHA/IL-6/IL-2 (**Supplementary Figure 2D**), further confirming that the inherent capacity of encapsulated cells for activation and proliferation is not affected by the dense RGD-PIC gel matrix.

Collectively, these data show that PIC and RGD-PIC hydrogels are biocompatible matrices that can be used to culture primary T cells and other primary immune cells in a 3D environment. The dense polymer network physically restricts immune cell migration but does not affect T cell ability to become activated or expand.

Primary T Cells Can be Expanded at High Cell Densities Within PIC Hydrogels

In adoptive T cell therapy approaches such as TIL therapy, T cells need to be expanded to high numbers of tumor-reactive T cells as typically 10^{10} cells are administered to patients (36). Therefore, we tested if PIC hydrogels could facilitate and promote the proliferation of pre-activated T cells embedded within the 3D scaffold. We incorporated human pan T cells into PIC hydrogels functionalized with RGD to support integrin-mediated T cell interaction with the matrix. Non-activated T cells cultured with RGD-PIC hydrogels did not proliferate, demonstrating that RGD-PIC scaffolds do not intrinsically induce T cell expansion (**Supplementary Figure 3A**). Next, we activated T cells with $\alpha\text{CD}3/\alpha\text{CD}28$ -coated dynabeads (**Supplementary Figure 3B**) and transferred these pre-activated T cells into 2D medium, collagen gels or RGD-PIC hydrogels at a density of 1×10^6 cells/mL and studied their proliferation. Pre-activated lymphocytes expanded rapidly and extensively in RGD-PIC gels similar to that of T lymphocytes embedded in collagen gels or in 2D medium, with proliferation rates ranging from 60 to 80% (**Figures 3A–C**, low cell density). We hypothesized that the 3D environment provided by the RGD-PIC gels could be beneficial for expansion of T cells at high

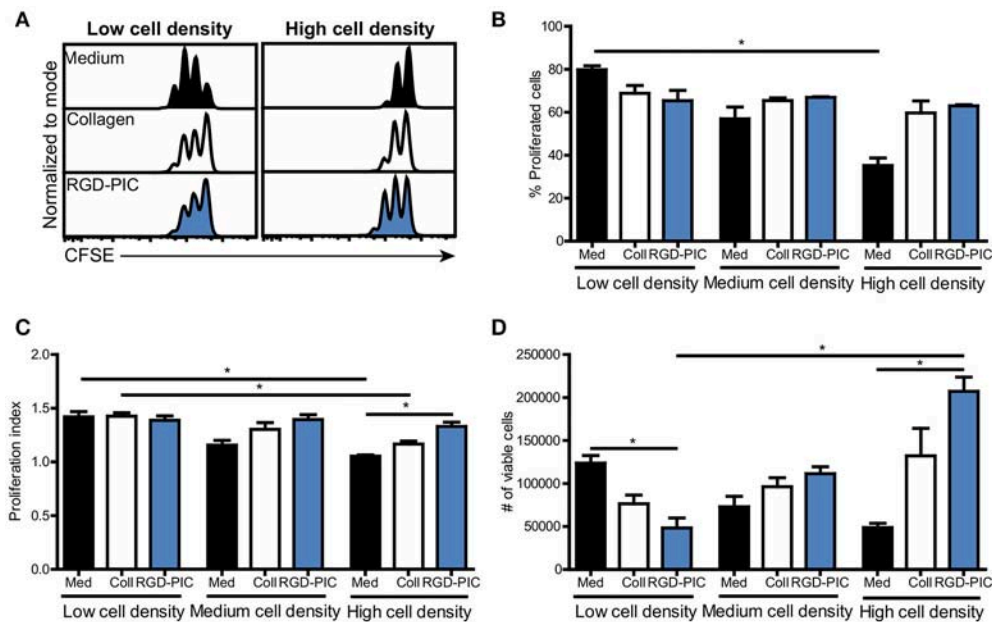


FIGURE 3 | Pre-stimulated T cells display extensive proliferation within RGD-PIC hydrogels, even at high cell densities. **(A)** Representative histograms of CFSE-labeled pre-activated pan T cells cultured in medium, collagen gels or RGD-PIC gels (1.5 mg/mL) for 3 days. Low cell density: 1×10^6 cells/mL, medium cell density: 2.5×10^6 cells/mL, high cell density: 5×10^6 cells/mL. **(B–D)** The percentage of proliferated T cells defined as cells that have undergone at least 1 proliferation cycle **(B)**, the proliferation index defined as the number of cycles of proliferating T cells **(C)** and a quantification of the absolute number of cell retrieved **(D)** 72 h after transferring pre-activated T cells to medium, collagen or RGD-PIC gels at varying cell densities. Low cell density: 1×10^6 cells/mL, medium cell density: 2.5×10^6 cells/mL, high cell density: 5×10^6 cells/mL. **(B–D)** Values represent mean and SEM. Data were analyzed with the Kruskal Wallis test and the Dunns post-test. $n = 2$ or 3 in at least two independent experiments. $*p \leq 0.05$.

quantities and therefore we increased the density of T cells within the gels with 2.5 times (“medium cell density”) and 5 times (“high cell density”). Notably, T lymphocytes propagated extensively in RGD-PIC gels and collagen gels at high cell densities, in contrast to T cells cultured in 2D in medium where cellular crowding inhibited T cell expansion (**Figures 3A–C**). Quantification of the number of T cells demonstrated that significantly more T cells could be retrieved from RGD-PIC gels compared to T cells cultured in 2D medium when they were cultured at high cell densities (**Figure 3D**). On the contrary, at low cell densities most cells were retrieved from 2D medium cultures, even though T cell proliferated at similar speed in collagen and RGD-PIC gels. This indicates that cells are lost during cell recovery, which might be caused by collagenase treatment required to retrieve cells from collagen. For RGD-PIC gels, this is probably the result of a higher viscosity compared to medium, indicating that optimization of the cell retrieval process could further increase the number of T cells that can be obtained. The percentage of IFN γ -producing T cells in RGD-PIC gels was comparable to that of T cells expanded in collagen and medium (**Supplementary Figure 3C**), again suggesting that RGD-PIC gels do not inhibit T cell functions including cytokine production. These data confirm that the RGD-PIC hydrogels are able to support the propagation of T cells and can promote the rapid proliferation of pre-stimulated T cells at high cell densities.

Polyisocyanopeptide Polymers Form Stable Gels *in vivo* and Are Non-immunogenic

After having established that PIC gels support the survival and proliferation of T cells as 3D culture systems *in vitro*, we wanted to explore the feasibility of applying PIC gels *in vivo*. One of the key advantages of the thermoresponsive properties of PIC hydrogels is the opportunity to introduce these into the body in a minimally invasive manner via *in vivo* gelation after needle-mediated injection of a cold PIC polymer solution, as opposed to pre-formed scaffolds that require implantation. We set out to explore the potential of PIC hydrogels for the subcutaneous (s.c.) delivery of immune cells via injection in order to deliver high numbers of cells concentrated in a relatively small volume into the tissue, whilst retaining the effector functions of the expanded T cells (**Figure 3C**, **Supplementary Figure 3C**). The PIC gel could in this way be used to dictate cellular localization and at the same time provide a supportive matrix that promotes cellular proliferation.

To investigate the gelation of cold PIC polymers *in vivo* after subcutaneous injection, we labeled azide-functionalized PIC polymers with DBCO-sulfo-Cy5. We injected 100 μ l of cold 1.5 mg/mL PIC polymers mixed with pan T cells suspended in medium s.c. into the dorsal flank of C57Bl/6J mice. Fluorescent imaging starting 2 h after injection (day 0) revealed that a confined structure was formed (**Figure 4A**),

suggesting that gelation *in vivo* occurs rapidly before polymers get dispersed over the subcutaneous space. The PIC gel remained localized in the dorsal flank for at least 4 weeks and significantly decreased in fluorescence intensity after 4 weeks (**Figures 4A,B**), which could suggest that the gel is degrading over time and washed away from the injection site when gel stability reaches a lower limit. Importantly, mice did not show any signs of distress nor weight loss during the entire 4 weeks after gel administration, indicating that PIC gels are well tolerated. To study whether the PIC gel induces inflammation, we resected the PIC gels and the surrounding tissue to compare the presence of neutrophils and macrophages to that of a similar sized piece of skin close to the injection site of control mice injected with T cells in medium without PIC polymers. PIC gels did not induce any recruitment or influx of inflammatory neutrophils or macrophages toward or into the PIC gels compared to control mice (**Figures 4C,D**), indicating that the scaffolds are non-immunogenic. This was confirmed by the observation that predominantly CD11b⁺CD11c⁻ macrophages and CD11b⁺CD11c⁺ myeloid DCs take up some of the Cy5-labeled PIC polymers (**Figure 4E**) but these Cy5⁺ DCs do not migrate toward the draining lymph nodes of these mice (**Figure 4F**), suggesting that DCs do not receive any activation cues as a result of polymer uptake. Moreover, we did not detect any differences in the serum levels of TNF α 1 day after injection between mice injected with PIC gels vs. mice injected with medium (**Figure 4G**). Thus, s.c. injection of PIC polymers together with T lymphocytes results in the formation of a stable PIC gel without induction of local or systemic immune activation. This suggests that PIC hydrogels are non-immunogenic, biocompatible and can be safely used for *in vivo* immunomodulation.

Pre-activated T Cells Delivered via PIC Gels Maintain Their Function and Are Slowly Released Into the Environment

Next, we investigated whether T cells could be encapsulated within PIC gels after s.c. injection and *in vivo* gelation. We mixed 1.5×10^6 CFSE-labeled pre-stimulated primary mouse T cells per 100 μ l of cold Cy5-labeled PIC polymers. After s.c. injection, we could see a clear colocalization of the Cy5 signal coming from the PIC gel together with the CFSE signal from the T lymphocytes (**Figure 5A**). We resected the PIC gels on various time points after injection and performed immunohistochemistry for CD3 to localize and quantify the number of CD3⁺ T cells within the gel. T cells could be identified as high-density clusters within the polymers of the PIC hydrogel and localized mainly within or in close proximity to the gel (**Figure 5B**), confirming that T cells are encapsulated within PIC gels after *in vivo* gelation. This implies that T cells can tightly interact with the PIC polymers after co-delivery. Multispectral image analysis through different sections at varying heights of the PIC gel revealed that the scaffold contains a relatively consistent number of T cells throughout the entire gel construct (**Figure 5C**). Immunohistochemistry indicated that 1 day after injection there was an average of

more than 4000 CD3⁺ T cells per mm² PIC gel. Over time, the number of T cells within PIC gels gradually diminished (**Figure 5D**).

In order to use PIC gels as cellular delivery vehicles of T lymphocytes for applications such as ACT, it is crucial that pre-activated T cells retain their proliferative capacity and functionality after administration while preserving their ability to move out of the scaffold into the environment. In particular, we were interested in the release kinetics of T lymphocytes co-delivered with PIC polymers, as we observed that PIC gels restrict T cell migration *in vitro*. To this end, we mixed pre-activated mouse T cells (CD45.1⁺) with cold PIC polymer and adoptively transferred these by s.c. injection in the dorsal flank of CD45.2⁺ mice, and compared it to injection of T cells without PIC gel (**Figure 5E**). We studied T cell migration away from the injection site by investigating the presence of CD45.1⁺ cells in the spleen, draining lymph node (dLN), non-draining lymph node (ndLN) and blood at various time points. The relative amount of CD45.1⁺ T cells in the PIC gel determined by flowcytometry decreased starting from day 1 after injection until day 28 (**Figure 5F**), confirming data obtained by CD3⁺ staining of PIC gel sections (**Figure 5D**). A decrease of T cells within PIC gels was accompanied by an accumulation of CD45.1⁺ T cells in the dLN at day 3 after injection (**Figure 5G**). Subsequently, we detected a steady increase of adoptively transferred T cells in the non-draining LN, spleen and blood of the recipient mice up to 4 weeks after injection (**Figure 5H**). Strikingly, the release kinetics for T cells mixed with PIC polymers vs. T cells alone was similar. This suggests that T cells can readily migrate out of the PIC gels even though they are clustered within the scaffold 1 day after injection (**Figure 5B**), and they demonstrated a significantly restricted cellular migration *in vitro* on the short term (**Figure 2**). The release of T cells from PIC gels *in vivo* is likely a result of a decrease in gel stability and finally degradation over time. Thus, PIC gels incorporate T cells after *in vivo* gelation and allow egress of T lymphocytes from the injection site *in vivo*.

Finally, we performed a detailed characterization of the phenotype and function of adoptively transferred CD45.1⁺ T cells alone or in the context of PIC gels. The PIC gels did not affect the proliferative capacity (**Supplementary Figure 4A**) or the effector/memory phenotype balance of transferred T lymphocytes (**Supplementary Figures 4B,C**). Moreover, PD-1 expression on these cells was not changed compared to T cells injected without PIC gel (**Supplementary Figure 4D**). Together, these results suggest that the PIC gel does not negatively impact the quantity, release kinetics or quality of transferred T lymphocytes after s.c. delivery.

DISCUSSION

The use of biomaterial-based scaffolds as 3D culture systems and cellular delivery vehicles is a promising approach to improve the efficacy of immunotherapy for cancer and reduce toxicity. Scaffold properties need to be selected and tested systematically to match these to future applications. Here, we characterize the potential of PIC hydrogels as 3D culture systems for

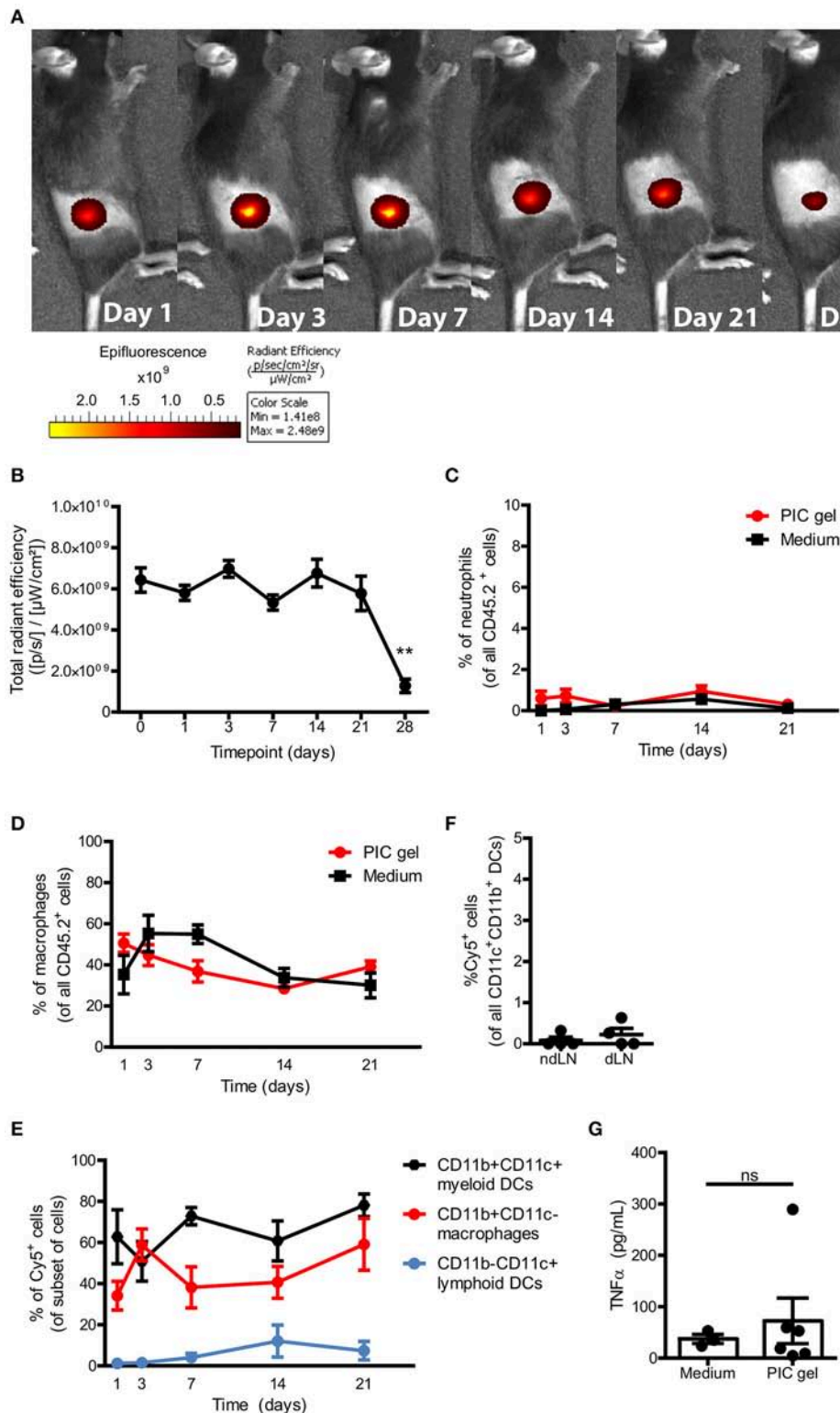


FIGURE 4 | PIC gels form stable gels *in vivo* that do not cause inflammation. **(A,B)** Representative example **(A)** and quantification **(B)** of the radiant efficiency of IVIS imaging of mice s.c. injected with 100 μL Cy5-labeled 1.5 mg/mL PIC polymers mixed with T cells in the dorsal flank. $N = 6\text{--}20$ in three independent experiments. **(C,D)** The percentage of SSC^{high}Ly6G⁺ neutrophils **(D)** and CD11b⁺CD11c⁻ macrophages **(E)** of all alive CD45.2⁺ cells that were surrounding or inside the PIC gel, compared to a similar region of skin in mice injected with medium and T cells. $N = 7\text{--}9$ in at least 3 independent experiments. **(F)** The percentage of Cy5⁺ cells of *(Continued)*

FIGURE 4 | the respective subset surrounding or inside the PIC gel of mice injected with Cy5-labeled PIC gel. $n = 7-9$ in at least 3 independent experiments. **(F)** The percentage of Cy5⁺ cells of all CD11c⁺CD11b⁺ DCs in the non-draining lymph node (ndLN) and draining lymph node (dLN) on day 7. $n = 4$ in 1 independent experiment. **(G)** The level of TNF α in serum 1 day after injecting mice with Cy5-labeled PIC polymers or medium mixed with T cells. **(B–G)** Values represent mean and SEM. **(B)** Data were analyzed with the Kruskal Wallis test and the Dunns post-test. $^{**}p \leq 0.01$ **(C–E)** Data were analyzed with the a two-way ANOVA and Bonferroni post-test. **(G)** Data were analyzed with the Mann Whitney test. ns, not significant.

primary T cells. We report that PIC gels support expansion of pre-stimulated T lymphocytes at high cell densities without affecting cell functionality, in contrast to less physiologically relevant 2D culture systems where crowding may hamper cellular proliferation (37). This demonstrates that PIC hydrogels are attractive 3D scaffolds to propagate pre-stimulated T cells to benefit T cell expansion protocols for ACT, as it can support extensive T cell expansion in a small volume while preserving T cell function. As such, we hypothesize that PIC hydrogels can be used to enhance efficiency of current T cell expansion protocols and that they could reduce the length of the *in vitro* culture period that precedes re-administration of tumor-reactive T cells, which positively affects the quality of the T cell infusion product (38, 39). The thermoreversible behavior of PIC hydrogels facilitates straight forward cell encapsulation into the 3D matrix and importantly, allows for rapid retrieval of cells. This is a great benefit over many other 3D culture systems where typically mechanical or enzymatic disruption is required to retrieve cells, which can affect cell survival, cell surface markers, phenotype or gene expression (40).

The mechanical properties of scaffolds are of high importance for stability during cell culture and may affect cellular behavior. We establish that the dense bundled network of PIC gels restricts primary T cell and DC migration, which is not mitigated by incorporating integrin-binding RGD motifs. This is likely caused by the physical restraints imposed on encapsulated cells by the tight bundled network with 200 nm pores (27). T cell sequestration does not affect their ability to become activated or proliferate, but has implications on how PIC gels can be applied as cells cannot freely interact with immobilized activating cues incorporated into the system. To fully apprehend the potential of PIC gels as 3D cell culture systems for various cell types, the precise relationship between gel stiffness, polymer concentration, the presence of adhesive ligands and migration propensity needs to be established and specified per cell subtype. It is important to take into account how gel stability and stiffness change over time, as prolonged use of PIC gels in 3D cell culture will decrease gel stiffness and permit cellular migration (41).

We exploit the thermosensitive behavior of PIC polymers to trigger gelation into 3D matrixes. This is highly advantageous as gelation occurs under physiological circumstances and does not require any crosslinkers, organic solvents or potentially toxic agents to induce gel formation (42, 43). The tri-ethylene glycol-substituted PIC polymers used in this study have a LCST of 16°C, which ensures stable gels at physiological temperatures of 37°C as gels become more stiff at higher temperatures (27). A consequence of this LCST is that polymer solutions need to be kept cool below 16°C during the handling time and prior to injection. The LCST of these PIC polymers can be tuned

for instance by the addition of salts (44), but this requires careful optimization as changing the LCST will also affect gel stiffness and stability at 37°C. Thermally-induced gelation permits minimally invasive delivery *in vivo* via needle-mediated injection which precludes the need for a surgical procedure for scaffold implantation. Injected cold PIC polymers are well tolerated, form stable gels after *in vivo* gelation and notably do not induce a local or systemic inflammatory response, suggesting that these polymers can be safely used *in vivo*. In particular, we observe no neutrophil or macrophage recruitment toward the gel, which is an important indicator for a lack of scaffold immunogenicity and a crucial factor for biocompatibility (45–48). The mode of delivery and precise scaffold formulation is central in this context, as implantation of RGD-PIC gels within silicon molds has previously been found to induce mild granulocyte recruitment (41). This results from a tissue damage response following implantation together with immune activation due to RGD peptide ligands immobilized onto the polymers (49, 50). Moreover, we observe that local *in vivo* gel fluorescence diminishes after 4 weeks, which implies PIC gel degradation and is favorable with respect to biocompatibility. Degradation is presumably a result of disruption of the non-covalent interactions that hold the PIC polymer bundles together, until the hydrogel is too weak to stay intact and is cleared from the subcutaneous space. Thus, our findings demonstrate that PIC polymers can be safely used *in vivo* and can efficiently be delivered via injection.

By mixing pre-activated T cells with PIC polymers, we could locally deliver high numbers of T cells *in vivo* within PIC gels. T cells move toward distant organs from PIC gels at a speed similar to that of T cells injected without scaffold, even though we observed restricted T cell migration *in vitro*. This can be explained by distinct gelation behavior after *in vitro* vs. *in vivo* gel formation (51, 52), as tissue pressure and fluid drainage may affect polymer gelation *in vivo*. Another factor is probably weakening of PIC gels over time, causing T cells to egress out of the scaffolds (41). T cells circulate systemically and move into distant organs, while preserving their proliferative capacity and functionality. This is critical to ensure that PIC-delivered T cells can migrate toward target sites and execute their (effector) functions. We demonstrate that cells are in close proximity of PIC polymers after injection and are migratory, suggesting that extensive cell-matrix interaction is possible. This provides the opportunity to exploit the azide handles present on PIC polymers using bio-orthogonal click chemistry to co-deliver a wide variety of biomolecules that can steer T cell survival, phenotype or function after administration. Covalent attachment of T cell survival factors or activating cues onto the polymers promotes sustained availability of these

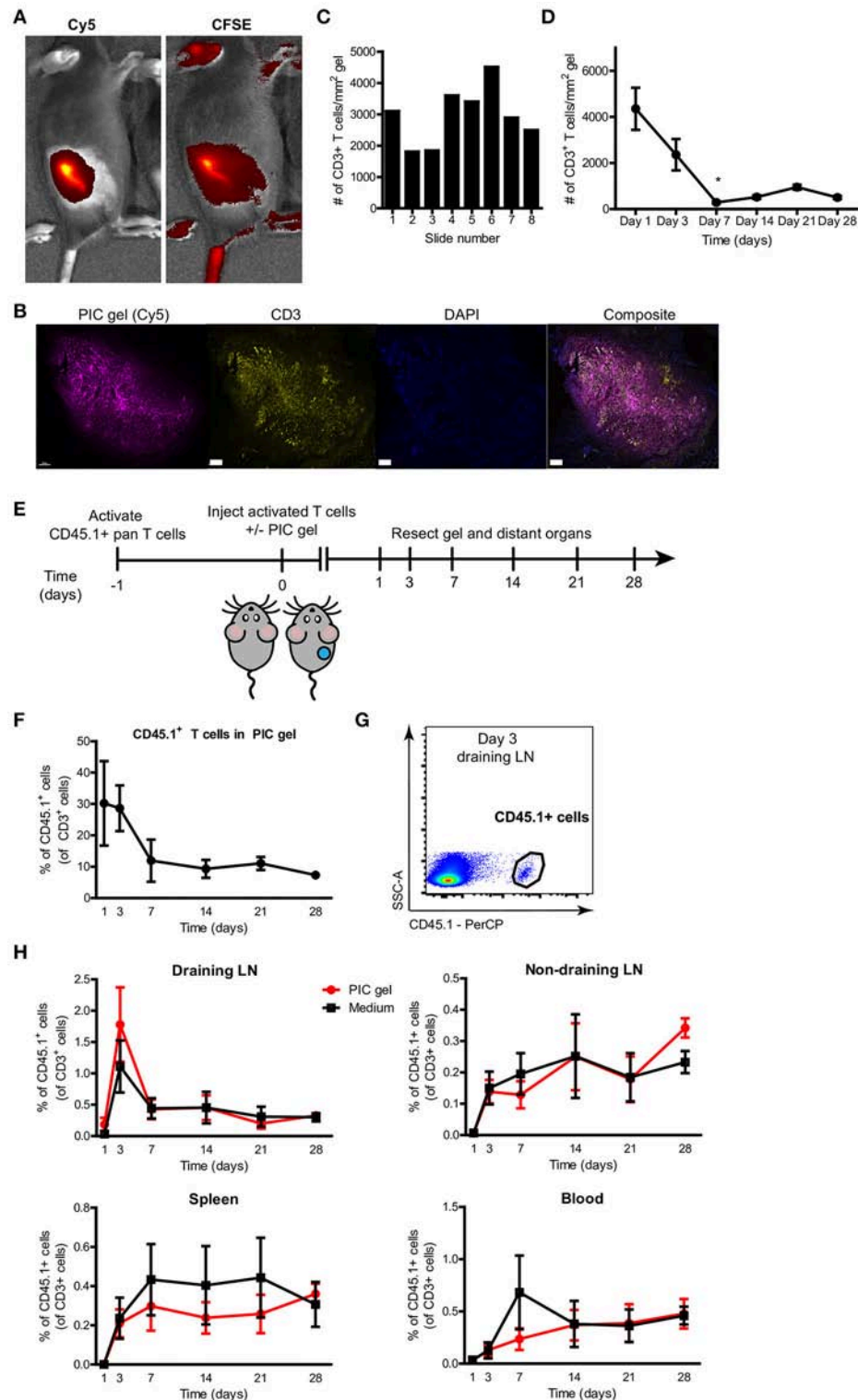


FIGURE 5 | T cells are encapsulated within PIC gels after *in vivo* gelation and are released into the blood and distant organs. **(A)** Representative IVIS fluorescent images of the radiant efficiency of a mouse injected s.c. with 100 µl Cy5-labeled 1.5 mg/mL PIC gel mixed with CFSE-labeled pan T cells in the dorsal flank. **(B)** Representative image of a 10 µm section of FFPE-treated PIC gel in the skin 1 day after injection. Scale bar = 400 µm. **(C)** Representative quantification of the number of CD3+ T cells per mm² PIC gel of 10 µm sections through the PIC gel with typically 20–30 µm in between each section (1 day after injection).

(Continued)

FIGURE 5 | (D) Quantification of the average number of CD3⁺ T cells per mm² PIC gel of 10 μ m sections through the PIC gel ($n = 2$ or 3 per timepoint in 2 independent experiments). Data analyzed with Kruskal Wallis test with Dunn's post test relative to day 1, $*p \leq 0.05$. **(E)** Setup of experiment to study release of T cells from PIC gels after injection. **(F)** Quantification of the percentage of CD45.1⁺ cells of all CD3⁺ T cells retrieved from PIC gels and surrounding skin. $n = 5$ – 7 in at least 3 independent experiments for timepoints day 1–21, $n = 2$ in 1 independent experiment for day 28. Data analyzed with Kruskal Wallis test with Dunn's post test relative to day 1, not significant. **(G)** Representative flow cytometry plot demonstrating CD45.1⁺ cells gated on CD3 expression in the draining lymph node (LN) on day 3 after injection of PIC gel with CD45.1⁺ T cells. **(H)** Quantification of the percentage of CD45.1⁺ cells of all CD3⁺ T cells in the draining lymph node (LN), non-draining lymph node, spleen and blood. $n = 7$ – 9 in at least three independent experiments for timepoints day 1–21, $n = 6$ in one independent experiment for timepoint day 28. **(D,F,H)** Values represent mean and SEM.

factors at a localized area, in contrast to rapid diffusion of biomolecules administered in a soluble fashion. We hypothesize that the introduction of T cell-stimulating cues in the PIC hydrogels may contribute to promoting T cell viability and functionality in order to outperform T cell delivery through bolus injection. This can be exploited by introducing IL-2 or IL-15 agonists into the PIC gels that may locally enhance T cell viability and persistence (18, 53), while circumventing the toxicity associated with high dose bolus IL-2 (24). Alternatively, scaffolds bearing T cell-activating cues such as agonistic CD3 and CD28 antibodies (54) alone or together with α CD137 and IL-15 agonists (55) can be used to promote vigorous *in situ* T cell expansion and improve functionality. Anti-tumor immune responses can be further boosted by presenting stimulator of interferon genes (STING) agonists (56). These strategies are promising to increase efficacy of ACT by locally stimulating and expanding adoptively transferred T lymphocytes in a tunable 3D environment compared to conventional bolus injection of pre-stimulated T cells (55, 56). In addition, this may reduce the need to support T cell engraftment using toxic co-treatments such as lymphodepleting chemotherapy and IL-2 (18).

As we describe that PIC hydrogels are not only suitable for cell culture of primary T cells but also support DC survival to a similar extent as medium, we speculate that PIC gels may be suitable for the localized delivery and local stimulation of DCs for DC-based cancer vaccination approaches. In this setting DC-stimulating cues such as covalently attached TLR ligands and tumor antigens can be grafted onto the polymers to create an immunostimulatory niche (10, 57, 58), although careful consideration with respect to the local persistence of TLR ligands and tumor antigens is pivotal to induce anti-cancer immunity rather than tolerance (59).

Our findings build on previous work that reported that PIC gels are biocompatible and can support the culture of various cell types including mesenchymal stem cells, adipocytes, melanoma

cells, fibroblasts and endothelial cells (28, 41). This highlights the biocompatible nature of PIC hydrogels and puts them forward as versatile scaffolds that are immunologically silent. Their synthetic nature and azide-handles create a flexible and controlled platform that is readily applied for immunomodulation, both *in vitro* and *in vivo*. As such, PIC hydrogels are highly valuable tools as 3D cell culture systems and cellular delivery vehicles, for which the choice and formulation need to be tailored to the desired application.

AUTHOR CONTRIBUTIONS

JW, YD, and LE performed the experiments. DV, RD, and RH prepared and characterized the polymers. AvD provided technical assistance and performed immunohistochemistry experiments together with JW. JW, YD, RH, and LE designed experiments and interpreted the data. AR, JT, and CF supervised the study. JW and CF wrote the manuscript with input from all authors.

FUNDING

We thank M. Ioannidis for technical assistance with ELISA and E.A. van Dinther for technical assistance with *in vivo* experiments. This work was supported by the Institute of Chemical Immunology (grant 024.002.009) and ERC advanced grant PATHFINDER (269019). CF received KWO grant 2009-4402 and the NWO Spinoza award.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02798/full#supplementary-material>

REFERENCES

- Schmeichel KL, Bissell MJ. Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci.* (2003) 116:2377–88. doi: 10.1242/jcs.00503
- Tolouei AE, Dülger N, Ghatte R, Kennedy S. A magnetically responsive biomaterial system for flexibly regulating the duration between pro- and anti-inflammatory cytokine deliveries. *Adv Healthc Mater.* (2018) 7:e1800227. doi: 10.1002/adhm.201800227
- Lee AS, Inayathullah M, Lijkwan MA, Zhao X, Sun W, Park S, et al. Prolonged survival of transplanted stem cells after ischaemic injury via the slow release of pro-survival peptides from a collagen matrix. *Nat Biomed Eng.* (2018) 2:104–13. doi: 10.1038/s41551-018-0191-4
- Roche ET, Hastings CL, Lewin SA, Shvartsman D, Brudno Y, Vasilyev NV, et al. Comparison of biomaterial delivery vehicles for improving acute retention of stem cells in the infarcted heart. *Biomaterials* (2014) 35:6850–8. doi: 10.1016/j.biomaterials.2014.04.114
- Wang P, Liu X, Zhao L, Weir MD, Sun J, Chen W, et al. Bone tissue engineering via human induced pluripotent, umbilical cord and bone marrow mesenchymal stem cells in rat cranium. *Acta Biomater.* (2015) 18:236–48. doi: 10.1016/j.actbio.2015.02.011

6. Weiden J, Tel J, Figdor CG. Synthetic immune niches for cancer immunotherapy. *Nature Reviews Immunology* (2018) 18:212–9. doi: 10.1038/nri.2017.89
7. Cheung AS, Mooney DJ. Engineered materials for cancer immunotherapy. *Nano Today* (2015) 10:511–31. doi: 10.1016/j.nantod.2015.06.007
8. Ali Oa, Huebsch N, Cao L, Dranoff G, Mooney DJ. Infection-mimicking materials to program dendritic cells *in situ*. *Nat Mater.* (2009) 8:151–8. doi: 10.1038/nmat2357
9. Bencherif SA, Warren Sands R, Ali OA, Li WA, Lewin SA, Brashler TM, et al. Injectable cryogel-based whole-cell cancer vaccines. *Nat Commun.* (2015) 6:7556. doi: 10.1038/ncomms8556
10. Li AW, Sobral MC, Badrinath S, Choi Y, Graveline A, Stafford AG, et al. A facile approach to enhance antigen response for personalized cancer vaccination. *Nat Mater.* (2018) 17:528–34. doi: 10.1038/s41563-018-0028-2
11. Wang C, Wang J, Zhang X, Yu S, Wen D, Hu Q, et al. *In situ* formed reactive oxygen species-responsive scaffold with gemcitabine and checkpoint inhibitor for combination therapy. *Sci Transl Med.* (2018) 10:eaa3682. doi: 10.1126/scitranslmed.aan3682
12. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res.* (2011) 17:4550–7. doi: 10.1158/1078-0432.CCR-11-0116
13. Dudley ME, Gross CA, Somerville RP, Hong Y, Schaub NP, Rosati SE, et al. Randomized selection design trial evaluating CD8+ enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. *J Clin Oncol.* (2013) 31:2152–9. doi: 10.1200/JCO.2012.46.6441
14. Stevanović S, Draper LM, Langhan MM, Campbell TE, Kwong ML, Wunderlich JR, et al. Complete regression of metastatic cervical cancer after treatment with human papillomavirus-targeted tumor-infiltrating T cells. *J Clin Oncol.* (2015) 33:1543–50. doi: 10.1200/JCO.2014.58.9093
15. Junker N, Andersen MH, Wenandy L, Dombrowsky SL, Kiss K, Sørensen CH, et al. Bimodal *ex vivo* expansion of T cells from patients with head and neck squamous cell carcinoma: a prerequisite for adoptive cell transfer. *Cytotherapy* (2011) 13:822–34. doi: 10.3109/14653249.2011.563291
16. Griffith KD, Read EJ, Carrasquillo JA, Carter CS, Yang JC, Fisher B, et al. *In vivo* distribution of adoptively transferred indium-111-labeled tumor infiltrating lymphocytes and peripheral blood lymphocytes in patients with metastatic melanoma. *J Natl Cancer Inst.* (1989) 81:1709–17. doi: 10.1093/jnci/81.22.1709
17. Fisher B, Packard BS, Read EJ, Carrasquillo JA, Carter CS, Topalian SL, et al. Tumor localization of adoptively transferred indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. *J Clin Oncol.* (1989) 7:250–61. doi: 10.1200/JCO.1989.7.2.250
18. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev.* (2014) 257:6–71. doi: 10.1111/imr.12132
19. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* (2002) 298:850–4. doi: 10.1126/science.1076514
20. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin. Cancer Res.* (2006) 12:6106–15. doi: 10.1158/1078-0432.CCR-06-1183
21. Lamers CH, Sleijfer S, van Steenberg S, van Elzakker P, van Krimpen B, Groot C, et al. Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. *Mol Ther.* (2013) 21:904–12. doi: 10.1038/mt.2013.17
22. Park JR, Digiusto DL, Slovak M, Wright C, Naranjo A, Wagner J, et al. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol Ther.* (2007) 15:825–33. doi: 10.1038/sj.mt.6300104
23. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: *in vivo* persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci USA.* (2002) 99:16168–73. doi: 10.1073/pnas.242600099
24. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol.* (2005) 23:2346–57. doi: 10.1200/JCO.2005.00.240
25. Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, et al. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol.* (2004) 173:7125–30. doi: 10.4049/jimmunol.173.12.7125
26. Huang J, Khong HT, Dudley ME, El-Gamil M, Li YF, Rosenberg SA, et al. Survival, persistence, and progressive differentiation of adoptively transferred tumor-reactive T cells associated with tumor regression. *J Immunother.* (2005) 28:258–67. doi: 10.1097/01.cji.0000158855.92792.7a
27. Kouwer PH, Koepf M, Le Sage VA, Jaspers M, van Buul AM, Eksteen-Akeroyd ZH, et al. Responsive biomimetic networks from polyisocyanopeptide hydrogels. *Nature* (2013) 493:651–5. doi: 10.1038/nature11839
28. Das RK, Gocheva V, Hammink R, Zouani OF, Rowan AE. Stress-stiffening-mediated stem-cell commitment switch in soft responsive hydrogels. *Nat Mater.* (2016) 15:318–25. doi: 10.1038/nmat4483
29. Mandal S, Eksteen-Akeroyd ZH, Jacobs MJ, Hammink R, Koepf M, Lambeck AJA, et al. Therapeutic nanoworms: towards novel synthetic dendritic cells for immunotherapy. *Chem Sci.* (2013) 4:4168. doi: 10.1039/c3sc51399h
30. Jaspers M, Dennison M, Mabesoone MF, MacKintosh FC, Rowan AE, Kouwer PH. Ultra-responsive soft matter from strain-stiffening hydrogels. *Nat Commun.* (2014) 5:5808. doi: 10.1038/ncomms6808
31. van Rijn A, Paulis L, te Riet J, Vasaturo A, Reinieren-Beeren I, van der Schaaf A, et al. Semaphorin 7A promotes chemokine-driven dendritic cell migration. *J. Immunol.* (2016) 196:459–68. doi: 10.4049/jimmunol.1403096
32. Wolf K, Te Lindert M, Krause M, Alexander S, Te Riet J, Willis AL, et al. Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J Cell Biol.* (2013) 201:1069–84. doi: 10.1083/jcb.201210152
33. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol.* (1996) 12:697–715. doi: 10.1146/annurev.cellbio.12.1.697
34. Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials* (2003) 24:4385–415. doi: 10.1016/S0142-9612(03)00343-0
35. Krummel MF, Friedman RS, Jacobelli J. Modes and mechanisms of T cell motility: roles for confinement and Myosin-IIA. *Curr Opin Cell Biol.* (2014) 30:9–16. doi: 10.1016/j.ceb.2014.05.003
36. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* (2015) 348:62–8. doi: 10.1126/science.aaa4967
37. Duval K, Grover H, Han LH, Mou Y, Pegoraro AF, Fredberg J, et al. Modeling physiological events in 2D vs. 3D cell culture. *Physiology* (2017) 32:266–77. doi: 10.1152/physiol.00036.2016
38. Tran KQ, Zhou J, Durlinger KH, Langhan MM, Shelton TE, Wunderlich JR, et al. Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy. *J Immunother.* (2008) 31:742–51. doi: 10.1097/CJI.0b013e31818403d5
39. Prieto PA, Durlinger KH, Wunderlich JR, Rosenberg SA, Dudley ME. Enrichment of CD8+ cells from melanoma tumor-infiltrating lymphocyte cultures reveals tumor reactivity for use in adoptive cell therapy. *J Immunother.* (2010) 33:547–56. doi: 10.1097/CJI.0b013e3181d367bd
40. Caliri SR, Burdick JA. A practical guide to hydrogels for cell culture. *Nat Methods* (2016) 13:405–14. doi: 10.1038/nmeth.3839
41. Zimoch J, Padial JS, Klar AS, Vallmajo-Martin Q, Meuli M, Biedermann T, et al. Polyisocyanopeptide hydrogels: a novel thermo-responsive hydrogel supporting pre-vascularization and the development of organotypic structures. *Acta Biomater.* (2018) 70:129–39. doi: 10.1016/j.actbio.2018.01.042
42. Koetting MC, Peters JT, Steichen SD, Peppas NA. Stimulus-responsive hydrogels: Theory, modern advances, and applications. *Mater Sci Eng R Rep.* (2015) 93:1–49. doi: 10.1016/j.mser.2015.04.001
43. Ruel-Gariépy E, Leroux J-C. *In situ*-forming hydrogels—review of temperature-sensitive systems. *Eur J Pharm Biopharm.* (2004) 58:409–26. doi: 10.1016/j.ejpb.2004.03.019

44. Jaspers M, Rowan AE, Kouwer PHJ. Tuning Hydrogel Mechanics Using the Hofmeister Effect. *Adv Funct Mater.* (2015) 25:6503–10. doi: 10.1002/adfm.201502241
45. Brown BN, Londono R, Tottey S, Zhang L, Kukla KA, Wolf MT, et al. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. *Acta Biomater.* (2012) 8:978–87. doi: 10.1016/j.actbio.2011.11.031
46. Brown BN, Valentin JE, Stewart-Akers AM, McCabe GP, Badylak SF. Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials* (2009) 30:1482–91. doi: 10.1016/j.biomaterials.2008.11.040
47. Badylak SF, Gilbert TW. Immune response to biologic scaffold materials. *Semin Immunol.* (2008) 20:109–16. doi: 10.1016/j.smim.2007.11.003
48. Williams DF. Biocompatibility pathways: biomaterials-induced sterile inflammation, mechanotransduction, and principles of biocompatibility control. *ACS Biomater Sci Eng.* (2017) 3:2–35. doi: 10.1021/acsbomaterials.6b00607
49. Bartneck M, Skazik C, Paul NE, Salber J, Klee D, Zwadlo-Klarwasser G. The RGD coupling strategy determines the inflammatory response of human primary macrophages *in vitro* and angiogenesis *in vivo*. *Macromol Biosci.* (2014) 14:411–8. doi: 10.1002/mabi.201300362
50. Yano A, Onozuka A, Matin K, Imai S, Hanada N, Nisizawa T. RGD motif enhances immunogenicity and adjuvanicity of peptide antigens following intranasal immunization. *Vaccine* (2003) 22:237–43. doi: 10.1016/S0264-410X(03)00561-9
51. Yu L, Ding J. Injectable hydrogels as unique biomedical materials. *Chem Soc Rev.* (2008) 37:1473–81. doi: 10.1039/b713009k
52. Guvendiren M, Lu HD, Burdick JA. Shear-thinning hydrogels for biomedical applications. *Soft Matter* (2012) 8:260–72. doi: 10.1039/C1SM06513K
53. Bailey CP, Budak-Alpdogan T, Sauter CT, Panis MM, Buyukgoz C, Jeng EK, et al. New interleukin-15 superagonist (IL-15SA) significantly enhances graft-versus-tumor activity. *Oncotarget* (2017) 8:44366–78. doi: 10.18632/oncotarget.17875
54. Cheung AS, Zhang DKY, Koshy ST, Mooney DJ. Scaffolds that mimic antigen-presenting cells enable *ex vivo* expansion of primary T cells. *Nat Biotechnol.* (2018) 36:160–9. doi: 10.1038/nbt.4047
55. Stephan SB, Taber AM, Jileeva I, Pegues EP, Sentman CL, Stephan MT. Biopolymer implants enhance the efficacy of adoptive T-cell therapy. *Nat Biotechnol.* (2015) 33:97–101. doi: 10.1038/nbt.3104
56. Smith TT, Moffett HF, Stephan SB, Opel CF, Dumigan AG, Jiang X, et al. Biopolymers codelivering engineered T cells and STING agonists can eliminate heterogeneous tumors. *J Clin Invest.* (2017) 127:2176–91. doi: 10.1172/JCI87624
57. Hori Y, Winans AM, Huang CC, Horrigan EM, Irvine DJ. Injectable dendritic cell-carrying alginate gels for immunization and immunotherapy. *Biomaterials* (2008) 29:3671–82. doi: 10.1016/j.biomaterials.2008.05.033
58. Verma V, Kim Y, Lee MC, Lee JT, Cho S, Park IK, et al. Activated dendritic cells delivered in tissue compatible biomatrices induce *in-situ* anti-tumor CTL responses leading to tumor regression. *Oncotarget* (2016) 7:39894–906. doi: 10.18632/oncotarget.9529
59. Hailemichael Y, Dai Z, Jaffarzad N, Ye Y, Medina MA, Huang XF, et al. Persistent antigen at vaccination sites induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. *Nat Med.* (2013) 19:465–72. doi: 10.1038/nm.3105

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPEN ACCESS

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 30 September 2018

Accepted: 20 November 2018

Published: 04 December 2018

TCR and CD28 Concomitant Stimulation Elicits a Distinctive Calcium Response in Naive T Cells

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T cell activation is initiated upon ligand engagement of the T cell receptor (TCR) and costimulatory receptors. The CD28 molecule acts as a major costimulatory receptor in promoting full activation of naive T cells. However, despite extensive studies, why naive T cell activation requires concurrent stimulation of both the TCR and costimulatory receptors remains poorly understood. Here, we explore this issue by analyzing calcium response as a key early signaling event to elicit T cell activation. Experiments using mouse naive CD4⁺ T cells showed that engagement of the TCR or CD28 with the respective cognate ligand was able to trigger a rise in fluctuating calcium mobilization levels, as shown by the frequency and average response magnitude of the reacting cells compared with basal levels occurred in unstimulated cells. The engagement of both TCR and CD28 enabled a further increase of these two metrics. However, such increases did not sufficiently explain the importance of the CD28 pathways to the functionally relevant calcium responses in T cell activation. Through the autocorrelation analysis of calcium time series data, we found that combined but not separate TCR and CD28 stimulation significantly prolonged the average decay time (τ) of the calcium signal amplitudes determined with the autocorrelation function, compared with its value in unstimulated cells. This increase of decay time (τ) uniquely characterizes the fluctuating calcium response triggered by concurrent stimulation of TCR and CD28, as it could not be achieved with either stronger TCR stimuli or by co-engaging both TCR and LFA-1, and likely represents an important feature of competent early signaling to provoke efficient T cell activation. Our work has thus provided new insights into the interplay between the TCR and CD28 early signaling pathways critical to trigger naive T cell activation.

Keywords: T cell activation, naive T cells, co-stimulation, CD28, TCR–T cell receptor, calcium signaling

INTRODUCTION

T cell activation involves two types of signal transmitted by surface receptors upon engagement with their respective ligands, which are present on antigen-presenting cells (APCs) (1). The first signal is induced via the T cell receptor (TCR) upon binding to antigenic peptide–major histocompatibility complex (pMHC); the second signal is induced via costimulatory receptors, the prototype of which is CD28, binding to either B7-1 (also known as CD80) or B7-2 (also known as CD86). CD28 is the only B7 receptor constitutively expressed on naive T cells. Costimulation through CD28 is critically required for naive T cells to achieve clonal expansion, cytokine production and for protection against apoptosis and anergy (2, 3). However, despite many years of study, the contributions from and relationship between the TCR and CD28-mediated pathways remains to be elucidated. In particular, a long-debated question in the field is: does CD28 mainly work to increase the activation of TCR-induced signaling pathways, or does it allow triggering of some unique intracellular events that are absent when only the TCR is stimulated? In this context, it has been shown that the CD28-mediated signaling—that is, signal 2—can decrease the threshold of sensitivity of TCR signaling—that is, signal 1—upon binding to pMHC, but this seems due to the modulation of TCR signaling events downstream of receptor-proximal steps (4). Moreover, CD28 stimulation in general has no effect on the “down-regulation” of TCRs upon binding to antigen (5), a surrogate marker for TCR engagement (6). In addition, engagement of CD28 at surface of T cells appears not modifying formation of the immunological synapse (IS) (7), but could impact TCR *in situ* location at T cell/APC contacts (8–11). Nevertheless, increase of signal-1-pathway activation by signal 2 does not easily explain how the presence of signal 2 prevents naive T cells from the anergy that occurs following activation of signal 1 alone. It is therefore considered that CD28 contributes both quantitatively and qualitatively to the signaling pathways driving T cell activation (2). On the other hand, recent studies conducted in antigen-experienced T cells suggested that TCR engagement can facilitate CD28–B7 interactions (12, 13) and consequently favors the costimulatory signal initiation. Sanchez-Lockhart et al. (12, 13) found that TCR stimulation, in previously activated T cells, could enhance the avidity of CD28–B7 binding via a mechanism involving a possible rotation of the ligand binding interface of the extracellular domain of CD28 homodimer. In the context of the regulation of CD28 ligand avidity, Bromley et al. (7) showed that the interactions between CD28 on naive T cells and B7 molecules on APCs are extremely weak. It was also proposed that TCR triggering produces a microenvironment at the immunological synapse that favors the interactions of potent secondary signaling molecules, such as CD28.

In many of the previous studies, the CD28-mediated (and to some degree also the TCR-mediated) signaling pathways were investigated in T cell lines or antigen-experienced T cells, but this was rarely performed in naive T cells. However, it is not clear to what extends the information obtained from these studies can apply to the activation of naive T cells. Here, we investigated the interactions between TCR- and CD28-mediated

early signaling pathways upon engagement with their respective ligands and evaluated their contribution to T cell activation in mouse naive CD4⁺ T cells. By analyzing the autocorrelation function of the signal, we showed for the first time that concurrent TCR and CD28 stimulation, but not the individual stimuli, significantly prolonged the average decay time (τ) of calcium signal amplitudes, as compared with its value found in unstimulated cells. This unique costimulatory function likely contributes to TCR- and CD28-mediated signaling responses leading to efficient T cell activation. Thus, we showed calcium fluxes as a potentially important step through which TCR and CD28 early signaling pathways interact and cooperate each other for the effective initiation of naive T cell activation.

MATERIALS AND METHODS

Mice and Ethics Statement

This study has been approved by the following Animal Care and Use Committee: Departmental Direction of Veterinary Services of Bouches du Rhône (Direction Départementale des Services Vétérinaires des Bouches du Rhône), and the approval number is F13-055-10. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals the French Ministry of Agriculture and of the European Union. Mice were housed under specific pathogen-free conditions and handled in accordance with French and European directives. Animals were housed in the CIML animal facilities accredited by the French Ministry of Agriculture to perform experiments on alive mice. All efforts were made to minimize animal suffering. 3A9 TCR transgenic (TCRtg) mice (14) maintained on a CBA/J background were bred onto a CBA/J x C3H/HeN before used in the experiments (15). C3H/HeN mice were purchased from Janvier Labs SAS (Le Genest St Isle, France). Sex and age of mice were varying according to the experiment performed.

Reagents and Antibodies

4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo [3,4-d] pyrimidine (PP2) was purchased from Calbiochem. The anti-CD3 ϵ mAb (clone145-2C11) and the CTLA4-Fc fusion protein were supplied by Becton Dickinson. The antibodies used for FACS analysis were as follows: anti-CD28-PE (37.51) and anti-CD80-PE (16-10A1) from eBioscience, anti-CD80-APC (16-10A1), anti-CD54-APC (YN1/1.7.4), anti-CD4-Pacific Blue (GK1.5), anti-CD25-APC (PC61) and anti-CD62L-Pecy7 (MEL-14) from BioLegend, anti-CD44-PE (IM7), anti-I-A^k-PE (11-5.2) and anti-CD69-FITC (H1 2F3), anti-CD11a-PE (Clone 2D7) from BD Biosciences.

Transfection of B7-1 and ICAM-1

pSelectBlastimB7.1 and pUNO1-mICAM1 plasmids (from Invivogen) encoding for mouse B7-1 and mouse ICAM-1, respectively, were used for transfecting (Amaya, V solution, A024) the COS-A^k cells (16). Cells were sorted based on their staining with specific antibodies. The resulting stable transfectants were referred as COS-A^k/B7-1 and COS-A^k/ICAM-1, respectively. The surface density of B7-1 in COS-A^k/B7-1 was

estimated to be ~ 10 molecules/ μm^2 by using anti-CD80-PE and QuantiBRITE beads (BD Biosciences), which was within the range of that found on professional APCs *in vivo* (17).

Flow Cytometry

Cells were analyzed on a BD LSR II flow cytometer using the FlowJo software. Cells sorting were performed on BD Aria III. To quantify the antigen molecules on cell surface, BD Quantibrite™ PE Beads (BD Biosciences) were used according to the manufacturer's manual.

T Cell Isolation

CD4⁺ T cells were purified from the total cell population of pooled lymph nodes and spleens from 3A9 TCRtg mice using Dynabeads Untouched Mouse CD4 cell kits (Life Technologies), after cell extraction on nylon membrane and lysis of erythrocytes with NH₄Cl. All cells were 3A9 TCR positive. The naive T cell (CD44^{lo}CD62L^{hi}CD25⁻CD69⁻) population was generally >95%.

Measurement of IL-2 Production From T Cell Stimulated

Purified CD4⁺ T cells from 3A9 TCRtg mice were co-cultured at 37°C with COS APC cells, with different doses of HEL 48-63 peptides. T cells were harvested after 20 h. The concentration of IL-2 in the supernatants was measured by Mouse IL-2 ELISA Ready-SET-Kit (eBioscience).

The Recording of the T Cells Stained With Calcium Indicator Dye

The measurement of CD4⁺ T cell intracellular calcium was performed using MAAACS (methods for automated and accurate analysis of cell signals) (15). In brief, T cells were first loaded with calcium indicator dye (BD™ PBX Calcium Assay Kit) at 37°C for 30 min protected from light. After washing twice in HBSS (Hank's buffer salt solution) containing 1 mM HEPES (pH 7.4), T cells resuspended in the same solution plus CaCl₂ and MgCl₂ were added onto the monolayer of COS APCs in Lab-Tek chamber slides (Nalge Nunc International). Time-lapse movies of the cells, composed of 420 images with each taken every 7 s, were performed on a Zeiss LSM 510 Meta confocal microscope equipped with a C-Apochromat 40X/1.2 water immersion objective as well as an argon laser with a 488 nm dichroic and a 505 nm long pass filter. During the movie recording, cells were kept at 37°C.

Analyses of the T Cell Movement

From recorded movies, T cell instantaneous speeds were calculated as previously described using MAAACS (15). The cell trajectories analyzed by MAAACS were also used to calculate the mean square displacement (MSD) values, which were computed using MSDAnalyzer freely available on GitHub, a Matlab (The MathWorks, Natick, MA) package accordingly to Tarantino et al. (18). The log-log MSD curves were fitted with a linear function to retrieve the so-called α value reflecting the mean cell motion behavior. α close to 1 means a normal diffusive movement, while

a value < 1 indicates a subdiffusive movement and values > 1 a superdiffusive movement of the cells.

The Analysis of the T Cell Calcium Response Magnitude and Types

Experimental raw fluorescence images were first median-filtered and then correlated with a cylindrical mask, transforming pseudo round objects into Gaussian peaks while retaining the overall initial intensity. Note that the mask width is set to match the average radius of the cell. The trajectories are rebuilt by connecting frame to frame cell positions using the MTT algorithm (19). The construction of the raw fluorescence intensity distribution histogram prior to the maximal amplitude allowed to compute a median that is defined as the individual baseline value to which raw intensities then are normalized. The mean amplitude (MA) of the fluorescence intensities corresponds to the average normalized intensity over the whole trace. From the average MA of the cell population, an activation threshold could be established by comparing the stimulating and non-stimulating conditions. This was determined as a combination of maximizing the probability of detection (PD), and minimizing the probability of false alarm (PFA). Based on our previous work using MAAACS to study the calcium responses in 3A9 TCRtg CD4⁺ T cells (15), we set this threshold to 2, and verified that when performing analyses with this threshold, while the PD was very high (>0.96), the PFA was kept low (<0.04) for reacting cells under all stimulation conditions (data not shown). A number of parameters were then established for the calcium response. The activated (or reacting) cells are cells whose normalized fluorescent intensity spikes reached the threshold value in a given condition. The magnitude of calcium response for each reacting cell was quantified using two parameters, i.e., MA as already mentioned, and the response fraction (RF), corresponding to the ratio between the time when the intensity is above the threshold and the total time during which the intensity is detected. It could be noted that MA may be <2, because MA is the mean normalized intensity value of a recording. Finally, the calcium responses displayed by the active cells were classified into three types according to the level of the RF (15). The “maintained” type was defined as RF higher than 0.8, while the “unique” type was defined as RF lower than 0.2 with a single burst. In other cases, the type is “oscillatory.”

The Autocorrelation Analysis of the Calcium Signal Amplitudes

The autocorrelation analysis was performed with the following steps:

a: data detrending

In our analysis, the lagged difference was used as the data detrending method to ensure that the detrended time series were first-order and second-order stationary after removing the trend of the raw data (y_t) along with time. We obtained the stationary process X_t by the first (or higher) order differences, for instance, for the first order differences calculation:

$$X_t = y_t - y_{t-1}$$

And higher order difference is needed until the stationary condition is satisfied.

b: *Autoregressive model*

Each time series was then decomposed into the deterministic and purely stochastic parts by fitting an autoregressive (AR) model with the following form:

$$X_t = \sum_{l=1}^p \alpha_l X_{t-l} + \varepsilon_t \quad (1)$$

Where parameter α_p represents the correlation between X_t and X_{t-p} , and the error term ε_t refers to the disturbance with mean of zero. The optimal lag order p is chosen according to the Schwartz Bayesian Criterion (SBC). We obtained the correlation coefficients via Yuler-Walker method (which is invulnerable to non-normality of the error term but requires the zero mean) and then use them to analyze the typical oscillation and decay times of signals. The Equation (1) could be rewritten into a compact form so that:

$$\tilde{X}_t = \tilde{A}\tilde{X}_{t-1} + \tilde{\varepsilon}_t \quad (2)$$

Where:

$$\tilde{X}_t = \begin{pmatrix} X_t \\ X_{t-1} \\ \vdots \\ X_{t-p+1} \end{pmatrix} \in \mathbb{R}^p \text{ and } \tilde{\varepsilon}_t = \begin{pmatrix} \varepsilon_t \\ 0 \\ \vdots \\ 0 \end{pmatrix} \in \mathbb{R}^p \quad (3)$$

c: *Construction of the $\tilde{A}$ matrix* (20)

$$\text{With } \tilde{A} = \begin{bmatrix} \alpha_1 & \alpha_2 & \cdots & \alpha_{p-1} & \alpha_p \\ 1 & 0 & \cdots & 0 & 0 \\ 0 & 1 & \cdots & 1 & 0 \\ 0 & 0 & \ddots & 0 & 0 \\ 0 & 0 & \cdots & 1 & 0 \end{bmatrix} \in \mathbb{R}^{p \times p} \quad (4)$$

The dynamics of the coefficients $\tilde{X}_t^{(p)}$ are then characterized by the combination of p univariate AR (1) models given as:

$$\tilde{X}_t^{(l)} = \lambda_l \tilde{X}_{t-1}^{(l)} + \tilde{\varepsilon}_t^{(l)}, \quad l = 1, \dots, p$$

By taking the temporal average, we obtain the dynamic of the expanded values of the coefficients:

$$\langle \tilde{X}_t^{(l)} \rangle = \lambda_l \langle \tilde{X}_{t-1}^{(l)} \rangle$$

and which describes a spiral

$$\langle \tilde{X}_t^{(l)} \rangle = \lambda_l^t \langle \tilde{X}_{t-1}^{(l)} \rangle = e^{-t/\tau_l} e^{(i \arg \lambda_l) t} \langle \tilde{X}_{t-1}^{(l)} \rangle$$

d: *Decomposition*

Thus, we could decompose the coefficient matrix $\tilde{A}$

$$\tilde{A} = \tilde{S} \Lambda \tilde{S}^{-1}$$

where $\tilde{S}$ is a non-singular matrix with the columns of which correspond to the eigenvectors and Λ is the associated diagonale matrix whose diagonal is composed by the eigenvalues λ_l

e: *Decaying*

Decaying is a function of λ_l and the maximum value of decay time is what we need

With decay times:

$$\tau_l = -1/\log |\lambda_l|$$

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7.0. A non-parametric two-tailed unpaired Mann-Whitney test was used for comparison between two groups.

RESULTS

Concomitant Stimulation of the TCR and CD28 With Their Cognate Ligands Expressed on COS-7 Cells Trigger an Efficient Activation of Naive CD4⁺ T Cells

To analyze the TCR and CD28 interactions with their respective ligands, we used naive CD4⁺ T cells from 3A9 TCRtg mice (14), and COS-7 fibroblasts (derived from monkey kidney tissue) stably transfected with mouse MHC class II (MHCII) I-A^k (designated as COS-A^k) alone or together with mouse B7-1 (COS-A^k/B7-1) as the antigen-presenting cells (APCs). 3A9 TCR is specific for the hen egg lysozyme (HEL) peptides, such as HEL48-63, presented by I-A^k. We have previously shown that the I-A^k-expressing COS-A^k cells enabled efficient presentation of HEL peptides to T cell hybridoma cells expressing the 3A9 TCR (15). Moreover, given the fact that COS-7 cells are known to be devoid of ascribed ligands for T cell costimulatory or adhesion receptors (21–23), or T cell costimulation promoting activity (24), COS-A^k/B7-1 cells allowed us to analyze the contribution of CD28-B7 interactions in T cell activation. In addition, we generated COS-A^k/ICAM-1 in order to compare the effects of B7-1 and ICAM-1 in T cell activation as ICAM-1 binding to LFA-1 was known to facilitate TCR-pMHC interactions and strengthen TCR-triggered signaling pathways. The different cell lines were sorted according to their surface expression of I-A^k, B7-1, and ICAM-1. As a result (**Figure S1**), all the cell lines expressed the same level of I-A^k on their surface. In addition, the relative surface expression of B7-1 on COS-A^k/B7-1 and ICAM-1 on COS-A^k/ICAM-1 were comparable. On the other hand, and as expected, mouse naïve CD4 T cells had a much higher surface level of LFA-1 than CD28 (**Figure S1**).

The incubation with COS-A^k cells loaded with HEL peptides resulted in inefficient activation of naive 3A9 TCRtg CD4⁺ T cells, as measured by IL-2 secretion. However, the incubation with COS-A^k/B7-1 cells loaded with HEL peptides to concurrently trigger TCR and CD28 signaling pathways elicited strong activation of 3A9 TCRtg CD4⁺ T cells (**Figure 1A**). In addition, we observed that COS-A^k/ICAM-1 cells also could support the activation of 3A9 TCRtg CD4⁺ T cells when pulsed with HEL peptides, even if the strength of activation appeared to be less strong than with COS-A^k/B7-1 cells. Interestingly, video microscopic examination revealed that 3A9 TCRtg CD4⁺ T cells patrolled quickly on the surface of the COS APCs with or without

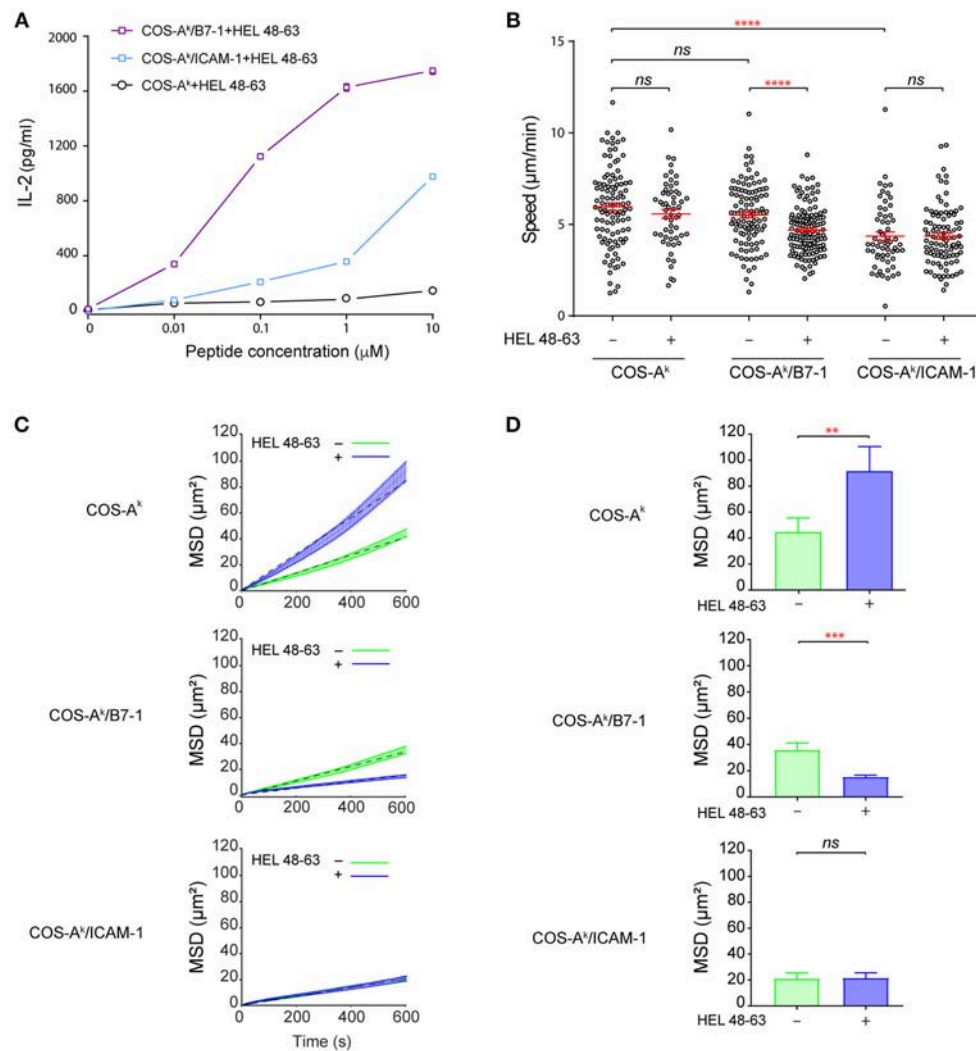


FIGURE 1 | B7-1 expression on COS APCs provides efficient costimulatory signaling to naive CD4⁺ T cells. **(A)** Activation of naive CD4⁺ T cells upon contact with COS APCs loaded with HEL 48–63 peptides. 5×10^5 naive CD4⁺ T cells from 3A9 TCRtg mice were cultured at 37°C with COS-A^k, COS-A^k/B7-1, COS-A^k/ICAM-1 cells, loaded or not with different doses of HEL 48–63. T cells were harvested after 20 h. IL-2 concentration in the supernatants was measured by ELISA. Data represented the mean of three individual experiments. Data: mean $\pm$ s.d. **(B)** Naive CD4⁺ T cells form mobile contacts with COS APCs loaded or not with HEL 48–63. Naive CD4⁺ T cells from 3A9 TCRtg mice were loaded onto APCs monolayers of COS-A^k, COS-A^k/B7-1, COS-A^k/ICAM-1 cells grown to confluency on Lab-Tek chamber slide, loaded or not with 1 μ M HEL 48–63. T cell trajectories were recorded for 45 min at 37°C using confocal video-microscope. Average migration speed over the whole trajectory was calculated as described in Materials and Methods. Data: mean $\pm$ SEM. Their numerical values were 5.96 ± 0.21 μ m/min ($n = 105$), 5.57 ± 0.24 μ m/min ($n = 56$), 5.59 ± 0.18 μ m/min ($n = 111$), 4.68 ± 0.11 μ m/min ($n = 134$), 4.36 ± 0.23 μ m/min ($n = 60$), and 4.36 ± 0.17 μ m/min ($n = 93$) for the T cells contacting with COS-A^k, COS-A^k plus HEL 48–63, COS-A^k/B7-1, and COS-A^k/B7-1 plus HEL 48–63, COS-A^k/ICAM-1, and COS-A^k/ICAM-1 plus HEL 48–63, respectively. **(C)** Mean square displacement over time and **(D)** average mean square displacement over 10 min of T cells seeded onto COS-A^k, COS-A^k/B7-1, and COS-A^k/ICAM-1 cells, loaded or not with 1 μ M HEL 48–63, respectively. Mann-Whitney tests were performed using Graphpad prism 7.0 for comparisons between two groups. Statistical significance was set at 0.05, and p -values < 0.05 were denoted in red. ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

antigenic peptides, forming transient, and mobile contacts (Figure 1B and Figure S2A) with the average instantaneous speed values that were higher than 4.0 μ m/min. Such mobile and short-lasting contacts between T cell and APCs where the TCR binds to pMHC have been referred as immunological kinapses (25) [in which the average T cell speed > 2.5 μ m/min (26)], differentiating them from immunological synapses, which are long-lived and stable [average T cell speed < 2.5 μ m/min (26)].

Various studies have shown that for naive T cells, immunological kinapses are probably the most frequent type observed *in vivo* (26–30) and *in vitro* (26), at least during the early phase of T cell activation. Our analysis also showed that the average speed of 3A9 TCRtg CD4⁺ T cells was the same with COS-A^k cells loaded or not with HEL peptides; a slight reduction was found with COS-A^k/B7-1 cells after HEL peptide loading, and the lowest speed was observed with 3A9 TCRtg CD4⁺ T cells interacting

with COS-A^k/ICAM-1 cells, either before or after peptide loading (**Figure 1B**). We next conducted the mean square displacement (MSD) vs time analyses (31) of the CD4⁺ T cell movements on different COS APCs between non-stimulating and stimulating conditions (**Figures 1C,D** and **Figure S2B**). Of interests, these analyses revealed that the antigen stimulation affected the MSD of CD4⁺ T cells that interacted with COS-A^k and COS-A^k/B7-1 cells, respectively, but in an opposite direction (**Figure 1D**). Moreover, while these movements all contained heterogeneous diffusion modalities, they were all also largely dominated by the constrained (subdiffusive) type. However, an increase in the percentage of trajectories with an α value significantly > 1 suggested that a directed-like motion (superdiffusive) type was present in the movement of T cells upon stimulation by antigen in the absence of CD28 or LFA-1 signaling (**Figure S2B**). Finally, the free-like (normal diffusive) motion was essentially absent in all cases (**Figure S2B**).

TCR Stimulation With pMHC Raises the Fluctuating Calcium Mobilization Level in Naive CD4⁺ T Cells

The calcium response is a major signaling event downstream to receptor-proximal signaling pathways in T cell activation (32, 33). Previous works have established that the TCR early signaling enables elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) that could be further potentiated upon CD28 coengagement (4, 10). Moreover, the results from several studies have suggested that enhanced calcium response plays an essential role in the costimulatory function of CD28 in T cell activation (2, 4, 10, 34). However, its involvement in the TCR- and CD28-mediated stimulation currently remains poorly known for the naive T cell activation. One important reason is that study models were built up either by inferring from observations made in other T cell types, or experimental data by using antibodies against the TCR and CD28, which may mimic some properties but certainly miss others of the corresponding natural ligands. We therefore decided to examine the calcium responses of 3A9 TCRtg CD4⁺ T cells upon interactions with COS APCs. For this, we used the MAAACS approach (15), an inclusive method that we previously developed, which enabled comprehensive analyses of the calcium dynamics in T cells that are constantly moving and forming kinapses with APCs. The **Figure 2** and **Figure S3** depict how the use of MAAACS to analyze the calcium mobilization in 3A9 TCRtg CD4⁺ T cells allowed the efficient determination of its frequency and average response magnitude that was determined by the average mean amplitude (MA) and response fraction (RF). Consistent with the previous study (15), we observed that when seeded onto COS-A^k cells, some 3A9 TCRtg CD4⁺ cells exhibited weak and transient calcium elevation over a period of several tens of minutes (**Figure 3**). A factor that could account for such basal fluctuating $[Ca^{2+}]_i$ rises is T cells interacting with APCs, either with or without the involvement of MHC molecules (35–37). When 3A9 TCRtg CD4⁺ T cells were seeded onto COS-A^k cells loaded with HEL peptides, 3A9 TCRtg CD4⁺ T cells continued to experience fluctuating $[Ca^{2+}]_i$ elevations, in line with previous studies on

naive CD4⁺ T cells upon TCR stimulation (15, 36, 38); however, there was an increase in both percentage and average response magnitude, as estimated by the MA and RF of the reacting cells, respectively (**Figures 3, 4**). In addition, we observed that these increases took place in a dose-dependent manner when peptides were loaded at concentrations of 0.1, 1.0, and 10 μ M, respectively. Finally, for all the stimuli conditions, the main type of calcium response for activated cells continued to be “oscillatory,” the proportion of which increased with the peptide concentration (**Figure 3**).

TCR and CD28 Concomitant Stimulation Induces a Stronger Calcium Mobilization Than Single-Receptor Stimulation in Naive CD4⁺ T Cells

We next examined the calcium mobilization of 3A9 TCRtg CD4⁺ T cells activated by concurrent TCR and CD28 stimulation. When 3A9 TCRtg CD4⁺ T cells were seeded onto COS-A^k/B7-1 cells without HEL peptides, a weak but detectable increase in calcium elevations, in terms of both frequency and average response magnitude of the reacting cells, was observed compared with 3A9 TCRtg CD4⁺ T cells seeded onto COS-A^k cells, indicating that the engagement alone of CD28 enabled a low level of calcium mobilization (**Figures 3, 4**). When 3A9 TCRtg CD4⁺ T cells were incubated with COS-A^k/B7-1 cells loaded with HEL peptides, with both TCR and CD28 being engaged, the calcium mobilization level was higher than that obtained with each receptor alone, as expected. In addition, the type of response with regards to the presence of antigen stimulus was consistent with that expected—that is, the response increased with the increased concentration of the HEL peptide, and the response attained its maximum level at the concentration of 10 μ M. However, we noticed that the observed augmentation in the percentage and average mean response magnitude of reacting cells did not characterize a functional aspect that could account for the high costimulatory activity of CD28. Indeed, the costimulation of CD28 together with TCR stimulation triggered by a low concentration of HEL peptide (0.1 μ M) resulted in a weaker calcium response in 3A9 TCRtg CD4⁺ cells than that observed with TCR stimulation alone triggered by a higher concentration of HEL peptide (10 μ M); however, a much higher secretion rate of IL-2, was found in the first case than in the second (**Figures 1A, 4**).

Our following comparison between CD28 and LFA-1 in their cooperation with the TCR to elicit the calcium response further supported the above remark. We observed that the engagement of LFA-1 by its ligand ICAM-1 alongside the stimulation of TCR by pMHC resulted in a significant increase in calcium mobilization, whereby both frequency and average response magnitude of reacting cells reached plateau level with 0.1 μ M peptides. Comparatively, co-triggering LFA-1 along with TCR stimulation produced a stronger calcium response in 3A9 TCRtg CD4⁺ cells than did CD28 (**Figures 3, 4**). However, co-engagement of CD28 caused a greater TCR-induced IL-2 secretion than the LFA-1. Indeed, co-engaging CD28 with the TCR that was stimulated with HEL peptides

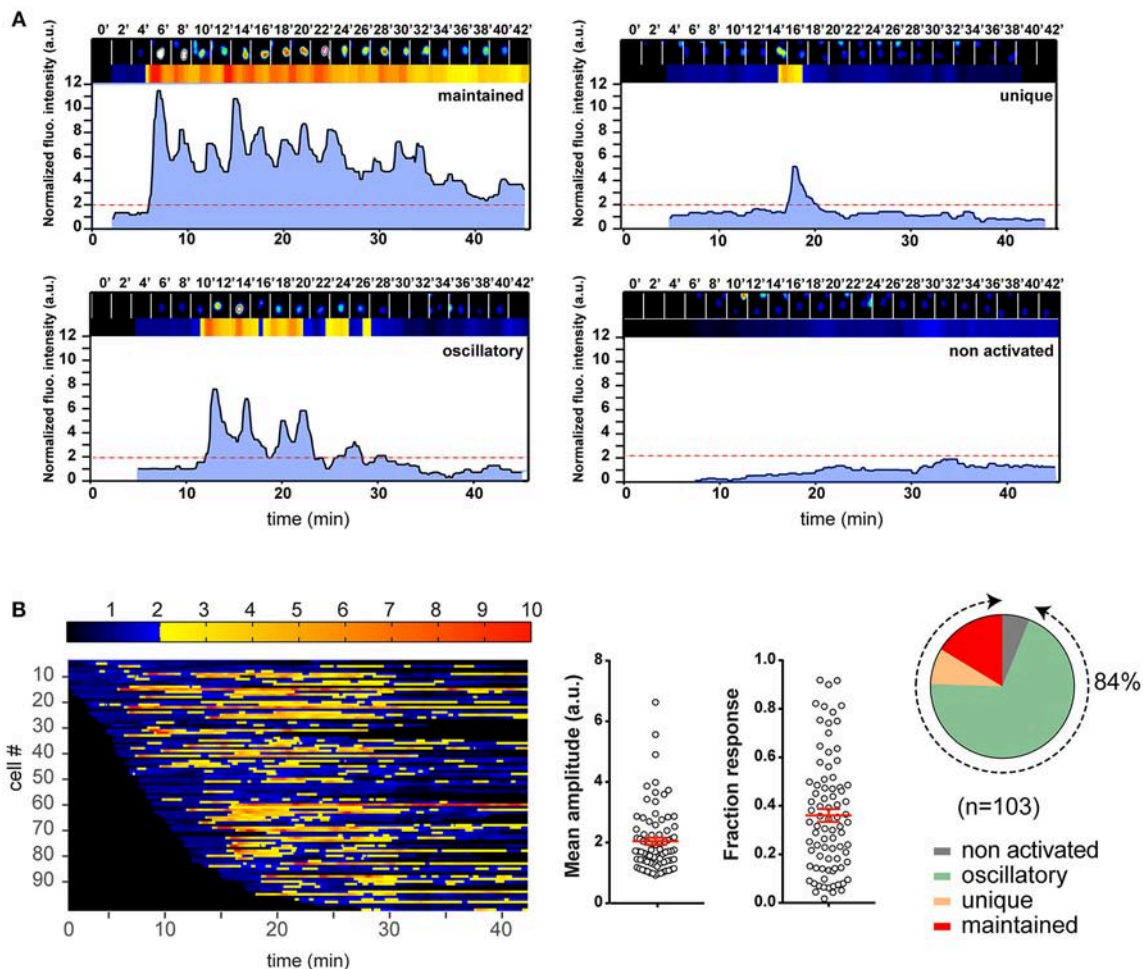


FIGURE 2 | Illustration of the T cell calcium response analysis with an example experiment. Naive 3A9 TCRtg CD4⁺ T cells were loaded with Fluo-4 PBX before incubation at 37°C for 45 min with COS-A^K pulsed with 10 μ M HEL 48–63. Time-lapse movies of T cells were made using confocal microscope as described in Materials and Methods. **(A)** Single cell fluorescence recordings analyzed by MAAACS and categorized into different classes according to the magnitude and shape of fluorescence signals. The examples of four different response types are shown. In each panel, the top row is made up of 2 min-delayed snapshots of raw images of a cell. Just below are the normalized fluorescence intensities displayed in the form of a bar code and a line profile, respectively. The non-activated cells are defined as cells whose normalized fluorescence intensities have never reached the activation threshold (set at 2.0, red dotted line, see Methods and Materials for more details) along the whole trace. For the activated cells, we defined a response as “maintained” when the response fraction (RF) was higher than 0.8, and “unique” when the response fraction was lower than 0.2 with a single burst. In all other situations, calcium responses were “oscillatory.” **(B)** Color barcoding and calculation of the analytical parameters of the calcium response. The normalized fluorescence amplitude of each cell is plotted along a horizontal line as a function of time with a color-coded intensity (dark to blue below the threshold of activation and yellow to red above the threshold of activation). The analytical parameters of the calcium response, i.e., the mean amplitude (MA) and RF calculated by MAAACS are plotted (mean $\pm$ SEM). The global overview of the cell response heterogeneity is summarized in the form of a pie chart.

at 0.1 μ M resulted in a higher IL-2 production than co-engaging LFA-1 with TCR stimulation with HEL peptides at any concentration (Figure 1A), despite the more potent calcium response magnitude occurred in the cases of LFA-1 co-engagement (Figure 4). Altogether, our data showed that both CD28 and LFA-1 stimulation could significantly potentiate the calcium response induced by the TCR-pMHC interactions. However, the increase of the frequency and average response magnitude in the calcium mobilization is probably not the most crucial facet of CD28 costimulatory function in T cell activation.

TCR and CD28 Concomitant Stimulation Triggers a Distinctive Increase in the Decay Time (τ) of $[Ca^{2+}]_i$ Elevations as Shown by Time Series Analysis

Our observations made above could reflect that CD28 functions through mechanisms that are partly dependent on calcium mobilization or, alternatively, that CD28 costimulation crucially relies calcium mobilization, but in addition to the classical aspects—namely the reacting cell frequency and average response magnitude—there could be other molecular features. Given

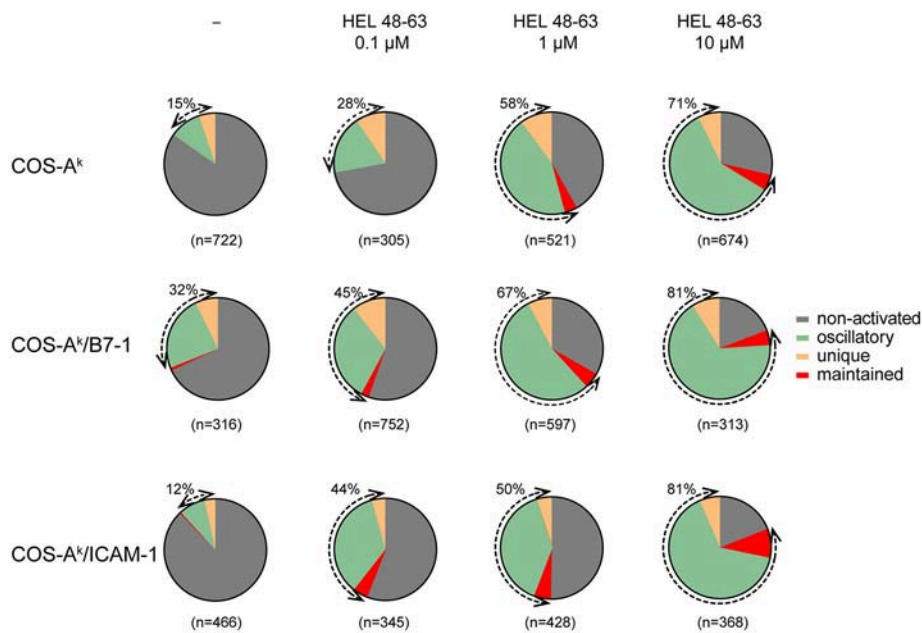


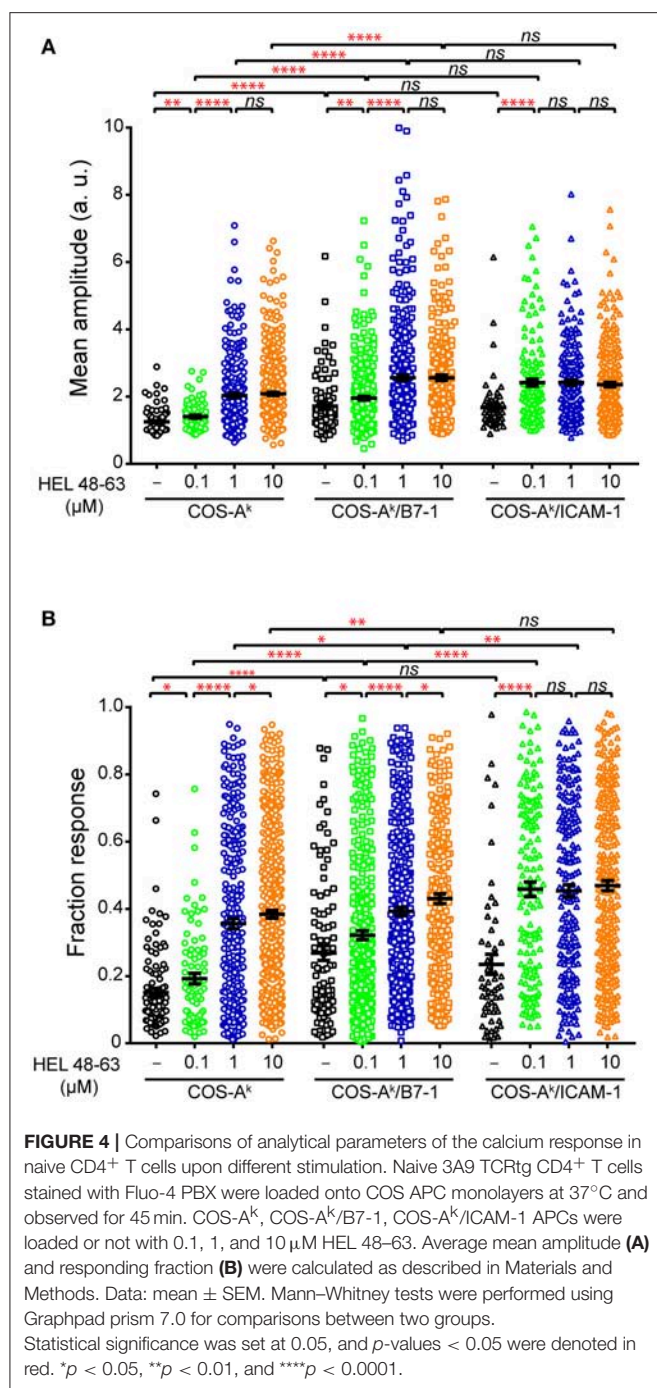
FIGURE 3 | Naive CD4⁺ T cells show various calcium response modes upon different stimulation. Naive 3A9 TCRtg CD4⁺ T cells were loaded with Fluo-4 PBX before incubation at 37°C for 45 min with COS APC cells pulsed without or with different doses of HEL 48–63 as indicated. Time-lapse movies of T cells were made by confocal microscope as described in Materials and Methods. For each antigenic peptide concentration, modes of calcium response are represented as a pie chart: non-activated in gray, maintained in red, oscillatory in green, and unique in yellow. The total number of cells analyzed is indicated in parentheses. The percentage of activated (reacting) cells is shown by the double arrows, and their values are indicated.

the results of previous studies (2, 4, 34), we assumed the latter to be the most likely possibility. We considered that CD28 costimulation could change the temporal dynamics of the calcium response induced with TCR stimulation alone (10, 34, 39). For instance, earlier investigations with human primary T cells using antibodies showed that concurrent TCR and CD28 ligation elicited a more-sustained calcium response than did each receptor individually (34, 39). However, such antibody-elicited T cell responses were not fluctuating, and their maintenance was generally characterized by a flatter slope in the signal decay, which differed from the calcium response found here. We therefore decided to conduct the autocorrelation analysis to reveal the dynamics of intracellular calcium mobilization in 3A9 TCRtg CD4⁺ T cells. The autocorrelation analysis examined the self-similarity of a time series signal (Figure S4), which can be characterized with a decay time (τ) that describes the persistence of the information that the signal carries. Such an approach was previously employed to examine the contribution of $[Ca^{2+}]_i$ oscillation during IFN- γ production by human cytotoxic T cell clones (40). Our autocorrelation analysis interestingly revealed that the calcium amplitude signal in the (reacting) 3A9 TCRtg CD4⁺ T cells at the basal conditions had an average characteristic decay time (τ) of ~ 46 s (Figure 5). The calcium signal decay time (τ) remained unchanged in 3A9 TCRtg CD4⁺ T cells upon stimulation of TCR, CD28, or LFA-1, with the corresponding ligands. However, stimulation of both TCR and CD28, but not both TCR and LFA-1, induced a striking increase of decay time (τ) to ~ 58 s (Figure 5). Moreover, the increase immediately

reached a plateau value with 0.1 μ M HEL peptide and kept unchanged at higher peptide concentrations, suggesting that the induction involves a very efficient mechanism that allows it to take place even when the level of antigen is low. Therefore, our analysis uncovered the augmented decay time (τ), representing a unique feature of the calcium response elicited by TCR and CD28 concurrent stimulation, and we suggest that the CD28 costimulatory activity thus contributes to producing a type of fluctuating intracellular calcium mobilization with greater incidence, higher magnitude, and longer memory of information it carries than TCR stimulation alone during naive T cell activation.

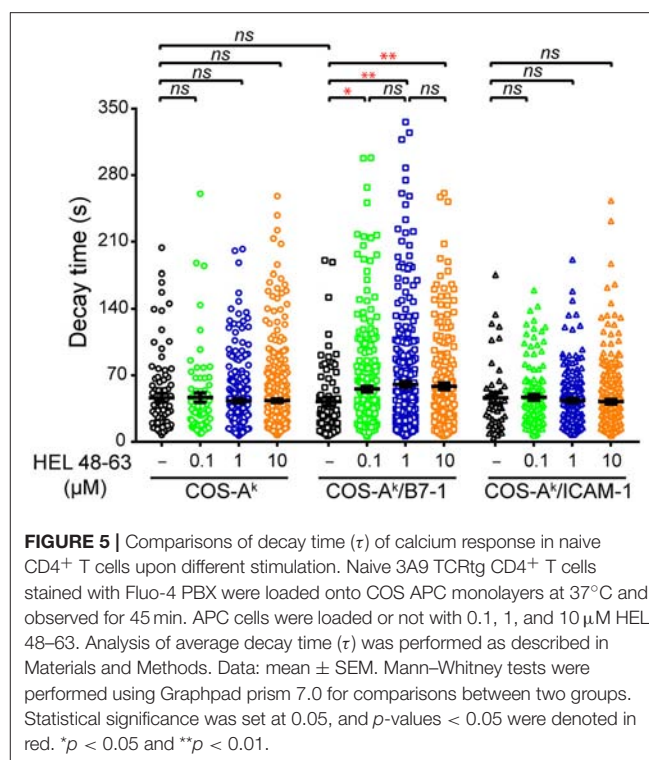
DISCUSSION

In this work, we investigated the mechanism of activation of naive CD4⁺ T cells mediated by the TCR and CD28. The T cell activation process has been the focus of numerous studies during past decades, but its mechanism is still only partially understood. Our current study has identified an important step in the TCR and CD28 signaling pathways where the two pathways interact and complement one another to promote the effective initiation of naive T cell activation—namely the regulation of the fluctuating intracellular calcium mobilization. Indeed, we have shown for the first time that simultaneous TCR and CD28 stimulation, but not stimulation of individual receptors, significantly extended the decay time (τ) of the calcium amplitude signal detected prior stimulation. This special



property, uniquely characterizing the costimulatory action of CD28, probably contributes to TCR- and CD28-mediated signaling pathways leading to efficient T cell activation. This finding supports the notion that it is only after initial antigen recognition by TCR that CD28 efficiently triggers signaling pathways in naive T cells, given the very weak basal interactions of CD28 with B7 molecules (7), and its low abundance in this cell population (2, 3).

CD28 is a major costimulatory receptor that is constitutively expressed on naive T cells and is essential for the activation of naive T cells by antigen recognition. However, much information



on the molecular mechanism of CD28 signaling in naive T cells is largely inferred from results obtained with T cell lines or antigen-experienced T cells but very rarely directly studied. This is mainly due to the fact that it remains technically more challenging to study early signaling events following stimulation of surface receptors in naive T cells. However, it is not clear to what extent the information obtained from these studies can be applied directly to the activation of naive T cells, as the early signaling steps induced upon T cell antigen recognition between naive T cells and stimulation-experienced T cells exhibit both qualitative and quantitative differences. In addition, there is also the problem that, in many of these studies, receptor stimulation has been achieved only by means of antibodies instead of physiological ligands. It is reasonable to consider that antibodies, especially in soluble form, can only mimic some, but not all, of the key actions of membrane-bound ligands (41, 42). It is therefore important to examine the mechanism of activation of naive T cells via TCR and CD28 by directly using naive cells that are triggered by natural ligands on APCs, which we have done here.

Calcium mobilization is known to be an essential signaling event in T cell activation (32, 33). Previous studies have suggested that the contribution of CD28 in calcium mobilization is essential to its co-stimulatory activity in promoting T cell activation. Naive T cells exhibit significant basal intracellular Ca²⁺ transients both *in vitro* and *in vivo* (35–37). The considerable fluctuation of [Ca²⁺]_i rises also feature the calcium responses in naive T cells when exposed to various types of external stimuli (15, 36, 38). We utilized in our study MAAACS, an inclusive method that we recently developed that allows efficient and robust analysis of the calcium responses in naive mouse CD4⁺ T cells. We observed that in the activation of naive T cells, the concurrent

engagement of the TCR and CD28 with their cognate ligands elicited an elevation in the fluctuating $[Ca^{2+}]_i$ with both higher frequency and magnitude than did TCR or CD28 engagement alone, which was not surprising. However, by examining the autocorrelation function of the calcium amplitude signal (40), we uncovered for the first time that there was an increase in the decay time (τ) of such signal from the basal level specifically after concurrent TCR and CD28 stimulation. The autocorrelation function normally describes the correlation between the signal observed at one time point with the observed at several previous time points and the decay time (τ) derived from this analysis characterizes the time length within which the signals at two time points show correlation. The decay time (τ) therefore provides information on the existence of a memory for the signal and its decay rate. It was found that the fluctuating calcium rises in mouse naive $CD4^+$ T cells show an average decay time of ~ 46 s under basal conditions, which remained unchanged when the same cells were stimulated via either TCR, CD28, LFA-1, or TCR plus LFA-1 by the corresponding ligands, respectively. The combined stimulation by TCR plus CD28, however, resulted in a significantly longer decay time of ~ 58 s. This unique capacity from the concurrent TCR and CD28 stimulation may be linked to the key co-stimulatory molecule statue of CD28 critical to TCR-induced naive T cell activation upon pMHC engagement.

The origin of the prolonged decay time currently is unknown. One possible cause is because of the existence of constrained diffusions of intracellular Ca^{2+} ions due to spatial confinements for local and specific activation processes (43, 44). This could be arisen from generation of specific calcium nano- or microdomains at the vicinity of Ca^{2+} channels in membranes (45–47), or calcium flux in subcellular organelles such as nuclei (43, 48) and mitochondria (46, 49), etc. Interestingly, our present work is reminiscent for several aspects to the previous characterization of the role of CD28 co-stimulatory signaling in calcium responses by the previously activated mouse $CD4^+$ T cells. In particular, Andres et al. (10) have found that CD28 co-stimulation upon binding to B7 strongly increased both the frequency and magnitude of TCR-induced calcium response upon binding to physiological levels of antigens during the T cell activation. In absence of CD28 co-stimulation, the predominant pattern of calcium influx triggered by the TCR was both attenuated in amplitude and transient in duration. It would therefore be interesting to examine whether the observed more persistent calcium response pattern associated with a productive activation signal in general displays an augmented decay time.

It was interesting to observe that, in our study, unlike the co-engagement of CD28 by B7-1, co-engagement of LFA-1 by ICAM-1 along with TCR engagement by pMHC did not extend the decay time (τ) of calcium amplitude signal as compared to the basal level in naive $CD4^+$ cells. This occurred despite the co-engagement of LFA-1 with TCR engagement produced a stronger calcium response, both in terms of the frequency and magnitude than the co-engagement of CD28 (Figures 3, 4). Future studies will be needed to see if this remarkable functional difference between CD28 and LFA-1 also applies to other T cell populations. In this context, it noteworthy that a previous work has shown that in mouse naive $CD8^+$ cells, that LFA-1 and CD28 exhibit distinct, non-overlapping ways to influence T cell activation (50).

Our data suggest that the interplays between the TCR and CD28 early signaling generate the coincidence detection mechanism for the initiation of naive T cell activation. This endows naive T cells with the ability to tightly control their activation, which is just as important as the ability to trigger activation. For instance, B7 molecules are expressed on dendritic cells and macrophages in lymphoid tissue, the levels of which are upregulated during infection and inflammation (17); their inappropriate binding to and stimulation of CD28 could have adverse pathological consequences. On the other hand, interplays at the early signaling steps could facilitate the TCR and CD28 to trigger balanced signaling pathways and their integration. At the cell population level, coincidence detection would be a mechanism that allows T cells to focus rapidly and efficiently on APC cells that have captured antigens and upregulated B7 and MHC molecules simultaneously, as in the case of dendritic cells stimulated with microbial products. Thus, our present work has provided strong support for a “privileged” costimulatory molecule role of CD28, the only B7 receptor constitutively expressed on naive T cells, in the activation of this T cell population.

AUTHOR CONTRIBUTIONS

X-JG, FX, C-RQ, and ZX designed the study, performed the experiments and analyzed the results. YH, A-MS, AF, SM, M-CP, CB, SJ, and JN assisted with realization and interpretation of the experiments, and provided several reagents. X-JG and H-TH supervised and directed the research. X-JG, FX, ZX, YH, SM, and H-TH wrote the manuscript. All authors discussed the results and commented on the manuscript.

FUNDING

This work was supported by institutional grants from INSERM and CNRS and by specific grants from the Agence Nationale de la Recherche (ANR-08-PCVI-0034; ANR-10-BLAN-1509; ANR-11-LABX-Investissement d'Avenir Labex-INFORM; ANR-10-INBS-04 France BioImaging). FX was awarded a fellowship from the Ligue Nationale Contre le Cancer. JN laboratory is supported by the Fondation pour la Recherche Médicale (Equipe FRM DEQ20180339209).

ACKNOWLEDGMENTS

The authors wish to thank Qian Wang, Rémi Lasserre, Didier Marguet for advice and discussion, Jean-Yves Tinevez from the Image Analysis Hub of the Institut Pasteur for this help on MSD analyses and AngloScribe for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02864/full#supplementary-material>

REFERENCES

- Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annu Rev Immunol.* (2009) 27:591–619. doi: 10.1146/annurev.immunol.021908.132706
- Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol.* (2003) 3:939–51. doi: 10.1038/nri1248
- Boomer JS, Green JM. An enigmatic tail of CD28 signaling. *Cold Spring Harb Perspect Biol.* (2010) 2:a002436. doi: 10.1101/cshperspect.a002436
- Michel F, Attal-Bonnefoy G, Mangino G, Mise-Omata S, Acuto O. CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. *Immunity* (2001) 15:935–45. doi: 10.1016/S1074-7613(01)00244-8
- Cai Z, Kishimoto H, Brunmark A, Jackson MR, Peterson PA, Sprent J. Requirements for peptide-induced T cell receptor downregulation on naive CD8+ T cells. *J Exp Med.* (1997) 185:641–51.
- Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* (1995) 375:148–51. doi: 10.1038/375148a0
- Bromley SK, Iaboni A, Davis SJ, Whitty A, Green JM, Shaw AS, et al. The immunological synapse and CD28-CD80 interactions. *Nat Immunol.* (2001) 2:1159–66. doi: 10.1038/ni737
- Wetzel SA, McKeithan TW, Parker DC. Live-cell dynamics and the role of costimulation in immunological synapse formation. *J Immunol.* (2002) 169:6092–101. doi: 10.4049/jimmunol.169.11.6092
- Wulfig C, Sumen C, Sjaastad MD, Wu LC, Dustin ML, Davis MM. Costimulation and endogenous MHC ligands contribute to T cell recognition. *Nat Immunol.* (2002) 3:42–7. doi: 10.1038/ni741
- Andres PG, Howland KC, Dresnek D, Edmondson S, Abbas AK, Krummel MF. CD28 signals in the immature immunological synapse. *J Immunol.* (2004) 172:5880–6. doi: 10.4049/jimmunol.172.10.5880
- Tseng SY, Liu M, Dustin ML. CD80 cytoplasmic domain controls localization of CD28, CTLA-4, and protein kinase C θ in the immunological synapse. *J Immunol.* (2005) 175:7829–36.
- Sanchez-Lockhart M, Kim M, Miller J. Cutting edge: A role for inside-out signaling in TCR regulation of CD28 ligand binding. *J Immunol.* (2011) 187:5515–9. doi: 10.4049/jimmunol.1102497
- Sanchez-Lockhart M, Rojas AV, Fettes MM, Bauserman R, Higa TR, Miao H, et al. T cell receptor signaling can directly enhance the avidity of CD28 ligand binding. *PLoS ONE* (2014) 9:e89263. doi: 10.1371/journal.pone.0089263
- Ho WY, Cooke MP, Goodnow CC, Davis MM. Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. *J Exp Med.* (1994) 179:1539–49.
- Salles A, Billadeau C, Serge A, Bernard AM, Phelipot MC, Bertaux N, et al. Barcoding T cell calcium response diversity with methods for automated and accurate analysis of cell signals (MAAACS). *PLoS Comput Biol.* (2013) 9:e1003245. doi: 10.1371/journal.pcbi.1003245
- Chouaki-Benmansour N, Ruminiski K, Sartre AM, Phelipot MC, Salles A, Bergot E, et al. Phosphoinositides regulate the TCR/CD3 complex membrane dynamics and activation. *Sci Rep* (2018) 8:4966. doi: 10.1038/s41598-018-23109-8
- Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol.* (2002) 20:29–53. doi: 10.1146/annurev.immunol.20.091101.091806
- Tarantino N, Tinevez JY, Crowell EF, Boisson B, Henriques R, Mhlana M, et al. TNF and IL-1 exhibit distinct ubiquitin requirements for inducing NEMO-IKK supramolecular structures. *J Cell Biol.* (2014) 204:231–45. doi: 10.1083/jcb.201307172
- Serge A, Bertaux N, Rigneault H, Marguet D. Dynamic multiple-target tracing to probe spatiotemporal cartography of cell membranes. *Nat Methods* (2008) 5:687–94. doi: 10.1038/nmeth.1233
- Honerkamp J. *Stochastic Dynamical Systems: Concepts, Numerical Methods, Data Analysis*. Hoboken, NJ: John Wiley & Sons (1994).
- Freeman GJ, Gribben JG, Boussiotis VA, Ng JW, Restivo VA Jr, Lombard LA, et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science* (1993) 262:909–11.
- Seder RA, Germain RN, Linsley PS, Paul WE. CD28-mediated costimulation of interleukin 2 (IL-2) production plays a critical role in T cell priming for IL-4 and interferon gamma production. *J Exp Med.* (1994) 179:299–304.
- Klickstein LB, York MR, Fougereolles AR, Springer TA. Localization of the binding site on intercellular adhesion molecule-3 (ICAM-3) for lymphocyte function-associated antigen 1 (LFA-1). *J Biol Chem.* (1996) 271:23920–7.
- Gaglia JL, Mattoo A, Greenfield EA, Freeman GJ, Kuchroo VK. Characterization of endogenous Chinese hamster ovary cell surface molecules that mediate T cell costimulation. *Cell Immunol.* (2001) 213:83–93. doi: 10.1006/cimm.2001.1867
- Dustin ML. T-cell activation through immunological synapses and kinapses. *Immunol Rev.* (2008) 221:77–89. doi: 10.1111/j.1600-065X.2008.00589.x
- Moreau HD, Lemaitre F, Terriac E, Azar G, Piel M, Lennon-Dumenil AM, et al. Dynamic in situ cytometry uncovers T cell receptor signaling during immunological synapses and kinapses in vivo. *Immunity* (2012) 37:351–63. doi: 10.1016/j.immuni.2012.05.014
- Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* (2004) 427:154–9. doi: 10.1038/nature02238
- Miller MJ, Safrina O, Parker I, Cahalan MD. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med.* (2004) 200:847–56. doi: 10.1084/jem.20041236
- Scholer A, Hugues S, Boissonnas A, Fetter L, Amigorena S. Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory. *Immunity* (2008) 28:258–70. doi: 10.1016/j.immuni.2007.12.016
- Friedman RS, Beemiller P, Sorensen CM, Jacobelli J, Krummel MF. Real-time analysis of T cell receptors in naive cells *in vitro* and *in vivo* reveals flexibility in synapse and signaling dynamics. *J Exp Med.* (2010) 207:2733–49. doi: 10.1084/jem.20091201
- Jacobelli J, Friedman RS, Conti MA, Lennon-Dumenil AM, Piel M, Sorensen CM, et al. Confinement-optimized three-dimensional T cell amoeboid motility is modulated via myosin IIA-regulated adhesions. *Nat Immunol.* (2010) 11:953–61. doi: 10.1038/ni.1936
- Lewis RS, Cahalan MD. Potassium and calcium channels in lymphocytes. *Annu Rev Immunol.* (1995) 13:623–53. doi: 10.1146/annurev.13.040195.003203
- Feske S, Giltman J, Dolmetsch R, Staudt LM, Rao A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat Immunol.* (2001) 2:316–24. doi: 10.1038/86318
- Skanland SS, Moltu K, Berge T, Aandahl EM, Tasken K. T-cell co-stimulation through the CD2 and CD28 co-receptors induces distinct signalling responses. *Biochem J.* (2014) 460:399–410. doi: 10.1042/BJ20140040
- Delon J, Bercovici N, Raposo G, Liblau R, Trautmann A. Antigen-dependent and-independent Ca²⁺ responses triggered in T cells by dendritic cells compared with B cells. *J Exp Med.* (1998) 188:1473–84.
- Le Borgne M, Raju S, Zinselmeyer BH, Le VT, Li J, Wang Y, et al. Real-Time analysis of calcium signals during the early phase of T cell activation using a genetically encoded calcium biosensor. *J Immunol.* (2016) 196:1471–9. doi: 10.4049/jimmunol.1502414
- Dong TX, Othy S, Greenberg ML, Jairaman A, Akunwafo C, Leverrier S, et al. Intermittent Ca²⁺ signals mediated by Orai1 regulate basal T cell motility. *Elife* (2017) 6:e27827. doi: 10.7554/eLife.27827
- Boutin Y, Leitenberg D, Tao X, Bottomly K. Distinct biochemical signals characterize agonist-and altered peptide ligand-induced differentiation of naive CD4+ T cells into Th1 and Th2 subsets. *J Immunol.* (1997) 159:5802–9.
- Zaru R, Berrie CP, Iurisci C, Corda D, Valitutti S. CD28 co-stimulates TCR/CD3-induced phosphoinositide turnover in human T lymphocytes. *Eur J Immunol.* (2001) 31:2438–47. doi: 10.1002/1521-4141(200108)31:8
- Utzny C, Faroudi M, Valitutti S. Frequency encoding of T-cell receptor engagement dynamics in calcium time series. *Biophys J.* (2005) 88:1–14. doi: 10.1529/biophysj.103.038216
- Liu B, Chen W, Evavold BD, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* (2014) 157:357–68. doi: 10.1016/j.cell.2014.02.053
- Pielak RM, O'Donoghue GP, Lin JJ, Alfieri KN, Fay NC, Low-Nam ST, et al. Early T cell receptor signals globally modulate ligand:receptor affinities during antigen discrimination. *Proc Natl Acad Sci USA.* (2017) 114:12190–5. doi: 10.1073/pnas.1613140114

43. Marangoni F, Murooka TT, Manzo T, Kim EY, Carrizosa E, Elpek NM, et al. The transcription factor NFAT exhibits signal memory during serial T cell interactions with antigen-presenting cells. *Immunity* (2013) 38:237–49. doi: 10.1016/j.immuni.2012.09.012
44. Courjaret R, Dib M, Machaca K. Spatially restricted subcellular Ca(2+) signaling downstream of store-operated calcium entry encoded by a cortical tunneling mechanism. *Sci Rep.* (2018) 8:11214. doi: 10.1038/s41598-018-29562-9
45. Rizzuto R, Pozzan T. Microdomains of intracellular Ca2+: molecular determinants and functional consequences. *Physiol Rev.* (2006) 86:369–408. doi: 10.1152/physrev.00004.2005
46. Quintana A, Pasche M, Junker C, Al-Ansary D, Rieger H, Kummerow C, et al. Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation. *EMBO J.* (2011) 30:3895–912. doi: 10.1038/emboj.2011.289
47. Wolf IMA, Guse AH. Ca(2+) Microdomains in T-Lymphocytes. *Front Oncol.* (2017) 7:73. doi: 10.3389/fonc.2017.00073
48. Monaco S, Jahraus B, Samstag Y, Bading H. Nuclear calcium is required for human T cell activation. *J Cell Biol.* (2016) 215:231–43. doi: 10.1083/jcb.201602001
49. Fracchia KM, Pai CY, Walsh CM. Modulation of T Cell metabolism and function through calcium signaling. *Front Immunol.* (2013) 4:324. doi: 10.3389/fimmu.2013.00324
50. Bachmann MF, McKall-Faienza K, Schmits R, Bouchard D, Beach J, Speiser DE, et al. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* (1997) 7:549–57.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Citation: Xia F, Qian C-R, Xun Z, Hamon Y, Sartre A-M, Formisano A, Mailfert S, Phelipot M-C, Billaudeau C, Jaeger S, Nunès JA, Guo X-J and He H-T (2018) TCR and CD28 Concomitant Stimulation Elicits a Distinctive Calcium Response in Naive T Cells. *Front. Immunol.* 9:2864. doi: 10.3389/fimmu.2018.02864

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Integrating Experiment and Theory to Understand TCR-pMHC Dynamics

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

Rainer A. Böckmann,
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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 19 September 2018

Accepted: 26 November 2018

Published: 07 December 2018

Citation:

Buckle AM and Borg NA (2018)
Integrating Experiment and Theory to
Understand TCR-pMHC Dynamics.
Front. Immunol. 9:2898.
doi: 10.3389/fimmu.2018.02898

The conformational dynamism of proteins is well established. Rather than having a single structure, proteins are more accurately described as a conformational ensemble that exists across a rugged energy landscape, where different conformational sub-states interconvert. The interaction between $\alpha\beta$ T cell receptors (TCR) and cognate peptide-MHC (pMHC) is no exception, and is a dynamic process that involves substantial conformational change. This review focuses on technological advances that have begun to establish the role of conformational dynamics and dynamic allostery in TCR recognition of the pMHC and the early stages of signaling. We discuss how the marriage of molecular dynamics (MD) simulations with experimental techniques provides us with new ways to dissect and interpret the process of TCR ligation. Notably, application of simulation techniques lags behind other fields, but is predicted to make substantial contributions. Finally, we highlight integrated approaches that are being used to shed light on some of the key outstanding questions in the early events leading to TCR signaling.

Keywords: TCR, MHC, conformational dynamics, T cell activation, immune synapse, TCR recognition

INTRODUCTION

Self or foreign intracellular peptides are presented on the surface of antigen presenting cells (APC) by major histocompatibility complex (MHC) class I molecules. These peptide-bound MHC (pMHC) molecules undergo surveillance by CD8⁺ cytotoxic T lymphocytes (CTLs) via the $\alpha\beta$ T cell receptors (TCR) that are expressed on their surface. TCR engagement of the pMHC leads to the formation of an immune synapse that is central to T cell activation (**Figure 1A**). The outcome of T cell engagement with the pMHC influences T cell fate, playing a role in the defense against infection and cancer, but on the flip side, allergic reactions, autoimmune disease, transplant rejection, and drug hypersensitivity. Despite the importance of T cell activation, we have a poor understanding of how the TCR-pMHC initiates an intracellular signal and this impedes our ability to manipulate the T cell response to target infection and cancer. What is clear however is that there is enormous complexity to the overall response and dissecting it requires the integration of a diverse suite of both experimental and computational tools and techniques.

Due to the relatively small size of the TCR-pMHC, X-ray crystallography has led the way in the structural determination of the extracellular domains of pMHC and TCR alone or in complex with one another at near-atomic resolution. These studies detail the conformation of the peptide, its interactions with MHC as well as the TCR and structural changes the MHC and/or peptide undergoes upon TCR binding. It was long-anticipated that these accrued X-ray structures would also reveal how the information at the pMHC interface is communicated from the variable domains to the membrane proximal constant domains and via the CD3 subunits necessary for signal transduction. A clear mechanism however has not been revealed, exemplified by instances where

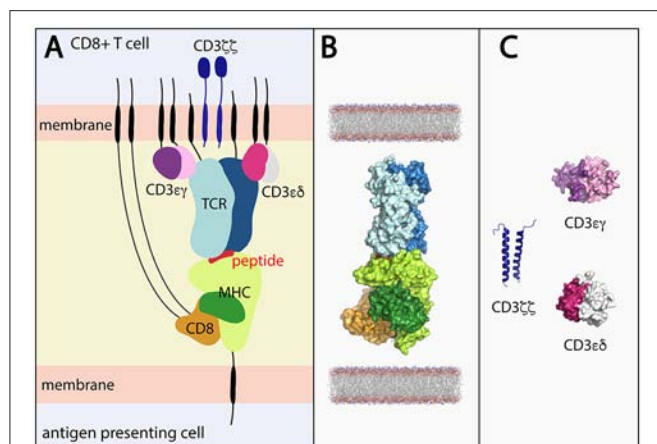


FIGURE 1 | (A) Stylized view of the pMHC-TCR and core components required for T cell signaling. **(B)** Components of the pMHC-TCR complex for which structures have been determined in combination with one another. The complex depicts peptide-bound HLA-A*02 in complex with CD8 and the B7 TCR (derived by superimposing components of PDB ID's 1AKJ and 1BD2). **(C)** Portions of the CD3εγ (ectodomain; PDB ID 1SY6), CD3εδ (ectodomain; PDB 1XIW) and CD3ζζ (TM domains; PDB ID 2MAC) signaling components have been structurally determined, but not in complex with the TCR. Black lines represent regions of conformational flexibility. TCR α and β chain shown in dark and light blue, respectively. MHC class I heavy and light (β₂-microglobulin) chain shown in light and dark green, respectively. Peptide shown in red. CD8αα shown in orange.

single amino acid changes in a peptide produce near-identical structural snapshots but different T cell outcomes (1–3). This indicates additional factors, concealed by X-ray crystallographic snapshots, are at play.

There is emerging evidence that conformational dynamics and dynamic allostery influences T cell recognition and activation (4–6), yet until recently, this has been overlooked in our effort to understand the structural basis of TCR recognition of the pMHC. The importance of conformational dynamics at the immune synapse has been the subject of excellent recent reviews [see, for example (7–12)]. In this review, we instead focus on relevant methodologies (highlighted in **Figure 2**), and specifically recent advances in computational, structural and biophysical techniques, and how they can be integrated to provide powerful insights into the key early stages of the TCR-pMHC interaction. Finally, we highlight integrated approaches that are being used to shed light on some of the key outstanding questions in the early events leading to TCR signaling.

X-RAY CRYSTALLOGRAPHY: PIONEERING ATOMIC RESOLUTION DETAILS

Typically, the structural flexibility of a TCR-pMHC system is solely interpreted from a single set of coordinates derived from X-ray diffraction data. In this case flexibility is merely inferred mainly by comparing structural differences between TCR-pMHC and their unbound constituents (13, 14) and the consideration of atomic temperature (B) factors. B-factors can be used to estimate atomic displacements that arise from static

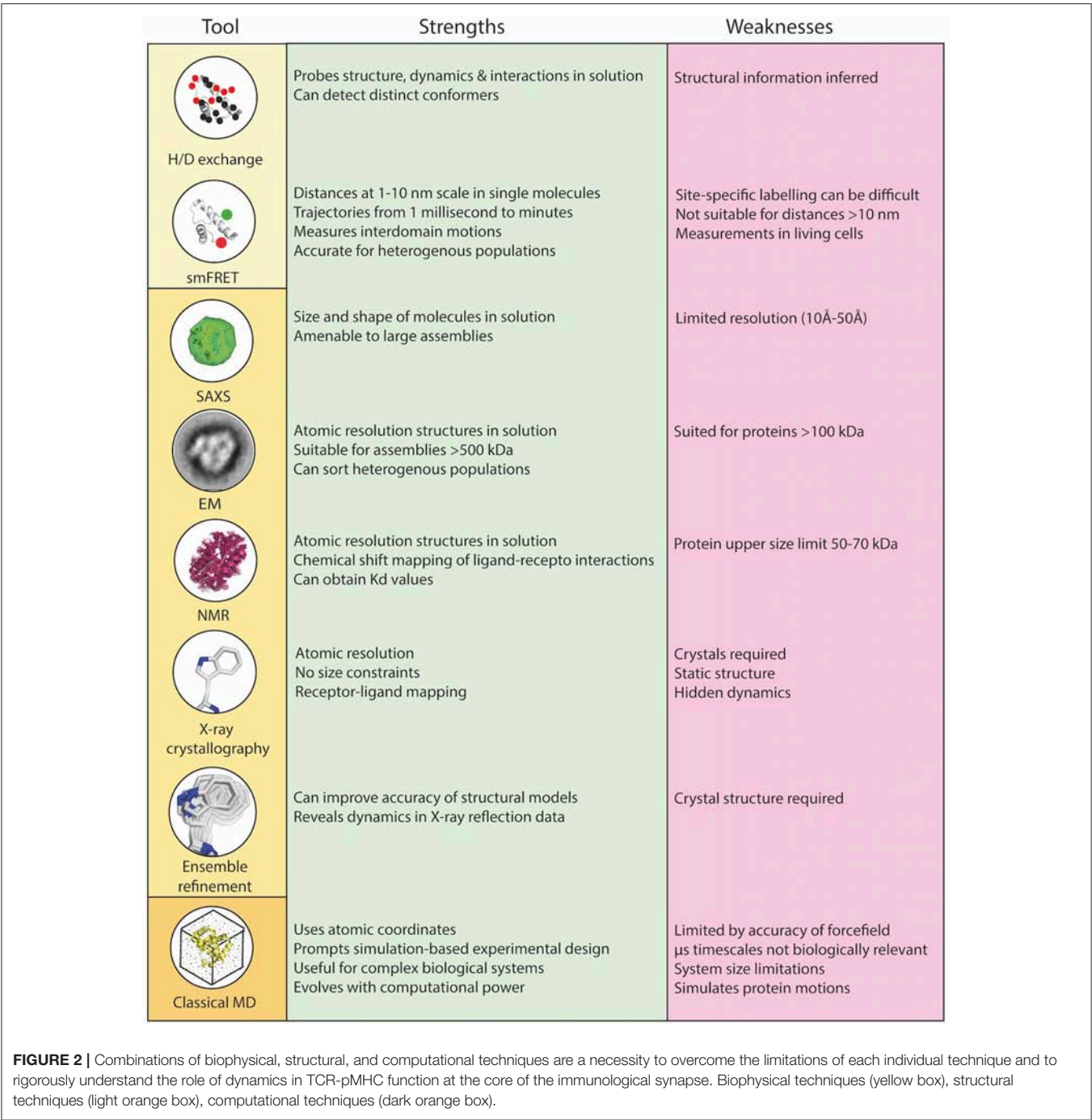
and dynamic disorder (alternative conformations in the crystal lattice, and atomic fluctuations in the crystal, respectively). By comparing identical molecules in different crystal lattices, the influence of crystal packing on protein structure can be analyzed (15–17). Indeed, crystal packing can select radically different conformations from a heterogeneous ensemble, giving clues to conformational dynamics (16). For example, structural variation of HLA-B*35:08-LPEP with SB27 TCR within two crystal forms suggested a “scanning” motion of the TCR on pMHC that was further supported and extended by molecular dynamics (MD) studies (3, 18).

Due to crystal packing and data collection at cryogenic temperatures, B-factors underrepresent the amplitude of conformational populations (19). Thus, due to the failure of current refinement algorithms to model structural heterogeneity, the analysis of single, static crystallographic models can reveal limited information on the dynamics of the system in solution (20–22). However, there are examples where flexibility insight has been successfully obtained and useful correlations made. For example, B-factor analysis of structures of HLA-B*35:01 and HLA-B*35:08, that differ by a single amino acid, but are bound to the same Epstein-Barr virus (EBV) peptide (HPVG) provided insights into the influence of MHC polymorphism on peptide mobility and the T cell response (23). Likewise, the structural comparison of a TCR in its unbound vs. pMHC-bound state revealed a conformational change in the A-B loop of the Cα domain that borders the CD3ε binding site (24) that was later verified to correlate with pMHC ligation (25).

In addition to concealing flexibility, the X-ray structures available are also incomplete and lack the core components necessary for signal transduction (**Figures 1B,C**). While the structures of TCR-pMHC, pMHC-CD8 (26–29), CD3 heterodimers (30–34), and CD8 homo/heterodimers (35, 36) have been determined, critically informative complexes such as CD8 and/or CD3 in complex with TCR-pMHC or even just TCR-CD3 are lacking due to the poor affinity of soluble CD3 and CD8 for the TCR and MHC, respectively (33, 37, 38). Also absent, due to technical challenges, are the stalk regions, membrane-spanning domains, and intracellular tails of the TCR, MHC, and CD8 and CD3 molecules, which play a role in complex assembly, the spatial organization of the components and signal transmission (39–46). Therefore, whilst X-ray crystallography can yield highly informative, high-resolution structures, its limitations necessitate the use of clever engineering and complementary techniques to make the next leap in terms of T cell signaling.

MOLECULAR DYNAMICS SIMULATIONS: PRODUCING TESTABLE HYPOTHESES AND PLACING EXPERIMENTAL FINDINGS INTO A THEORETICAL FRAMEWORK

Although conventional X-ray crystallographic analysis provides little information regarding dynamics, the exquisite resolution as well as model completeness has provided a solid data foundation that has spurred an increasing amount of MD



simulation studies. All-atom MD simulations probe the flexibility of the system by computing iterative solutions of Newton's equations of motion over time (47). The raw output of MD simulations—trajectories, describe the atomic positions at time-points during the simulation. Unfortunately however, MD is computationally demanding and limits the technique to examination of relatively short time spans (e.g., a microsecond), orders of magnitude shorter than more biologically relevant timescales over which many larger motions occur. The signal, for example, produced following T cell recognition of a

pMHC is of the seconds to minutes timescale (48–52). Several approaches have emerged that allow this limitation to be mitigated somewhat and here we discuss briefly below the most popular and useful approaches. Replica exchange MD (REMD) can improve the sampling by simulating multiple copies of the same molecule at different temperatures, allowing an unbiased way of improving conformational sampling (53). REMD is computationally intensive and therefore currently limited to relatively small systems, for example to investigate how polymorphic amino acid differences between allotypes alter

the conformational plasticity of the MHC class I binding pocket (54). Alternatively, steered MD (SMD) applies an external force to the protein to study its mechanical response, analogous to atomic force microscopy (55). This method is well suited to studying protein-protein interactions, and has been used to investigate TCR-pMHC dissociation (56–58). A novel use of SMD simulations based on agonist and non-agonist complex crystal structures was to develop a molecular model of TCR-pMHC “catch bond” formation (12). Catch bonds represent a net accumulation of molecular interactions under force, revealing an additional level of dynamic diversity built-in as a proofreading mechanism to link TCR recognition and subsequent activation. The related approaches of targeted MD (TMD) and umbrella sampling apply forces in order to promote new conformations, and are used to predict a pathway between two known conformations. Such “pulling” simulations, although not used to study dynamics *per se*, have been used to estimate the binding free energies between peptides and MHC (59). The atomic complexity of the system can be reduced significantly using coarse-grained (CG) methods in which groups of atoms are replaced by beads, allowing longer simulations at the cost of fine details (60). This approach is therefore useful for studying larger complexes such as TCR-pMHC in membrane (61) and TCR-pMHC-CD4 complexes (62). CG methods are complimentary to atomistic simulations and offer a feasible approach to tackling the combined challenges of large immune assemblies and long timescales associated with changes in membrane morphology. Accuracy of MD simulations are dependent upon available force fields—mathematical-physical descriptions of a system used to calculate the forces acting upon all atoms in order to solve Newton’s equations of motion. Force fields, though improving constantly, have known imperfections (63–65), so currently it is preferable to seek experimental validation. Getting stuck in local energy minima is a particular limitation, but energy landscapes can be sampled more efficiently using advanced adaptive sampling techniques such as Markov state models (66), allowing the identification of metastable states.

In summary, MD simulation is an increasingly important member of the toolbox, since it is particularly well suited to producing experimentally testable hypotheses as well as placing existing experimental findings into a theoretical framework. Despite the advances in enhanced sampling methods and possible simulation of larger complexes, MD simulations of experimentally-determined TCR-pMHC structures have not been widely adopted.

ENSEMBLE REFINEMENT: USING MD SIMULATIONS TO EXTRACT DYNAMICS FROM THE CRYSTALLINE STATE

Another way to explore protein dynamics from experimental diffraction data is by simultaneously performing short molecular dynamics simulations with structure refinement (20, 67, 68). This method, known as ensemble refinement, produces an ensemble of models of the same structure that provides extended biological insight, often whilst improving the refinement statistics [i.e., free

R-factor (R_{free})]. Although the ensemble refinement method has long been established and is well-validated (69–72), it is not routinely incorporated as a tool to analyze the X-ray structures of pMHC class I systems. This prompted our re-analysis of 11 published systems to reveal the dynamics present in the X-ray data, revealing the benefits of incorporating ensemble refinement to the structural interpretation (73).

A pertinent example of how ensemble refinement can extend and enrich existing crystallographic interpretations relates to the induced fit vs. conformational selection model of TCR binding to pMHC. In the induced fit model, the TCR undergoes a conformational change upon binding the pMHC, whereas in the conformational selection model a conformation that is compatible with binding is selected from an ensemble of conformations (74). Borbulevych et al. (75) sought to understand the causes of the cross-recognition of self- and non-self-peptides by the A6 TCR, by comparing the structures of the self-peptide HuD and the non-self-peptide Tax, both when bound to MHC HLA-A*02 alone and in a MHC-TCR complex. While the bound conformations are very similar for both peptides, differences in the orientation of the p3 and p5 side chains necessitate that the HuD peptide must undergo a conformational change in order to bind to the TCR (**Figure 3A**), while the Tax peptide does not. Our ensemble results, however, show that the Tyr3 and Phe5 residues in the HuD peptide are flexible enough to convert between the two conformations (**Figure 3B**). This indicates that the differences found between the MHC and TCR-pMHC conformations may be due to intrinsic flexibility rather than any change elicited by binding itself, and suggests that differences between static pMHC and TCR-pMHC may be due to a combination of the inherent flexibilities of each system, and the complexation process. Static structures may bias the interpretation in favor of an induced fit mechanism, whereas analysis as a conformational ensemble can allow also for conformational selection. Furthermore, reliance on single crystallographic structures of pMHC, with or without TCR, entails pitfalls for understanding the rules of productive TCR ligation, particularly for static interpretations involving fine details such as interaction networks and side chain orientation. Since in the worst cases, it may fail to properly distinguish real results from noise, it supports a view that biological observations should be explained through the properties of ensembles rather than isolated structures, as these are less prone to observer bias.

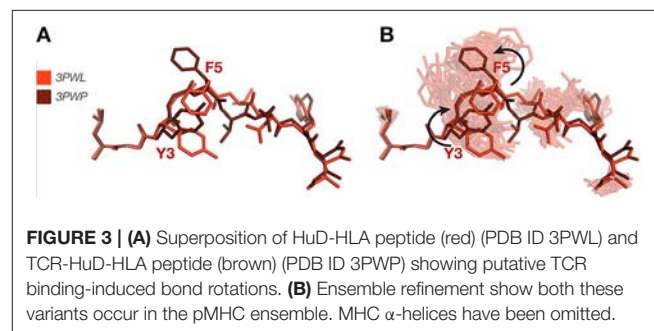


FIGURE 3 | (A) Superposition of HuD-HLA peptide (red) (PDB ID 3PWL) and TCR-HuD-HLA peptide (brown) (PDB ID 3PWP) showing putative TCR binding-induced bond rotations. **(B)** Ensemble refinement show both these variants occur in the pMHC ensemble. MHC α -helices have been omitted.

While we tend to describe TCR binding to the pMHC as either undergoing induced fit or conformational selection, the likelihood is that the TCR binds through a combination of both models. Scott et al. (6), highlight using time resolved fluorescence measurements, MD and structural and thermodynamic data that the CDR3 loops of a TCR can have varying degrees of flexibility. In the case of the A6 TCR the CDR3 β loop is highly flexible and can rapidly sample ligands with a range of conformations, whereas the CDR3 α has a slower motion that restricts the repertoire of peptides that it can bind. Therefore, the two models are not necessarily mutually exclusive, but instead describe a continuum (74).

Computing requirements of ensemble refinement are typically much greater than for single structure analysis, likely explaining the relatively slow adoption of this technique. However, continual improvements in refinement software [notably Phenix, which allows straightforward and user-friendly ensemble refinement from a PC (76)] and hardware have now placed this technique easily within the grasp of most structural immunologists. For example, in a recent ensemble refinement analysis of pMHC structures, refinement could be completed in <3 h for most systems, using a typical off-the-shelf desktop computer (73).

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR): ATOMISTIC DYNAMICS IN SOLUTION

NMR measures the absorbance and re-emission of electromagnetic radiation by nuclei in a magnetic field, and has provided significant information on protein structure and dynamics for the past 30 years. The use of NMR spectroscopy to study pMHC dynamics is complicated by the relatively large size of these systems (77), however several recent studies that have characterized TCR-pMHC binding have all found that significant conformational variation exists in the TCR, peptide, and MHC (78). In addition to noting conformational changes at the TCR-pMHC interface, conformational variation was also observed at remote sites, including within the membrane-proximal C β domain of the TCR, that implies an allosteric mechanism in TCR signaling (79), and the β_2m (β_2 -microglobulin) binding site on the MHC that was sensitive to MHC polymorphism and the bound peptide (80). Notably, each of these NMR studies benefitted from mapping chemical shift perturbations onto available X-ray structures, but revealed flexible regions not otherwise observed from the X-ray structures alone. NMR has also been used to validate MD predictions that show long-range allosteric communication between the TCR binding sites for pMHC and CD3, a key step in early T-cell activation (81). These NMR studies reiterate the need to characterize the TCR-pMHC system as an allosteric ensemble in which ligand binding alters the energy landscape of the entire ensemble. In an allosteric ensemble conformational changes that concurrently occur at distal sites, but do not necessarily dominate the ensemble, can be mapped to reveal cooperativity between sites, or dynamically-driven allostery, revealing previously hidden and unforeseen insights into signal transmission (82–84).

FLUORESCENCE SPECTROSCOPY: PROBING ENVIRONMENTAL DYNAMICS AND DISTANCES

The intrinsic fluorescence of aromatic (usually tryptophan) residues is sensitive to their environment, and can therefore be used to monitor dynamics. Fluorescence anisotropy, in particular, has become a powerful method with which to study pMHC-TCR dynamics, especially when coupled with other techniques (85–87). Dynamic insight has also been gained by Förster resonance energy transfer (FRET), in which energy transferred between a donor and an acceptor chromophore is used to measure distances between chromophore-labeled residues as a function of time, particularly powerful when combined with structural techniques (88–90). An elegant example of integrating experimental and computational approaches, from the protein folding field, combined small molecule (sm) FRET with advanced MD simulations and machine learning (91). Such a combined approach has not yet been reported for pMHC-TCR systems, but clearly holds much promise. Nevertheless, there are other examples pertaining to the value of the use of fluorescence to study pMHC-TCR systems. For example, site-directed fluorescence labeling, in which an extrinsic fluorescent probe is attached to a cysteine residue, has been used to note that the A-B loop within the TCR C α domain undergoes a conformational change upon pMHC ligation (25). FRET has also been used to measure intermolecular associations in live cells. Yachi et al. (92) measured the molecular interaction between TCR-CD3 ζ and CD8 on antigen presenting cells loaded with different peptides, revealing structurally similar peptides alter the kinetics of the CD8-TCR interaction and translate into differential T cell responses. Another study used a FRET sensor to map the spatiotemporal dynamics of protein clustering in live T cells, linking the molecular density of TCR clusters with TCR triggering (93). Clearly, our understanding of TCR-pMHC systems could benefit from the further integrated use of intramolecular and intermolecular FRET sensors, particularly when coupled with structural data.

HYDROGEN/DEUTERIUM (H/D) EXCHANGE: SOLVENT ACCESSIBILITY AND LOCAL DYNAMICS

H/D exchange involves a steady-state reaction in which deuterium atoms replace covalently bonded hydrogen atoms that are not participating in H-bonds. The rate of that exchange is usually measured by mass spectrometry, providing information on solvent accessibility and local dynamics. A study combining H/D exchange, fluorescence anisotropy, and structural analyses, showed that the flexibility of the peptide binding groove of the class I MHC protein HLA-A*02:01 varies significantly with different peptides (85). Further evidence for the role of conformational plasticity in peptide selection by MHC Class I was revealed by comparing H/D exchange of two allotypes in peptide-bound and free states (94). H/D exchange has also been used to probe the dynamics at the TCR-pMHC interface,

with several studies highlighting that conformational flexibility is contingent upon the MHC allele (95), the bound peptide (85), and upon TCR ligation (96) and all of which have implications for T cell signaling.

SMALL ANGLE X-RAY SCATTERING (SAXS): LOW RESOLUTION STRUCTURE IN SOLUTION

Despite inherent limitations in resolution that can be achieved, SAXS can be used to study the size, shape and assembly of proteins, without the size limitations of other techniques such as NMR (97). In a monodisperse solution, geometric parameters such as the maximum particle dimension (D_{max}), radius of gyration (R_g), and the forward scattering intensity, $I(0)$, can be calculated from SAXS data; these values can serve as a point of comparison with the dimensions provided by a crystal structure or when studying the same protein under various experimental conditions or in a liganded vs. unliganded state. For example, in conjunction with other techniques and by comparing the R_g of HLA-DR1 (MHC class II) bound to a wild-type peptide, or a weak- or tight-binding peptide variant Yin et al. (98, 99) correlated pMHC conformational differences with susceptibility to peptide exchange by the non-classical MHC class II molecule HLA-DM. In another pMHC class II system, SAXS was used to show the pathogen-derived proteins, Salp15 and gp120, caused binding-induced conformational changes in CD4 that subsequently influence CD4+ T cell activation during infection (100, 101).

SAXS can also be used to characterize polydisperse systems such as modular proteins with flexible linkers or proteins bearing disordered regions. The ensemble optimization method (EOM) (102) is one approach to describe this experimental SAXS data. It generates a pool of n independent models based upon the sequence and structural information of the target and then selects a subset of ensembles that best describe the experimental SAXS data. The distributions of the properties of the selected ensembles, including R_g (radius of gyration), D_{max} (maximum particle dimension), R_{flex} (measure of flexibility) and R_σ (variance of the ensemble distribution with respect to the original pool), can then be compared to those of the pool of independent models to assess the flexibility of the system. To the best of our knowledge EOM has not yet been used to study TCR-pMHC systems, despite both MHCs and TCRs being multidomain proteins with flexible linkers. It is thus highly feasible the interdomain motions of these proteins are coupled to binding events and are linked to signal transduction. On that note, the flexible stalks of the TCR, MHC, CD8, and CD3 molecules also likely play a role.

CONCLUSIONS AND FUTURE PERSPECTIVES

Protein flexibility is inherent to protein structure and function, and TCR-pMHC systems are no exception. Despite this the systematic analysis of the flexibility of TCR-pMHC systems is

lagging far behind that of other fields (103–105), particularly when it comes to integration of computational and experimental techniques.

We propose that to advance our mechanistic understanding of how TCR-pMHC engagement initiates intracellular signaling, and the influence of the peptide on the signal, that there needs to be a shift in our approach, both in terms of the suite of techniques used to assess flexibility, and use of creative engineering to surpass limitations specific to the molecules in question and their applicability to a technique. Elegant examples that illustrate the strength of this marriage are now emerging. For example, Natarajan et al. (38) overcame the size barrier that limits the study of the soluble TCR by NMR through the use of perdeuteration, and concomitantly simplified the NMR spectra using partial subunit labeling. This NMR approach combined with mutagenesis, computational docking, and validation using cell-based assays has enhanced our understanding of how the extracellular engagement of the TCR-CD3 complex transmits a signal. Likewise, Birnbaum et al. (106) implemented clever strategies to circumvent size limitations and issues pertaining to sample heterogeneity to use electron microscopy to observe the molecular architecture of the membrane-associated TCR-CD3 complex bound to pMHC. Using this approach combined with SAXS they put forward a ligand-dependent dimerization mechanism for TCR signaling in which flexibility plays a key role.

We also propose the ensemble refinement technique be used routinely in the X-ray crystallographic analysis of TCR-pMHC systems. The routine extraction of this data, and validation/interpretation in conjunction with other experimental techniques, some of which are summarized here, will provide previously hidden insights into the scope of conformational changes permissible by peptides when bound to MHC that influence TCR binding and T cell activation and will also reveal insights into how TCR flexibility and dynamically-driven allostery play a role. This hitherto missing information will enable us to more fully consider how a signal is transduced from the pMHC interface via the CD3 subunits and to determine how flexibility at the interface correlates with the degree of T cell stimulation (79, 107). This may provide new insights into how the T cell response can be therapeutically manipulated to fight infections or cancer. For example, by considering the flexibility of an MHC-bound peptide in conjunction with other peptide characteristics (such as amino acid sequence, prominence, solvent exposure, and affinity for MHC) we may more accurately predict epitope immunogenicity, particularly for neoantigen-based vaccine design (108–112). The use of polypeptide vaccines bearing HLA-restricted CD8+ T cell epitopes is fast gaining traction for cancer immunotherapy (108, 113, 114). The aim is to vaccinate individuals with mutated tumor-associated epitopes (mimotopes) that are then presented by MHC and in doing so stimulate CD8+ T cells that prevent tumor growth. Often mimotopes with enhanced binding to MHC and/or altered TCR interactions elicit a more effective tumor-specific T cell response (115–119) and so their rational design, facilitated by accurately predicting peptide immunogenicity (108, 120, 121), would be highly beneficial.

Likewise, the rational design, or engineering, of the antigen-binding site of TCRs with the same specificity, but enhanced affinity and kinetics for tumor antigens (which are mostly self-derived), has practical implications for soluble TCR-based therapy (113, 122) and adoptive T cell immunotherapies for cancer (123, 124). Both approaches require the considered engineering of high affinity TCRs that maintain their specificity for target tumor antigens. Although this has been accomplished using techniques such as directed evolution (125–130) and structure-based design (58) these experimental approaches are, in tandem, informing the development of computational approaches to predict how to manipulate TCR binding properties (58, 128), and there are indications that the accuracy of these computational approaches is enhanced by incorporating MD simulations for the consideration of protein flexibility (129, 131). However, application of simulation techniques lags markedly

behind other fields, so conceptual advances will require highly integrated experimental and computational approaches to fully understand, and exploit, the dynamics of the system.

AUTHOR CONTRIBUTIONS

AB and NB wrote the manuscript and NB produced **Figures 1,2**.

FUNDING

NB is funded by an ARC Future Fellowship (110100223).

ACKNOWLEDGMENTS

We thank James Fodor and Blake Riley for assistance with **Figure 3**.

REFERENCES

- Ding YH, Baker BM, Garboczi DN, Biddison WE, Wiley DC. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity* (1999) 11:45–56. doi: 10.1016/S1074-7613(00)80080-1
- Tynan FE, Borg NA, Miles JJ, Beddoe T, El-Hassen D, Silins SL, et al. High resolution structures of highly bulged viral epitopes bound to major histocompatibility complex class I. Implications for T-cell receptor engagement and T-cell immunodominance. *J Biol Chem*. (2005) 280:23900–9. doi: 10.1074/jbc.M503060200
- Reboul CF, Meyer GR, Porebski BT, Borg NA, Buckle AM. Epitope flexibility and dynamic footprint revealed by molecular dynamics of a pMHC-TCR complex. *PLoS Comput Biol*. (2012) 8:e1002404. doi: 10.1371/journal.pcbi.1002404
- Borbulevych OY, Piepenbrink KH, Gloor BE, Scott DR, Sommesse RF, Cole DK, et al. T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility. *Immunity* (2009) 31:885–96. doi: 10.1016/j.immuni.2009.11.003
- Insaiddo FK, Borbulevych OY, Hossain M, Santhanagopalan SM, Baxter TK, Baker BM. Loss of T cell antigen recognition arising from changes in peptide and major histocompatibility complex protein flexibility: implications for vaccine design. *J Biol Chem*. (2011) 286:40163–73. doi: 10.1074/jbc.M111.283564
- Scott DR, Borbulevych OY, Piepenbrink KH, Corcelli SA, Baker BM. Disparate degrees of hypervariable loop flexibility control T-cell receptor cross-reactivity, specificity, and binding mechanism. *J Mol Biol*. (2011) 414:385–400. doi: 10.1016/j.jmb.2011.10.006
- Ayres CM, Corcelli SA, Baker BM. Peptide and peptide-dependent motions in mhc proteins: immunological implications and biophysical underpinnings. *Front Immunol*. (2017) 8:935. doi: 10.3389/fimmu.2017.00935
- Van Hateren A, Bailey A, Elliott T. Recent advances in Major Histocompatibility Complex (MHC) class I antigen presentation: plastic MHC molecules and TAPBPR-mediated quality control. *Fl1000Res*. (2017) 6:158. doi: 10.12688/fl1000research.10474.1
- Wieczorek M, Abualrous ET, Sticht J, Alvaro-Benito M, Stolzenberg S, Noe F, et al. Major Histocompatibility Complex (MHC) Class I and MHC class II proteins: conformational plasticity in antigen presentation. *Front Immunol*. (2017) 8:292. doi: 10.3389/fimmu.2017.00292
- Yanaka S, Sugase K. Exploration of the conformational dynamics of major histocompatibility complex molecules. *Front Immunol*. (2017) 8:632. doi: 10.3389/fimmu.2017.00632
- Natarajan K, Jiang J, May NA, Mage MG, Boyd LE, Mcshan AC, et al. The role of molecular flexibility in antigen presentation and T cell receptor-mediated signaling. *Front Immunol*. (2018) 9:1657. doi: 10.3389/fimmu.2018.01657
- Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell* (2018) 174:672–687.e627. doi: 10.1016/j.cell.2018.06.017
- Reiser JB, Grégoire C, Darnault C, Mosser T, Guimezanes A, Schmitt-Verhulst AM, et al. A T cell receptor CDR3beta loop undergoes conformational changes of unprecedented magnitude upon binding to a peptide/MHC class I complex. *Immunity* (2002) 16:345–54. doi: 10.1016/S1074-7613(02)00288-1
- Tynan FE, Reid HH, Kjer-Nielsen L, Miles JJ, Wilce MC, Kostenko L, et al. A T cell receptor flattens a bulged antigenic peptide presented by a major histocompatibility complex class I molecule. *Nat Immunol*. (2007) 8:268–76. doi: 10.1038/ni1432
- Ely KR, Herron JN, Harker M, Edmundson AB. Three-dimensional structure of a light chain dimer crystallized in water. Conformational flexibility of a molecule in two crystal forms. *J Mol Biol*. (1989) 210:601–15. doi: 10.1016/0022-2836(89)90135-6
- Faber HR, Matthews BW. A mutant T4 lysozyme displays five different crystal conformations. *Nature* (1990) 348:263–6. doi: 10.1038/348263a0
- Eigenbrot C, Randal M, Kossiakoff AA. Structural effects induced by mutagenesis affected by crystal packing factors: the structure of a 30-51 disulfide mutant of basic pancreatic trypsin inhibitor. *Proteins* (1992) 14:75–87. doi: 10.1002/prot.340140109
- Tynan FE, Burrows SR, Buckle AM, Clements CS, Borg NA, Miles JJ, et al. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. *Nat Immunol*. (2005) 6:1114–22. doi: 10.1038/ni1257
- García AE, Krumhansl JA, Frauenfelder H. Variations on a theme by Debye and Waller: from simple crystals to proteins. *Prot Struct Funct Bioinformat*. (1997) 29:153–60. doi: 10.1002/(SICI)1097-0134(199710)29:2<153::AID-PROT3>3.0.CO;2-E
- Furnham N, Blundell TL, Depristo MA, Terwilliger TC. Is one solution good enough? *Nat Struct Mol Biol*. (2006) 13:184–5. doi: 10.1038/nsmb0306-184
- Woldeyes RA, Sivak DA, Fraser JS. E pluribus unum, no more: from one crystal, many conformations. *Curr Opin Struct Biol*. (2014) 28:56–62. doi: 10.1016/j.sbi.2014.07.005
- Van Den Bedem H, Fraser JS. Integrative, dynamic structural biology at atomic resolution [mdash] it's about time. *Nat Methods* (2015) 12:307–18. doi: 10.1038/nmeth.3324
- Miles JJ, Borg NA, Brennan RM, Tynan FE, Kjer-Nielsen L, Silins SL, et al. TCRa genes direct MHC restriction in the potent human T cell response to a class I-bound viral epitope. *J Immunol*. (2006) 177:6804–14. doi: 10.4049/jimmunol.177.10.6804
- Kjer-Nielsen L, Clements CS, Purcell AW, Brooks AG, Whisstock JC, Burrows SR, et al. A structural basis for the selection of dominant

- alphabeta T cell receptors in antiviral immunity. *Immunity* (2003) 18:53–64. doi: 10.1016/S1074-7613(02)00513-7
25. Beddoe T, Chen Z, Clements CS, Ely LK, Bushell SR, Vivian JP, et al. Antigen ligation triggers a conformational change within the constant domain of the alphabeta T cell receptor. *Immunity* (2009) 30:777–88. doi: 10.1016/j.immuni.2009.03.018
 26. Gao GF, Tormo J, Gerth UC, Wyer JR, McMichael AJ, Stuart DI, et al. Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature* (1997) 387:630–4. doi: 10.1038/42523
 27. Kern PS, Teng MK, Smolyar A, Liu JH, Liu J, Hussey RE, et al. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8alphaalpha ectodomain fragment in complex with H-2Kb. *Immunity* (1998) 9:519–30. doi: 10.1016/S1074-7613(00)80635-4
 28. Wang R, Natarajan K, Margulies DH. Structural basis of the CD8 alpha beta/MHC class I interaction: focused recognition orients CD8 beta to a T cell proximal position. *J Immunol.* (2009) 183:2554–64. doi: 10.4049/jimmunol.0901276
 29. Shi Y, Qi J, Iwamoto A, Gao GF. Plasticity of human CD8alphaalpha binding to peptide-HLA-A*2402. *Mol Immunol.* (2011) 48:2198–202. doi: 10.1016/j.molimm.2011.05.009
 30. Sun ZJ, Kim KS, Wagner G, Reinherz EL. Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. *Cell* (2001) 105:913–23. doi: 10.1016/S0092-8674(01)00395-6
 31. Arnett KL, Harrison SC, Wiley DC. Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. *Proc Natl Acad Sci USA.* (2004) 101:16268–73. doi: 10.1073/pnas.0407359101
 32. Kjer-Nielsen L, Dunstone MA, Kostenko I, Ely LK, Beddoe T, Mifsud NA, et al. Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. *Proc Natl Acad Sci USA.* (2004) 101:7675–80. doi: 10.1073/pnas.0402295101
 33. Sun ZY, Kim ST, Kim IC, Fahmy A, Reinherz EL, Wagner G. Solution structure of the CD3epsilon/delta ectodomain and comparison with CD3epsilon/gamma as a basis for modeling T cell receptor topology and signaling. *Proc Natl Acad Sci USA.* (2004) 101:16867–72. doi: 10.1073/pnas.0407576101
 34. Berry R, Headey SJ, Call MJ, McCluskey J, Tregaskes CA, Kaufman J, et al. Structure of the chicken CD3epsilon/delta/gamma heterodimer and its assembly with the alphabeta T cell receptor. *J Biol Chem.* (2014) 289:8240–51. doi: 10.1074/jbc.M113.544965
 35. Chang HC, Tan K, Ouyang J, Parisini E, Liu JH, Le Y, et al. Structural and mutational analyses of a CD8alphabeta heterodimer and comparison with the CD8alphaalpha homodimer. *Immunity* (2005) 23:661–71. doi: 10.1016/j.immuni.2005.11.002
 36. Liu Y, Li X, Qi J, Zhang N, Xia C. The structural basis of chicken, swine and bovine CD8alphaalpha dimers provides insight into the co-evolution with MHC I in endotherm species. *Sci Rep.* (2016) 6:24788. doi: 10.1038/srep24788
 37. He Y, Rangarajan S, Kerzic M, Luo M, Chen Y, Wang Q, et al. Identification of the docking site for CD3 on the T cell receptor beta chain by solution NMR. *J Biol Chem.* (2015) 290:19796–805. doi: 10.1074/jbc.M115.663799
 38. Natarajan A, Nadarajah V, Felsovalyi K, Wang W, Jeyachandran VR, Wasson RA, et al. Structural model of the extracellular assembly of the TCR-CD3 complex. *Cell Rep.* (2016) 14:2833–45. doi: 10.1016/j.celrep.2016.02.081
 39. Blumberg RS, Alarcon B, Sancho J, McDermott FV, Lopez P, Breitmeyer J, et al. Assembly and function of the T cell antigen receptor. Requirement of either the lysine or arginine residues in the transmembrane region of the alpha chain. *J Biol Chem* (1990) 265:14036–43.
 40. Call ME, Pyrdol J, Wiedmann M, Wucherpfennig KW. The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell* (2002) 111:967–79. doi: 10.1016/S0092-8674(02)01194-7
 41. Burrows SR, Chen Z, Archbold JK, Tynan FE, Beddoe T, Kjer-Nielsen L, et al. Hard wiring of T cell receptor specificity for the major histocompatibility complex is underpinned by TCR adaptability. *Proc Natl Acad Sci USA.* (2010) 107:10608–13. doi: 10.1073/pnas.1004926107
 42. Yin Y, Wang XX, Mariuzza RA. Crystal structure of a complete ternary complex of T-cell receptor, peptide-MHC, and CD4. *Proc Natl Acad Sci USA.* (2012) 109:5405–10. doi: 10.1073/pnas.1118801109
 43. Li Y, Yin Y, Mariuzza RA. Structural and biophysical insights into the role of CD4 and CD8 in T cell activation. *Front Immunol.* (2013) 4:206. doi: 10.3389/fimmu.2013.00206
 44. Gras S, Chadderton J, Del Campo CM, Farenc C, Wiede F, Josephs TM, et al. Reversed T cell receptor docking on a major histocompatibility class I complex limits involvement in the immune response. *Immunity* (2016) 45:749–60. doi: 10.1016/j.immuni.2016.09.007
 45. Merkle PS, Irving M, Hongjian S, Ferber M, Jorgensen TJD, Scholten K, et al. The T-cell receptor can bind to the peptide-bound major histocompatibility complex and uncomplexed beta2-microglobulin through distinct binding sites. *Biochemistry* (2017) 56:3945–61. doi: 10.1021/acs.biochem.7b00385
 46. Sullivan LC, Walpole NG, Farenc C, Pietra G, Sum MJW, Clements CS, et al. A conserved energetic footprint underpins recognition of human leukocyte antigen-E by two distinct alphabeta T cell receptors. *J Biol Chem.* (2017) 292:21149–58. doi: 10.1074/jbc.M117.807719
 47. Kass I, Buckle AM, Borg NA. Understanding the structural dynamics of TCR-pMHC complex interactions. *Trends Immunol.* (2014) 35:604–12. doi: 10.1016/j.it.2014.10.005
 48. Boniface JJ, Reich Z, Lyons DS, Davis MM. Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. *Proc Natl Acad Sci USA.* (1999) 96:11446–51. doi: 10.1073/pnas.96.20.11446
 49. Huse M, Klein LO, Girvin AT, Faraj JM, Li Q-J, Kuhns MS, et al. Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. *Immunity* (2007) 27:76–88. doi: 10.1016/j.immuni.2007.05.017
 50. Dustin ML, Groves JT. Receptor signaling clusters in the immune synapse. *Annu Rev Biophys.* (2012) 41:543–56. doi: 10.1146/annurev-biophys-042910-155238
 51. Brodovitch A, Bongrand P, Pierres A. T lymphocytes sense antigens within seconds and make a decision within one minute. *J Immunol.* (2013) 191:2064–71. doi: 10.4049/jimmunol.1300523
 52. Guedj C, Abraham N, Jullie D, Randriamampita C. T cell adhesion triggers an early signaling pole distal to the immune synapse. *J Cell Sci.* (2016) 129:2526–37. doi: 10.1242/jcs.182311
 53. Zhou R. Replica exchange molecular dynamics method for protein folding simulation. *Methods Mol Biol.* (2007) 350:205–23. doi: 10.1385/1-59745-189-4:205
 54. Ostermeir K, Springer S, Zacharias M. Coupling between side chain interactions and binding pocket flexibility in HLA-B*44:02 molecules investigated by molecular dynamics simulations. *Mol Immunol.* (2015) 63:312–9. doi: 10.1016/j.molimm.2014.07.021
 55. Izrailev S, Stepaniants S, Israilewitz B, Kosztin D, Lu H, Molnar F., et al. Steered molecular dynamics. In: Denfahrd P, Hermans J, Leimkuhler B, Mark A, Skeel R, Reich S, editors. *Computational Molecular Dynamics: Challenges, Methods, Ideas.* Berlin: Springer-Verlag (1998). p. 39–65.
 56. Cuendet MA, Michielin O. Protein-protein interaction investigated by steered molecular dynamics: the TCR-pMHC complex. *Biophys J.* (2008) 95:3575–90. doi: 10.1529/biophysj.108.131383
 57. Cuendet MA, Zoete V, Michielin O. How T cell receptors interact with peptide-MHCs: a multiple steered molecular dynamics study. *Proteins* (2011) 79:3007–24. doi: 10.1002/prot.23104
 58. Zoete V, Irving M, Ferber M, Cuendet MA, Michielin O. Structure-based, rational design of T cell receptors. *Front Immunol.* (2013) 4:268. doi: 10.3389/fimmu.2013.00268
 59. Huang M, Huang W, Wen F, Larson RG. Efficient estimation of binding free energies between peptides and an MHC class II molecule using coarse-grained molecular dynamics simulations with a weighted histogram analysis method. *J Comput Chem.* (2017) 38:2007–19. doi: 10.1002/jcc.24845
 60. Marrink SJ, Risselada HJ, Yefimov S, Tieleman DP, De Vries AH. The MARTINI force field: coarse grained model for biomolecular simulations. *J Phys Chem B* (2007) 111:7812–24. doi: 10.1021/jp071097f
 61. Chen J, Xie ZR, Wu Y. Study of protein structural deformations under external mechanical perturbations by a coarse-grained simulation method. *Biomech Model Mechanobiol.* (2016) 15:317–29. doi: 10.1007/s10237-015-0690-0

62. Wan S, Flower DR, Coveney PV. Toward an atomistic understanding of the immune synapse: large-scale molecular dynamics simulation of a membrane-embedded TCR-pMHC-CD4 complex. *Mol Immunol.* (2008) 45:1221–30. doi: 10.1016/j.molimm.2007.09.022
63. Lange OF, Van Der Spoel D, De Groot BL. Scrutinizing molecular mechanics force fields on the submicrosecond timescale with NMR data. *Biophys J.* (2010) 99:647–55. doi: 10.1016/j.bpj.2010.04.062
64. Piana S, Lindorff-Larsen K, Shaw DE. How robust are protein folding simulations with respect to force field parameterization? *Biophys J.* (2011) 100:L47–L49. doi: 10.1016/j.bpj.2011.03.051
65. Lindorff-Larsen K, Maragakis P, Piana S, Eastwood MP, Dror RO, Shaw DE. Systematic validation of protein force fields against experimental data. *PLoS ONE* (2012) 7:e32131. doi: 10.1371/journal.pone.0032131
66. Pande VS, Beauchamp K, Bowman GR. Everything you wanted to know about Markov State Models but were afraid to ask. *Methods* (2010) 52:99–105. doi: 10.1016/j.ymeth.2010.06.002
67. Levin EJ, Kondrashov DA, Wesenberg GE, Phillips GN Jr. Ensemble refinement of protein crystal structures: validation and application. *Structure* (2007) 15:1040–52. doi: 10.1016/j.str.2007.06.019
68. Burnley BT, Afonine PV, Adams PD, Gros P. Modelling dynamics in protein crystal structures by ensemble refinement. *Elife* (2012) 1:e00311. doi: 10.7554/eLife.00311
69. Forneris F, Burnley BT, Gros P. Ensemble refinement shows conformational flexibility in crystal structures of human complement factor D. *Acta Crystallogr Sect D* (2014) 70:733–43. doi: 10.1107/S1399004713032549
70. Weerth RS, Michalska K, Bingman CA, Yennamalli RM, Li H, Jedrzejczak R, et al. Structure of a cupin protein Plu4264 from *Photobacterium luminescens* subsp. *laumondii* TTO1 at 1.35 Å resolution. *Proteins* (2015) 83:383–8. doi: 10.1002/prot.24705
71. Cao H, Tan K, Wang F, Bigelow L, Yennamalli RM, Jedrzejczak R, et al. Structural dynamics of a methionine gamma-lyase for calicheamicin biosynthesis: rotation of the conserved tyrosine stacking with pyridoxal phosphate. *Struct Dyn.* (2016) 3:034702. doi: 10.1063/1.4948539
72. Correy GJ, Carr PD, Meirles T, Mabbitt PD, Fraser NJ, Weik M, et al. Mapping the accessible conformational landscape of an insect carboxylesterase using conformational ensemble analysis and kinetic crystallography. *Structure* (2016) 24:977–87. doi: 10.1016/j.str.2016.04.009
73. Fodor J, Riley BT, Borg NA, Buckle AM. Previously hidden dynamics at the TCR-Peptide-MHC interface revealed. *J Immunol.* (2018) 200:4134–45. doi: 10.4049/jimmunol.1800315
74. Cserrmely P, Palotai R, Nussinov R. Induced fit, conformational selection and independent dynamic segments: an extended view of binding events. *Trends Biochem Sci.* (2010) 35:539–46. doi: 10.1016/j.tibs.2010.04.009
75. Borbulevych OY, Piepenbrink KH, Baker BM. Conformational melting permits a conserved binding geometry in TCR recognition of foreign and self molecular mimics. *J Immunol.* (2011) 186:2950–8. doi: 10.4049/jimmunol.1003150
76. Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. Sect D* (2010) 66:213–21. doi: 10.1107/S0907444909052925
77. Insaído FK, Zajicek J, Baker BM. A general and efficient approach for NMR studies of peptide dynamics in class I MHC peptide binding grooves. *Biochemistry* (2009) 48:9708–10. doi: 10.1021/bi9008787
78. Hawse WF, De S, Greenwood AI, Nicholson LK, Zajicek J, Kovrigin EL, et al. TCR scanning of peptide/MHC through complementary matching of receptor and ligand molecular flexibility. *J Immunol.* (2014) 192:2885–91. doi: 10.4049/jimmunol.1302953
79. Natarajan K, Mcshan AC, Jiang J, Kumirov VK, Wang R, Zhao H, et al. An allosteric site in the T-cell receptor C β domain plays a critical signalling role. *Nat Commun.* (2017) 8:15260. doi: 10.1038/ncomms15260
80. Beerbaum M, Ballaschk M, Erdmann N, Schnick C, Diehl A, Uchanska-Ziegler B, et al. NMR spectroscopy reveals unexpected structural variation at the protein-protein interface in MHC class I molecules. *J Biomol NMR* (2013) 57:167–78. doi: 10.1007/s10858-013-9777-z
81. Rangarajan S, He Y, Chen Y, Kerzic MC, Ma B, Gowthaman R, et al. Peptide-MHC (pMHC) binding to a human antiviral T cell receptor induces long-range allosteric communication between pMHC- and CD3-binding sites. *J Biol Chem.* (2018) 293:15991–6005. doi: 10.1074/jbc.RA118.003832
82. Cooper A, Dryden DT. Allostery without conformational change. A plausible model. *Eur Biophys J.* (1984) 11:103–9. doi: 10.1007/BF00276625
83. Smock RG, Gierasch LM. Sending signals dynamically. *Science* (2009) 324:198–203. doi: 10.1126/science.1169377
84. Motlagh HN, Wrabl JO, Li J, Hilser VJ. The ensemble nature of allostery. *Nature* (2014) 508:331–9. doi: 10.1038/nature13001
85. Hawse WF, Gloor BE, Ayres CM, Kho K, Nuter E, Baker BM. Peptide modulation of class I major histocompatibility complex protein molecular flexibility and the implications for immune recognition. *J Biol Chem.* (2013) 288:24372–81. doi: 10.1074/jbc.M113.490664
86. Kozono H, Matsushita Y, Ogawa N, Kozono Y, Miyabe T, Sekiguchi H, et al. Single-Molecule Motions of MHC class II rely on bound peptides. *Biophys J.* (2015) 108:350–9. doi: 10.1016/j.bpj.2014.12.004
87. Pohlmann T, Bockmann RA, Grubmüller H, Uchanska-Ziegler B, Ziegler A, Alexiev U. Differential peptide dynamics is linked to major histocompatibility complex polymorphism. *J Biol Chem.* (2004) 279:28197–201. doi: 10.1074/jbc.C400128200
88. Gakamsky DM, Lewitzki E, Grell E, Saulquin X, Malissen B, Montero-Julian F, et al. Kinetic evidence for a ligand-binding-induced conformational transition in the T cell receptor. *Proc Natl Acad Sci USA.* (2007) 104:16639–44. doi: 10.1073/pnas.0707061104
89. Huppa JB, Axmann M, Mortelmaier MA, Lillemeier BF, Newell EW, Brameshuber M, et al. TCR-peptide-MHC interactions *in situ* show accelerated kinetics and increased affinity. *Nature* (2010) 463:963–7. doi: 10.1038/nature08746
90. Schutz GJ, Huppa JB. Förster resonance energy transfer to study TCR-pMHC interactions in the immunological synapse. *Methods Mol Biol.* (2017) 1584:207–29. doi: 10.1007/978-1-4939-6881-7_14
91. Matsunaga Y, Sugita Y. Linking time-series of single-molecule experiments with molecular dynamics simulations by machine learning. *Elife* (2018) 7:e32668. doi: 10.7554/eLife.32668
92. Yachi PP, Ampudia J, Zal T, Gascoigne NR. Altered peptide ligands induce delayed CD8-T cell receptor interaction—a role for CD8 in distinguishing antigen quality. *Immunity* (2006) 25:203–11. doi: 10.1016/j.immuni.2006.05.015
93. Ma Y, Pandzic E, Nicovich PR, Yamamoto Y, Kwiatek J, Pigeon SV, et al. An intermolecular FRET sensor detects the dynamics of T cell receptor clustering. *Nat Commun.* (2017) 8:15100. doi: 10.1038/ncomms15100
94. Van Hateren A, Anderson M, Bailey A, Werner JM, Skipp P, Elliott T. Direct evidence for conformational dynamics in major histocompatibility complex class I molecules. *J Biol Chem.* (2017) 292:20255–69. doi: 10.1074/jbc.M117.809624
95. Fabian H, Huser H, Narzi D, Misselwitz R, Loll B, Ziegler A, et al. HLA-B27 subtypes differentially associated with disease exhibit conformational differences in solution. *J Mol Biol.* (2008) 376:798–810. doi: 10.1016/j.jmb.2007.12.009
96. Hawse WF, Champion MM, Joyce MV, Hellman LM, Hossain M, Ryan V, et al. Cutting edge: evidence for a dynamically driven T cell signaling mechanism. *J Immunol.* (2012) 188:5819–23. doi: 10.4049/jimmunol.1200952
97. Putnam CD, Hammel M, Hura GL, Tainer JA. X-ray solution scattering (SAXS) combined with crystallography and computation: defining accurate macromolecular structures, conformations and assemblies in solution. *Q Rev Biophys.* (2007) 40:191–285. doi: 10.1017/S0033583507004635
98. Zarutskie JA, Sato AK, Rushe MM, Chan IC, Lomakin A, Benedek GB, et al. A conformational change in the human major histocompatibility complex protein HLA-DR1 induced by peptide binding. *Biochemistry* (1999) 38:5878–87. doi: 10.1021/bi983048m
99. Yin L, Trentham P, Guce A, Wiczorek M, Lange S, Sticht J, et al. Susceptibility to HLA-DM protein is determined by a dynamic conformation of major histocompatibility complex class II molecule bound with peptide. *J Biol Chem.* (2014) 289:23449–64. doi: 10.1074/jbc.M114.585539

100. Ashish JIJ, Garg R, Boone CD, Anguita J, Krueger JK. Conformational rearrangement within the soluble domains of the CD4 receptor is ligand-specific. *J Biol Chem.* (2008) 283:2761–72. doi: 10.1074/jbc.M708325200
101. Zimmermann K, Liechti T, Haas A, Rehr M, Trkola A, Gunthard HF, et al. The orientation of HIV-1 gp120 binding to the CD4 receptor differentially modulates CD4+ T cell activation. *J Immunol.* (2015) 194:637–49. doi: 10.4049/jimmunol.1401863
102. Tria G, Mertens HD, Kachala M, Svergun DI. Advanced ensemble modelling of flexible macromolecules using X-ray solution scattering. *IUCr.* (2015) 2:207–17. doi: 10.1107/S205225251500202X
103. Schaeffer RD, Fersht A, Daggett V. Combining experiment and simulation in protein folding: closing the gap for small model systems. *Curr Opin Struct Biol.* (2008) 18:4–9. doi: 10.1016/j.sbi.2007.11.007
104. Shaw DE, Maragakis P, Lindorff-Larsen K, Piana S, Dror RO, Eastwood MP, et al. Atomic-level characterization of the structural dynamics of proteins. *Science* (2010) 330:341–6. doi: 10.1126/science.1187409
105. Best RB. Atomistic molecular simulations of protein folding. *Curr Opin Struct Biol.* (2012) 22:52–61. doi: 10.1016/j.sbi.2011.12.001
106. Birnbaum ME, Berry R, Hsiao YS, Chen Z, Shingu-Vazquez MA, Yu X, et al. Molecular architecture of the alphabeta T cell receptor-CD3 complex. *Proc Natl Acad Sci USA.* (2014) 111:17576–81. doi: 10.1073/pnas.1420936111
107. Park S, Krshnan L, Call MJ, Call ME, Im W. Structural conservation and effects of alterations in T cell receptor transmembrane interfaces. *Biophys J.* (2018) 114:1030–5. doi: 10.1016/j.bpj.2018.01.004
108. Balachandran VP, Luksza M, Zhao JN, Makarov V, Moral JA, Remark R, et al. Identification of unique neoantigen qualities in long-term survivors of pancreatic cancer. *Nature* (2017) 551:512–6. doi: 10.1038/nature24462
109. Lauss M, Donia M, Harbst K, Andersen R, Mitra S, Rosengren F, et al. Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. *Nat Commun.* (2017) 8:1738. doi: 10.1038/s41467-017-01460-0
110. Wirth TC, Kuhnel F. Neoantigen targeting-dawn of a new era in cancer immunotherapy? *Front Immunol.* (2017) 8:1848. doi: 10.3389/fimmu.2017.01848
111. Aurisicchio L, Pallocca M, Ciliberto G, Palombo F. The perfect personalized cancer therapy: cancer vaccines against neoantigens. *J Exp Clin Cancer Res.* (2018) 37:86. doi: 10.1186/s13046-018-0751-1
112. Nogueira C, Kaufmann JK, Lam H, Flechtner JB. Improving cancer immunotherapies through empirical neoantigen selection. *Trends Cancer* (2018) 4:97–100. doi: 10.1016/j.trecan.2017.12.003
113. Boudousquie C, Bossi G, Hurst JM, Rygiel KA, Jakobsen BK, Hassan NJ. Polyfunctional response by ImmTAC (IMCgp100) redirected CD8(+) and CD4(+) T cells. *Immunology* (2017) 152:425–38. doi: 10.1111/imm.12779
114. Kuai R, Ochyl LJ, Bahjat KS, Schwendeman A, Moon JJ. Designer vaccine nanodiscs for personalized cancer immunotherapy. *Nat Mater.* (2017) 16:489–96. doi: 10.1038/nmat4822
115. Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J Immunol.* (1996) 157:2539–48.
116. Zaremba S, Barzaga E, Zhu M, Soares N, Tsang KY, Schlom J. Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. *Cancer Res.* (1997) 57:4570–7.
117. Salazar E, Zaremba S, Arlen PM, Tsang KY, Schlom J. Agonist peptide from a cytotoxic t-lymphocyte epitope of human carcinoembryonic antigen stimulates production of tcl-type cytokines and increases tyrosine phosphorylation more efficiently than cognate peptide. *Int J Cancer* (2000) 85:829–38. doi: 10.1002/(SICI)1097-0215(20000315)85:6<829::AID-IJC16>3.0.CO;2-K
118. Borbulevych OY, Baxter TK, Yu Z, Restifo NP, Baker BM. Increased immunogenicity of an anchor-modified tumor-associated antigen is due to the enhanced stability of the peptide/MHC complex: implications for vaccine design. *J Immunol.* (2005) 174:4812–20. doi: 10.4049/jimmunol.174.8.4812
119. McMahan RH, McWilliams JA, Jordan KR, Dow SW, Wilson DB, Slansky JE. Relating TCR-peptide-MHC affinity to immunogenicity for the design of tumor vaccines. *J Clin Invest.* (2006) 116:2543–51. doi: 10.1172/JCI26936
120. Rapin N, Lund O, Bernaschi M, Castiglione F. Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. *PLoS ONE* (2010) 5:e9862. doi: 10.1371/journal.pone.0009862
121. Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, et al. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* (2012) 482:400–4. doi: 10.1038/nature10755
122. McCormack E, Adams KJ, Hassan NJ, Kotian A, Lissin NM, Sami M, et al. Bi-specific TCR-anti CD3 redirected T-cell targeting of NY-ESO-1- and LAGE-1-positive tumors. *Cancer Immunol Immunother.* (2013) 62:773–85. doi: 10.1007/s00262-012-1384-4
123. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol.* (2011) 29:917–24. doi: 10.1200/JCO.2010.32.2537
124. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med.* (2015) 21:914–21. doi: 10.1038/nm.3910
125. Holler PD, Holman PO, Shusta EV, O'herrin S, Wittrup KD, Kranz DM. *In vitro* evolution of a T cell receptor with high affinity for peptide/MHC. *Proc Natl Acad Sci USA.* (2000) 97:5387–92. doi: 10.1073/pnas.080078297
126. Li Y, Moysey R, Molloy PE, Vuidepot AL, Mahon T, Baston E, et al. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat Biotechnol.* (2005) 23:349–54. doi: 10.1038/nbt1070
127. Dunn SM, Rizkallah PJ, Baston E, Mahon T, Cameron B, Moysey R, et al. Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide-MHC without increasing apparent cross-reactivity. *Protein Sci.* (2006) 15:710–21. doi: 10.1110/ps.051936406
128. Pierce BG, Hellman LM, Hossain M, Singh NK, Vander Kooi CW, Weng Z, et al. Computational design of the affinity and specificity of a therapeutic T cell receptor. *PLoS Comput Biol.* (2014) 10:e1003478. doi: 10.1371/journal.pcbi.1003478
129. Smith SN, Wang Y, Baylon JL, Singh NK, Baker BM, Tajkhorshid E, et al. Changing the peptide specificity of a human T-cell receptor by directed evolution. *Nat Commun.* (2014) 5:5223. doi: 10.1038/ncomms6223
130. Harris DT, Wang N, Riley TP, Anderson SD, Singh NK, Procko E, et al. Deep mutational scans as a guide to engineering high affinity T cell receptor interactions with peptide-bound major histocompatibility complex. *J Biol Chem.* (2016) 291:24566–78. doi: 10.1074/jbc.M116.748681
131. Riley TP, Ayres CM, Hellman LM, Singh NK, Cosiano M, Cimens JM, et al. A generalized framework for computational design and mutational scanning of T-cell receptor binding interfaces. *Protein Eng Des Sel.* (2016) 29:595–606. doi: 10.1093/protein/gzw050

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Structural Model of the mIgM B-Cell Receptor Transmembrane Domain From Self-Association Molecular Dynamics Simulations

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 05 September 2018

Accepted: 30 November 2018

Published: 17 December 2018

Citation:

Friess MD, Pluhackova K and
Böckmann RA (2018) Structural
Model of the mIgM B-Cell Receptor
Transmembrane Domain From
Self-Association Molecular Dynamics
Simulations. *Front. Immunol.* 9:2947.
doi: 10.3389/fimmu.2018.02947

Antigen binding to B-cell antigen receptors (BCRs) followed by signaling initiates the humoral immune response. The signaling is intimately coupled to nanoclustering of BCRs and their sorting to specific membrane domains, a process that is ruled by interactions between the BCR transmembrane domain and lipids. While the structure of the extracellular domains of BCRs has been resolved, little is known about the configuration of the constituting four immunoglobulin domains spanning the membrane. Here, we modeled the structure of the transmembrane (TM) domain of the IgM B-cell receptor using self-assembly coarse-grained molecular dynamics simulations. The obtained quaternary structure was validated against available experimental data and atomistic simulations. The IgM-BCR-TM domain configuration shows a 1:1 stoichiometry between the homodimeric membrane-bound domain of IgM (mIgM) and a Ig- α /Ig- β heterodimer. The mIgM homodimer is based on an asymmetric association of two mIgM domains. We show that a specific site of the Ig- α /Ig- β heterodimer is responsible for the association of IgM-BCRs with lipid rafts. Our results further suggest that this site is blocked in small-sized IgM-BCR clusters. The BCR TM structure provides a molecular basis for the previously suggested dissociation activation model of B-cell receptors. Self-assembly molecular dynamics simulations at the coarse-grained scale here proved as a versatile tool in the study of receptor complexes.

Keywords: B-cell receptor, transmembrane domain, nanodomains, self-assembly, molecular dynamics simulations, coarse-grained simulations, dissociation activation model

1. INTRODUCTION

As one of the main parts of the adaptive immune system, B cells play a key role in the protection against pathogens. Defects during B-cell development and selection may lead to resistance against healthy tissue resulting in autoimmunity, malignancy, or allergy (1). B cells recognize and fight pathogens by the help of proteins called immunoglobulins (Ig). The five immunoglobulin isotypes (IgA, IgD, IgE, IgG, and IgM) can either be secreted (sIgs) or membrane-bound (mIgs) on the cell surface. The membrane-bound immunoglobulins (mIgA, mIgD, mIgE, mIgG, and mIgM) are components of the so-called B-cell receptors (BCR).

The membrane-anchorage of mIgs, which are tetramers consisting of two identical heavy (μ) and two identical light chains, is granted only by the C-terminal ends of both heavy chains. In case of mIgM, the C-terminal parts can be further divided into three domains, namely the extracellular

Without any antagonistic force, evasion from the energetically unfavorable monomeric state and aggregation of hydrophilic areas is a plausible scenario (28, 29). The burial of polar TMD-areas could be controlled and stabilized by changes in the lipid environment, taking part in the BCR cluster formation and cluster opening processes. Alternatively, specific protein–lipid interactions may as well stabilize the BCR monomeric state and prevent reassembly, as it is required for BCR activation. This is in line with a recent *in silico* study of the dimerization of the G protein coupled receptor CXCR4, which revealed a cholesterol-dependent dimerization site that could be blocked by cholesterol (30).

Motivated by the reported major role of the TMD in mIgM – Ig- α /Ig- β assembly as well as in IgM-BCR oligomerization and the association of resting or active BCRs with different membrane nanodomains, this study focuses on the quaternary configuration of the IgM-BCR-TM domain and the underlying mechanisms of IgM-BCR-TMD – lipid raft association upon BCR activation. Ensembles of coarse-grained molecular dynamics (MD) simulations were employed to study the spontaneous association of the TM domains of mIgM and of Ig- α /Ig- β , accompanied by atomistic-scale MD simulations addressing the stability of obtained quaternary structures. This approach was shown before to yield excellent results for the dimerization and oligomerization of TM peptides (31–33) but as well for the homo- and heterodimerization of GPCRs (30, 34), or the adsorption of peptides to membrane interfaces (35).

We report a BCR-TMD configuration that is in agreement with the available experimental data. The Ig- α /Ig- β lipid interface is shown to drive the association of IgM-BCR-TMD to lipid raft-like domains. Shielding of this interface upon IgM-BCR oligomerization is suggested to suspend BCR lipid raft association; In turn, BCR cluster opening upon activation would shift the preferred BCR-TMD environment toward the disordered membrane phase.

2. MATERIALS AND METHODS

2.1. Coarse-Grained Simulations

The transmembrane domain of the B-cell receptor consists of four TM α -helices: the mIgM TMD homodimer and one α -helix of Ig- α and Ig- β , respectively. Association of the BCR-TMD was addressed in separate self-assembly MD simulations of the mIgM TMD monomers to a homodimer, of the Ig- α and Ig- β helices to a heterodimer, and finally of the pre-assembled mIgM TMD homodimer and the Ig- α /Ig- β heterodimer to the full IgM-BCR-TMD (see also **Figure 1**).

All coarse-grained simulations were prepared using the docking assay for transmembrane components (DAFT) scheme (31), combined with the Gromacs 4.6 simulation suite (36) and the coarse-grained MARTINI force field (37, 38). DAFT allows to efficiently setup a large number of oligomerization simulations starting from unbiased initial states. Thereby, ensembles of associating or non-associating proteins are obtained that mirror the underlying energy landscape and provide a converged view on protein-protein and protein-lipid interaction interfaces.

Input structures for self-association simulations of isolated TM α -helices were based on the sequences of the individual IgM-BCR components (**Table 1**). PyMOL (39) was used for modeling of the α -helical input structures (mIgM TMD, Ig- α and Ig- β TMDs; **Table 2**, Steps 1 and 2). Association of the full BCR TM complex was based on association simulations of pre-formed mIgM homodimer and the Ig- α /Ig- β heterodimer complexes (compare Results section, **Table 2**, Steps 3a, 3b, 5, and 6). Due to the high amount of charged residues surrounding the TMDs of Ig- α and Ig- β , all coarse-grained MD simulations containing Ig- α , Ig- β or an Ig- α /Ig- β heterodimer were carried out using the polarizable water model (40) (**Table 2**, Steps 2, 3a, 3b, 5, and 6) and the polarizable MARTINI protein force-field (41).

In simulations targeting the spontaneous self-assembly of two transmembrane domains, the TM helices/domains were embedded at a center of mass distance of 5 nm and random in-plane rotations in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane (**Table 2**, Steps 1, 2, 3a, 3b and 6). The membrane domain preference of the BCR was addressed for different BCR TMD models in simulations of the receptor embedded in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/1,2-di-(cis-cis-cis-9,12,15-octadecadienoyl)-sn-glycero-3-phosphocholine (DIPC)/cholesterol (proportions: 40:30:30) membranes as a model system for ordered/disordered membrane domains (**Table 2**, Step 4). The different lipids were initially randomly distributed within the membrane.

All systems were equilibrated according to the MARTINATE protocol (42). All production runs were then carried out in an (approximate) NpT ensemble with a timestep of 20 fs. The temperature was controlled by coupling to an external heat bath of 310 K with the aid of the Bussi velocity rescaling thermostat (43) and a coupling time constant of 1.0 ps. The Berendsen barostat (44) was used for semi-isotropic pressure coupling to an external pressure bath at 1 bar with a 3.0 ps coupling time constant and a compressibility of $3.0 \cdot 10^{-4} \text{ bar}^{-1}$. Lennard-Jones interactions were switched to zero between 0.9 and 1.2 nm. Bonds were constrained using LINCS (45).

In case of the non-polarizable MARTINI water model (**Table 2**, Step 1), the relative dielectric permittivity was set to 15 and electrostatic interactions were switched to zero between 0.0 and 1.2 nm. In contrast, in the case of the polarizable MARTINI water model (37) (**Table 2**, Steps 2, 3a, 3b, 5, and 6), a cut-off of 0.9 nm was applied for short-range electrostatic interactions and the PME method (46) was used for long-range electrostatics beyond the cutoff. Here, the relative dielectric permittivity was set to 2.5.

TABLE 1 | Amino acid sequences of the transmembrane domains of mIgM, Ig- α , and Ig- β studied in coarse-grained and atomistic MD simulations.

Molecule	Sequence
mIgM	442-GFENLWATASTFIVLFLSLFYSTTVTLFKVK-473
Ig- α	135-DMGEGTKNRIITAEGIILLFCVAVPGTLLLFRRKRWQ-170
Ig- β	151-LKQRNTLKDGIIMIQTLLIILFIIVPIFLLLDKDDS-186

TABLE 2 | List of coarse-grained (CG) and all-atom (AA) MD simulations performed to study the conformation of the IgM-BCR transmembrane domain.

Step ^a	Aim	Resolution ^b	Simulation time ^c	Number of (Simulations) ^d
1	mIgM homodimer assembly (resulting in BM-A and BM-B configurations)	CG	5000 ns	104
2	Ig- α /Ig- β heterodimer assembly (resulting in BM- α configuration)	CG	5000 ns	105
3a	IgM-BCR assembly of binding modes BM-A and BM- α	CG	5000 ns	176
3b	IgM-BCR assembly of binding modes BM-B and BM- α	CG	5000 ns	190
4	Stability assessment of BM-A, BM-B, BM- α , BM-A-1, BM-A-2, BM-B-1 and BM-B-2	AA	500 ns	7x3 ^e
5	Membrane domain preference of different IgM-BCR TM configurations (BM-A-1, BM-A-2, BM-B-1, and BM-B-2)	CG	2000 ns	4x10 ^f
6	Dimerization of IgM-BCR TM domains	CG	10000 ns	110
7	Stability assessment of IgM-BCR cluster	AA	500 ns	3

^aFor clarity, the workflow was divided into 7 parts.

^bSimulations were carried out either at all-atom (AA) or at coarse-grained (CG) resolution.

^cSimulation time of each simulation.

^dNumber of replica simulations.

^eThree all-atom simulations were performed for each IgM-BCR TM configuration (BM-A, BM-B, BM- α , BM-A-1, BM-A-2, BM-B-1, and BM-B-2).

^f10 CG simulations were performed for each IgM-BCR TM configuration (BM-A-1, BM-A-2, BM-B-1 and BM-B-2).

2.2. All-Atom Simulations

Atomistic simulations of BCR transmembrane domains were performed inside a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) membrane, in order to assure for a comparable membrane thickness between models at coarse-grained (CG) and atomistic resolution (Table 2, Steps 4 and 7; see Results section). The *insane* protocol (47, 48) was used to setup the lipid and solvent environment around input structures at CG resolution. Equilibration at CG resolution employing the *DAFT* scheme (31) was followed by conversion of the whole system to atomistic resolution employing the *backward* protocol (49). For all systems, the minimal distance between periodic images of the proteins never decreased below 3 nm.

Atomistic simulation production runs of 500 ns length, three replicas for each system, using Gromacs 5 (50) were preceded by an energy minimization using the steepest descent algorithm. The systems were simulated in the NpT ensemble for 10 ns with restraints on all heavy protein atoms, and additionally for 5 ns with restraints on the protein backbone atoms only. A combination of the AMBER14sb force field (51) for proteins and the LIPID14 (52) force field for lipids was chosen (53, 54). Water was described by the TIP3P water model (55) and ions were added at physiological concentration (150 mM Na⁺Cl⁻).

The temperature was controlled by coupling to an external heat bath at 310 K using the Bussi velocity rescaling thermostat (43) and a coupling time constant of 0.5 ps. A pressure of 1 bar was reached by semi-isotropic pressure coupling to an external pressure bath [Berendsen barostat (44)] with a time constant of 1 ps. The compressibility was set to $4.5 \cdot 10^{-5} \text{ bar}^{-1}$. Lennard-Jones interactions and short-range electrostatic interactions were taken into account until a cut-off of 1 nm, while the PME method was used for long-range electrostatics beyond the cutoff. The production runs were carried out with a timestep of 2 fs. Bonds to hydrogen atoms were constrained by LINCS (45).

2.3. Analysis

As a dimerization criterium both for α -helices (mIgM, Ig- α , and Ig- β) and for helical dimers in the formation of the full BCR TMD (mIgM TMD homodimer and the Ig- α /Ig- β heterodimer) the interaction energy (sum of Lennard-Jones and Coulomb interactions) between two monomers was set to -200 kJ/mol . This cutoff value was chosen from visual inspection of the compactness of the related complexes. For oligomerization of BCR TM complexes, the cutoff was increased to -800 kJ/mol . Here, in order to exclude less compact complexes, an additional cutoff criterium was employed for the buried surface area (BSA) between two BCR complexes ($> 10\%$ of the total protein surface).

Protein-protein binding interfaces were assessed by analysis of the average minimum distances between all interchain residue pairs during the last 50 ns of simulation time of all CG-simulations belonging to specific binding modes and visualized in contact maps. To that end, all simulation frames from the last 50 ns of those simulations showing a compact dimer at the end of the simulation were assigned to the different dimer configuration labels (i.e., the different binding modes) using a watershed transform (56) as described in detail in Pluhackova et al. (30). Additionally, in order to pinpoint residues that contribute most to direct helix-helix interactions, the average relative interaction energy contribution (sum of Lennard-Jones and Coulomb interactions) per residue during the last 50 ns of simulation time was computed (interaction-energy profiles).

3. RESULTS

Modeling of the TM domain of IgM-BCR (compare Figure 1) was addressed in spontaneous association simulations of its parts embedded in model 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayers. The association of the IgM-BCR TMD was investigated via extensive molecular dynamics simulations in three steps: First, the mIgM-TMD assembly was explored by analysis of the spontaneous dimerization of two

copies of a μ TM heavy chain (named μ -1 and μ -2; **Table 2**, Step 1). Second, the spontaneous formation of the Ig- α /Ig- β -TMD heterodimer was studied (Step 2). Third, the assembly of the full IgM-BCR-TMD was explored based on the dimers obtained in the first two steps (**Table 2**, Steps 3a and 3b). This sequential approach relies on the following three main assumptions: (i) The individual IgM-, Ig- α -, Ig- β -domains adopt an α -helical conformation both isolated and as part of the BCR complex (7). α -helices are the predominant structural motif to span the membrane hydrophobic core (57). (ii) The BCR TMD complex is formed of one mIg-dimer and one Ig- α /Ig- β heterodimer, as previously experimentally shown [1:1 stoichiometry (7, 8)]. (iii) mIgM TM domains and Ig- α /Ig- β -TM helices pre-assemble before formation of the full IgM-BCR TM complex. The latter assumption is supported by the observed association of Ig- α /Ig- β heterodimers to mIg-dimers but not to monomers (58), and the reported disulfide bonds between Ig- α and Ig- β adjacent to the membrane (5, 59) suggesting a close proximity and preassembly of Ig- α /Ig- β TM domains.

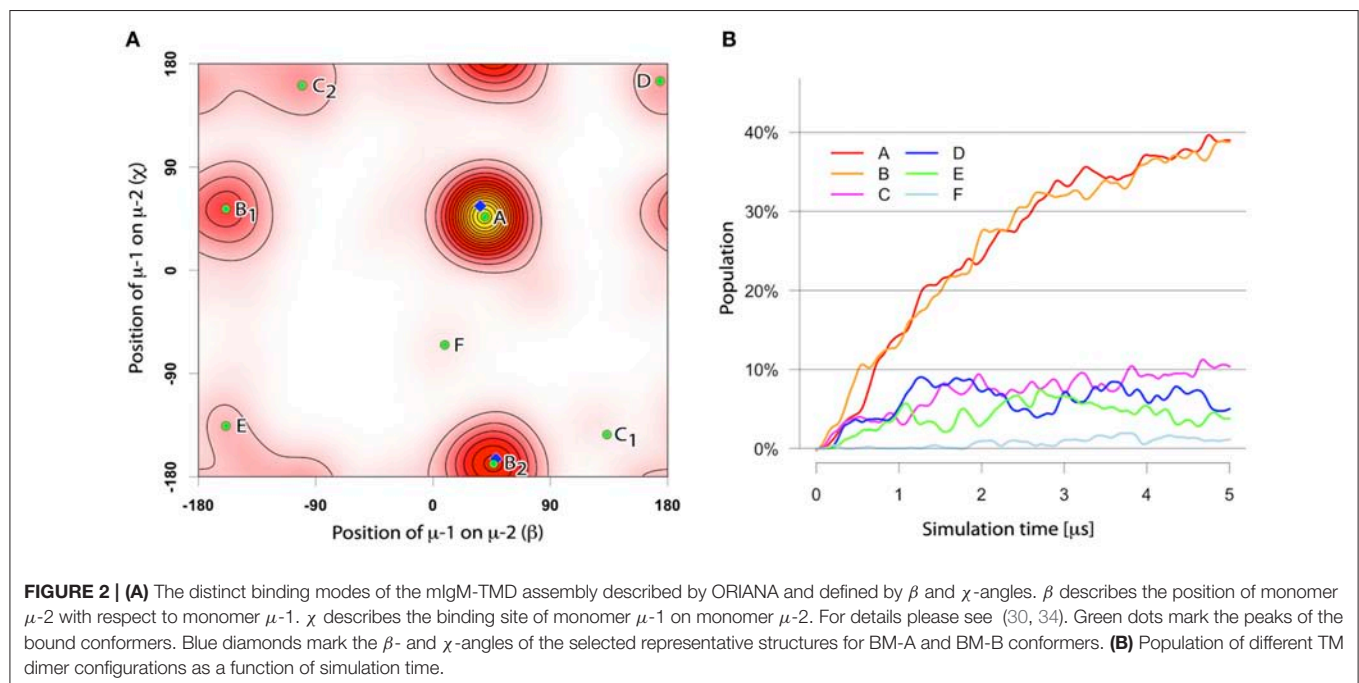
While the assembly of transmembrane domains was analyzed from a large number of coarse-grained MD simulations, the stability of obtained quaternary structures was further studied in atomistic simulations (**Table 2**, Step 4). The lateral partitioning of obtained IgM-BCR TM configurations to different membrane domains was addressed at coarse-grained resolution for a membrane with both ordered and disordered domains (**Table 2**, Step 5). Finally, we explored the dimerization/oligomerization of IgM-BCR TMDs (Step 6) and the stability of a IgM-BCR tetramer (Step 7).

3.1. Assembly of mIgM-TMD Homodimer

The assembly of two TM μ chains modeled in α -helical conformation was studied from in total 104 simulations of

each 5 μ s length, starting from two monomers (μ -1 and μ -2) initially separated by 5 nm. During the spontaneous self-assembly, μ -1 and μ -2 dimerized in 102 of 104 simulations within 5 μ s of simulation time (**Figure 2**). An orientation analysis (ORIANA) (30, 31, 34) revealed six distinct binding modes. The two dominant binding modes comprised each about 40% of the observed dimers at the end of the simulations (named BM-A and BM-B, see **Figure 3**). The following analysis focuses on these two major binding modes (for abbreviations of sampled binding configurations see **Table 3**).

Binding mode BM-A. BM-A describes a symmetric, right handed homodimer of the μ TM domains. The monomers are tilted by $\approx 40^\circ$ relative to each other, with a tilt angle of $\approx 20^\circ$ between the membrane normal and each μ -chain. The dimerization interface comprises the central part of the TM-C sites (conserved) of each monomer, whereas the TM-S sites (specific) are turned away from the interaction site and remain freely accessible (representative structure shown in **Figure 3A**). A contact map analysis based on all configurations sampled for this binding mode identifies residues Thr452, Phe453, Leu456, Phe457, Ser460, Leu461, Ser464, Thr465, and Thr468 as the main contributors to helix-helix association and the major interface-forming residues of the highly symmetric interface (see **Figures S1A, S2**). Thereby, except for Thr452 and Thr465, the interface is dominated by conserved sites, i.e., a TM-C/TM-C dimer interface is formed. Interestingly, besides of two phenylalanines and two leucines, several hydrophilic amino acids (colored yellow in **Figure 3B**) located within the central part of the TMDs are buried at the interface. While the central regions of the TMDs are in close contact, the intracellular and extracellular termini do not associate due to the relatively large tilt of the monomers.



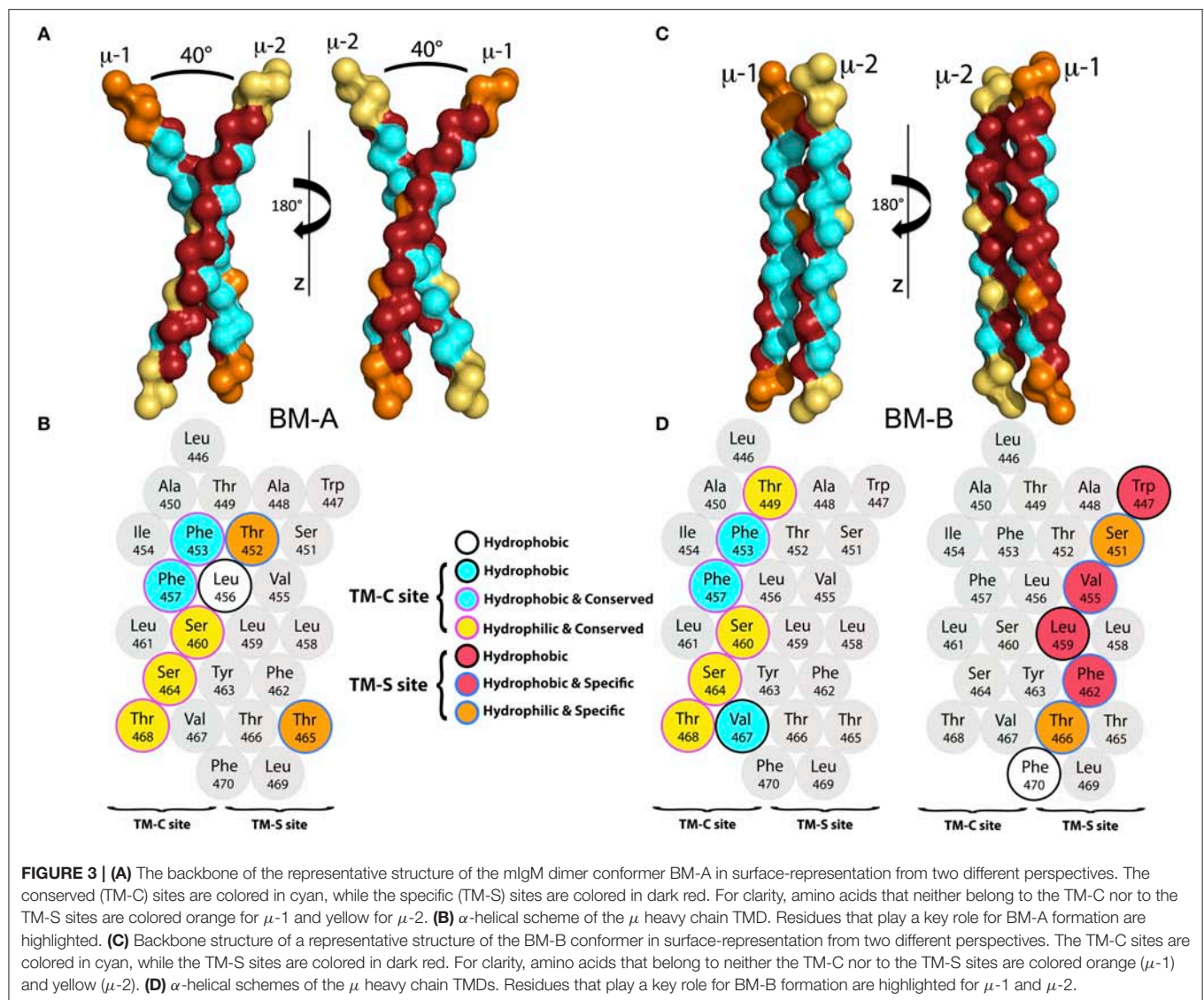


TABLE 3 | Dimer/oligomer configurations sampled in MD simulations and their abbreviations.

Molecules	Configuration	Abbreviation
mIgM homodimer	Symmetric homodimer	BM-A
	Asymmetric homodimer	BM-B
Ig- α /Ig- β	Heterodimer	BM- α
IgM-BCR TM domain	mIgM symmetric homodimer & Ig- α /Ig- β heterodimer	BM-A-1, BM-A-2
	mIgM asymmetr. homodimer & Ig- α /Ig- β heterodimer	BM-B-1, BM-B-2

Binding mode BM-B. In the second, asymmetric binding mode, the two helices assemble in a parallel fashion (Figures 3C,D), with a dimer tilt of 25° relative to the membrane surface normal. The interface of BM-B is roughly built by the

TM-C site of μ -1 and the TM-S site of μ -2. Consequently, one TM-S as well as one TM-C site are freely accessible on the surface of the mIgM TM dimer. Note that binding of the TM-C site of μ -1 to the TM-S site of μ -2 leads to the same dimer as binding of the TM-C site of μ -2 to the TM-S site of μ -1. For the sake of simplicity, the nomenclature introduced in Figure 3 is used throughout the manuscript to distinguish the positions of the BCR-forming μ chains.

The binding interface of BM-B is formed by Thr449, Phe453, Phe457, Ser460, Ser464, Val467, and Thr468 of μ -1, as identified by averaging of the interaction energies between both monomers over all configurations sampled within this binding mode. These amino acids also contribute significantly to the interaction energy of the two μ chains (Figure 3D, Figure S3). Interestingly, all of these seven residues are part of the conserved site of the helix (TM-C). In contrast, the interaction site on μ -2 is formed by the TM-S amino acids Trp447, Ser451, Val455, Leu459, Phe462, Tyr463, Thr466, and Phe470. Thus, the

BM-B binding mode is characterized by a TM-S/TM-C binding interface.

Summing up, two binding configurations of the mIgM-TMD were observed which have the burial of polar amino acids at the dimer interface in common. The significantly larger helical interface of the asymmetric BM-B conformer as compared to the symmetric BM-A conformer (see **Figure 3**), indicates an increased stability of BM-B as also observed in all-atom (AA) simulations of both homodimer configurations (see below). While BM-A is a symmetric TM-C—TM-C dimer, BM-B perfectly aligned one TM-C and one TM-S site. Neither fits the symmetrical TM-S—TM-S dimer previously suggested (17). Blocking of both TM-C sites in the BM-A conformer would likely not allow for TM-C—Ig- α /Ig- β association as implicated by the observation that all mIgs bind the same Ig- α /Ig- β heterodimer. In contrast, the accessibility of one TM-C and one TM-S site in the BM-B conformer can account for Ig- α /Ig- β association in a 1:1 stoichiometry (16, 60). Additionally, the exposed TM-S site within the BM-B conformer is compatible with a class-specific membrane localization of the IgM-BCR (26).

3.2. Assembly of the Ig- α /Ig- β -TMD

Of in total 105 self-assembly simulations of the Ig- α and Ig- β TM domains of each 5 μ s (**Table 2**, Step 2), 104 systems resulted in heterodimer formation. A majority (69%) of the dimers had the same binding mode termed BM- α (**Figures 4, 5**). The parallel dimer is characterized by a tilt of $\approx 20^\circ$ relative to the membrane normal.

Binding mode BM- α . The prevalent Ig- α /Ig- β dimer has a large binding interface, ranging from the intra- to the extracellular parts of the two helices and including a few charged residues located at the termini of the chains as well as a high amount of hydrophobic residues in the membrane spanning region (contact map **Figure 5A**). While the hydrophobic membrane-spanning residues moderately contribute to the

interaction energy, high contributions were observed for the charged residues (**Figure S4**) forming salt bridges between the helices. In detail, Ig- α -Lys167, Ig- α -Arg168, Ig- β -Asp184, and Ig- β -Asp185 at the extracellular part of the dimer as well as Ig- α -Glu138, Ig- β -Arg154, and Ig- β -Lys158 at the intracellular part of the dimer form salt bridge networks (**Figure 5B**).

3.3. Assembly of the Full IgM-BCR-TMD

The spontaneous association of the full IgM-BCR TM domain was studied as association of the IgM-homodimer—allowing for either of the preferred configurations (BM-A and BM-B)—and of the Ig- α /Ig- β heterodimer (BM- α) configuration (**Table 2**, Steps 3a, 3b). The mIgM TM dimer in BM-A conformation and the Ig- α /Ig- β heterodimer associated in 167 out of 176 simulations during 5 μ s simulation time. The two main obtained tetramer configurations (binding modes BM-A-1 and BM-A-2, **Figures 6A,B**, respectively) were considered further. Approximately 45% of the formed tetramers belong to BM-A-1 and $\approx 30\%$ of the tetramers were assigned to BM-A-2 (**Figure S5**). Similarly, mIgM in BM-B configuration assembled with Ig- α /Ig- β in 167 out of 190 simulations during 5 μ s of simulation time. The two main tetramer binding modes were observed with a population of 25% and 21%, respectively (BM-B-1 and BM-B-2, **Figures 6C,D**, respectively; **Figure S6**).

Only the asymmetrical BM-B conformer, i.e., the tetramer configurations BM-B-1 and BM-B-2 allow for the reported 1:1 stoichiometry of mIgM and Ig- α /Ig- β (16, 60). In turn, tetramers based on the symmetric BM-A conformer (BM-A-1 and BM-A-2) could equally enable a 2:1 stoichiometry (tentative structures following a 2:1 stoichiometry are shown in **Figure S7**). Noteworthy, the Ig- α /Ig- β dimer is rotated by 180° in the BM-A-2 and BM-B-1 conformers as compared to BM-A-1 and BM-B-2. The orientation of Ig- α /Ig- β with respect to mIgM has implications for the association of the BCR TM domain with lipid rafts (see below).

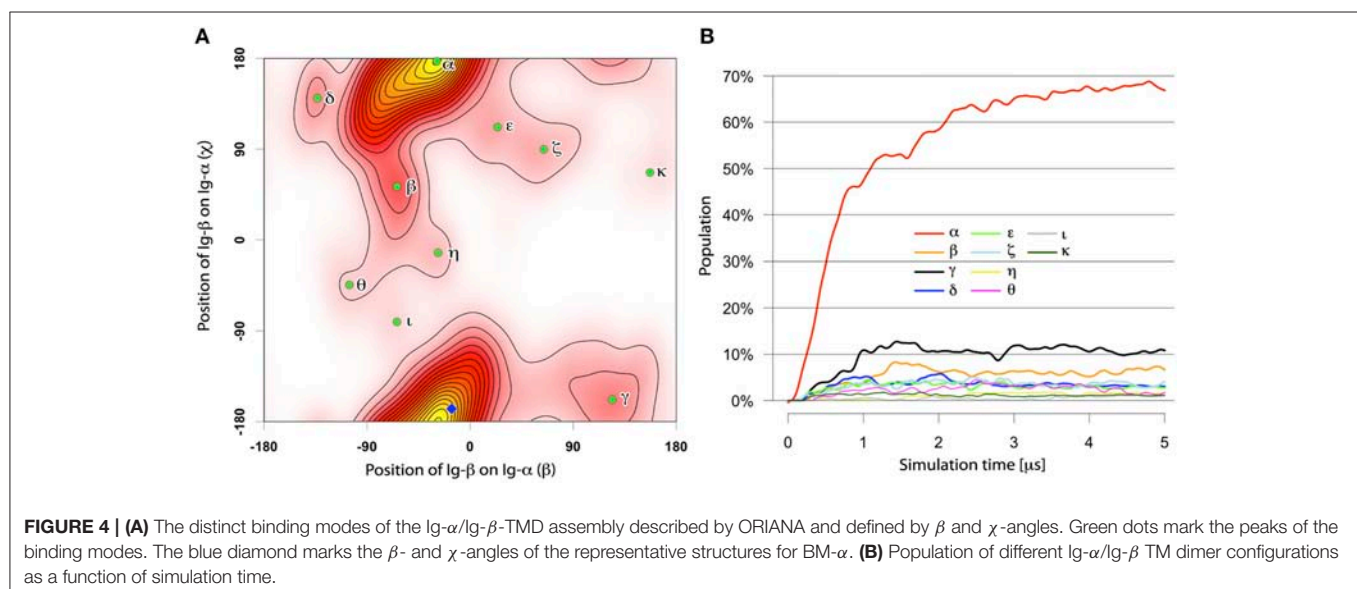
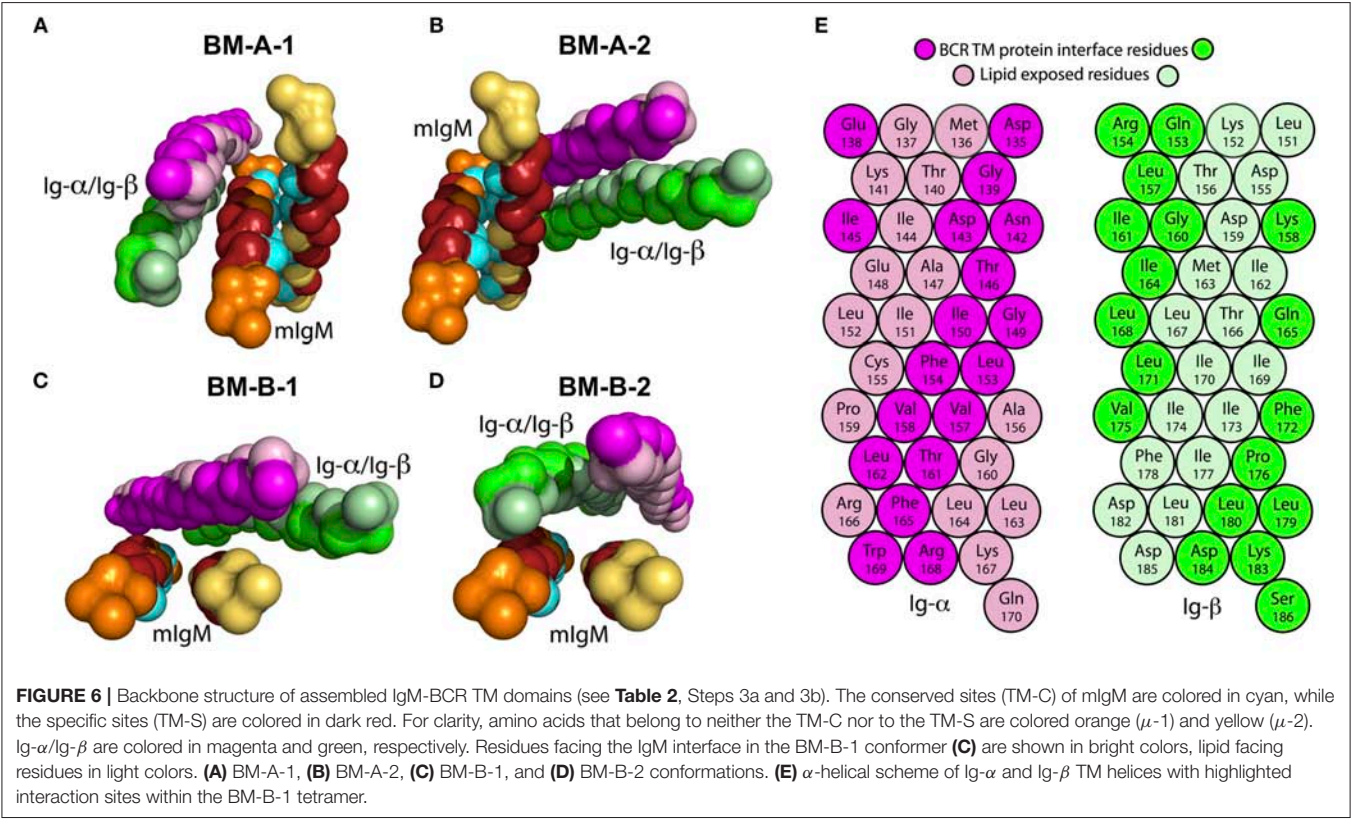
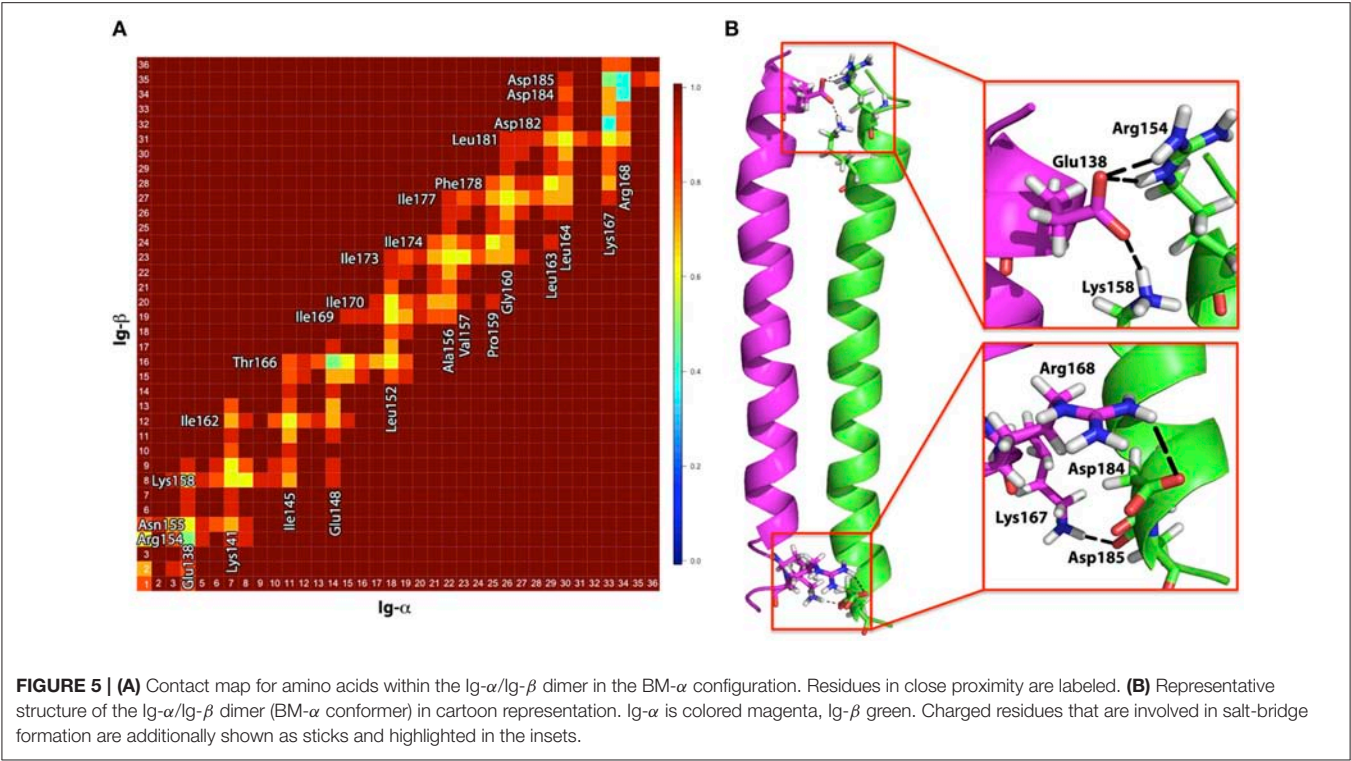


FIGURE 4 | (A) The distinct binding modes of the Ig- α /Ig- β -TMD assembly described by ORIANA and defined by β and χ -angles. Green dots mark the peaks of the binding modes. The blue diamond marks the β - and χ -angles of the representative structures for BM- α . **(B)** Population of different Ig- α /Ig- β TM dimer configurations as a function of simulation time.



Several experimental studies showed that mutation of mIgM-Tyr463 and mIgM-Ser464 to valines (YS/VV) results in uncoupling of mIgM from Ig- α /Ig- β (10, 11, 61, 62). Thus, at least one of these two residues probably plays a key role in mIgM–Ig- α /Ig- β association. While none of the two residues was shown to contribute significantly to IgM-BCR formation in BM-A-2, μ -1-Tyr463 strongly contributes to the interaction energy within BM-A-1 and μ -1-Tyr463 as well as μ -2-Tyr463 to the stability of the BM-B-1 and BM-B-2 conformers (Figure 7). Later in this study, all-atom simulations were used to show that BM-A-2, which is not stabilized by mIgM-Tyr463, does not result in a stable IgM-BCR TMD complex. These results underline the role of mIgM-Tyr463 in IgM-BCR TMD stabilization and suggest that it is the mutation of mIgM-Tyr463 and not the mutation of mIgM-Ser464 which is responsible for the uncoupling of mIgM from Ig- α /Ig- β in experiments. The IgM-BCR-TMD is additionally stabilized by the hydrophobic, aromatic residues

μ -Trp447, μ -Phe462, μ -Tyr463, and μ -Phe470, which anchor within the hydrophobic TMD of Ig- α /Ig- β in all four binding modes.

3.4. All-Atom Validation

The employed Martini coarse-grained model may overestimate the aggregation between proteins (63), in particular for soluble proteins (64). However, highly populated dimer configurations of integral membrane proteins observed in simulation ensembles have been shown to compare well to experimental findings (30, 31, 34). Here, to identify possibly artificial configurations, atomistic simulations were used to address the stability of all self-assembled transmembrane complexes, both of dimers and of tetramers. To that end, we performed a resolution-transformation of obtained representative conformations from coarse-grained resolution to atomistic detail, employing the *backmapping* scheme (49). The conformational stability was then

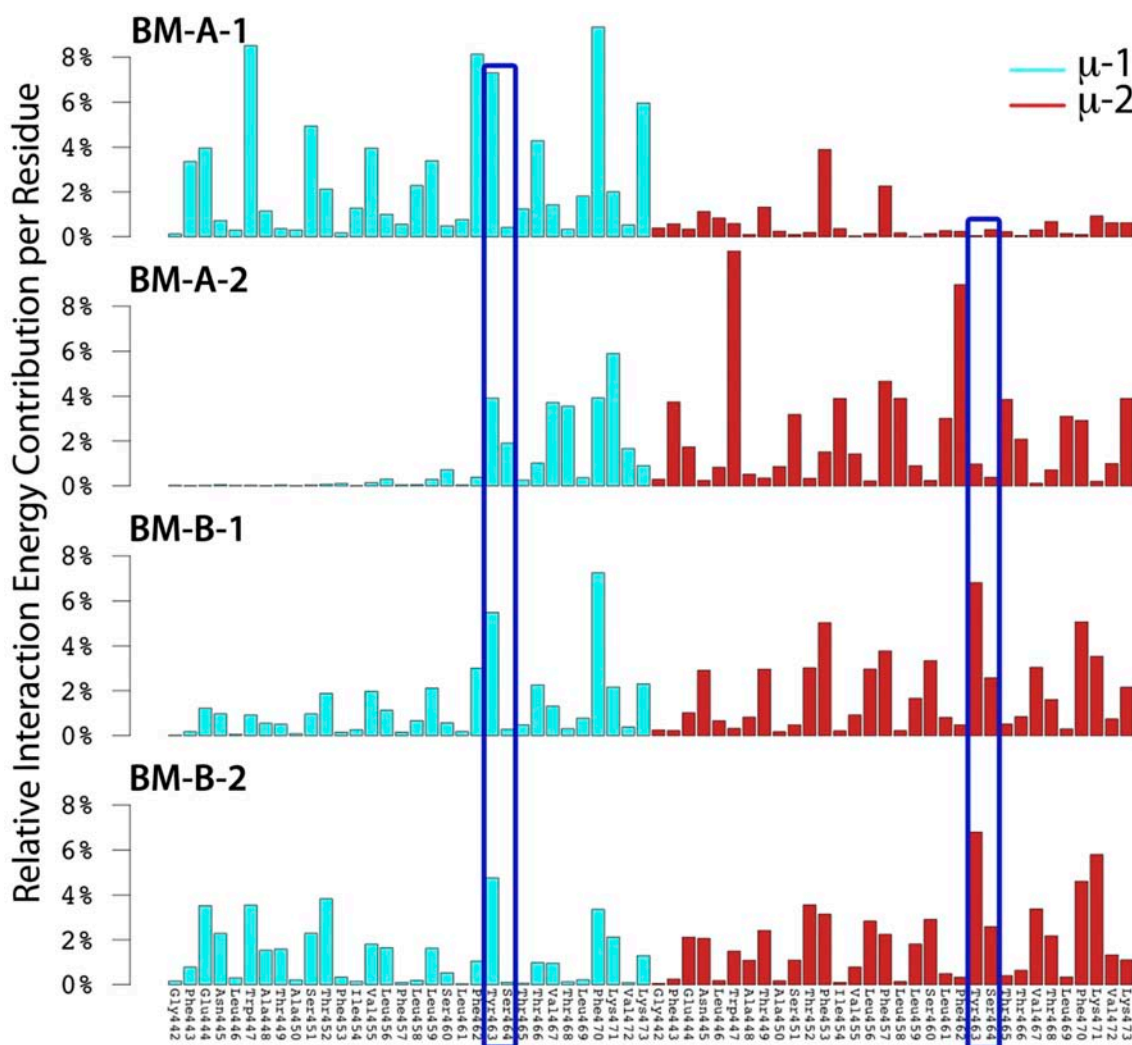


FIGURE 7 | The relative interaction energy contribution of mIgM to IgM-BCR-TM domain formation for the BM-A-1, BM-A-2, BM-B-1, and BM-B-2 conformers. Residues whose mutation led to uncoupling of mIgM from Ig- α /Ig- β in experiments (10, 11, 61, 62) are highlighted by dark blue frames (Tyr463, Ser464).

studied based on three 500 ns atomistic MD simulations each. All complexes were embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) membrane. A POPE membrane was chosen because the thickness of the POPE bilayer at atomistic resolution resembles the thickness of the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane at coarse-grained (CG) resolution used in the self-assembly simulations (compare **Table S1** for data on the membrane thickness of all investigated systems). Thereby, a comparable hydrophobic thickness was achieved between atomistic resolution simulations and CG simulations.

All studied TM dimers were stable on the studied timescale with root mean square deviations (RMSD) of approximately 3–4 Å (**Figure S8**). A comparison for the mIgM dimers reveals an enhanced stability of the asymmetric BM-B configuration that is probably related to the significantly larger interface area of the bound mIgM monomers in the asymmetric configuration of 13.7 nm² as compared to the symmetric structure (11.3 nm²).

For the full mIgM-BCR TM helical tetramers, the configurations based on the asymmetric mIgM TM dimer (BM-B conformer) were stable with RMSD values between 3 Å and 4 Å. In contrast, the tetramers based on the symmetric mIgM TM homodimer (BM-A-1, BM-A-2) were found to be overall less stable on the 500 ns timescale with RMSD values of 4–6 Å (**Figure S9**). Overall, our results suggest an increased stability of IgM-BCR TM domains that contain the mIgM TM homodimer in an asymmetric configuration with a TM-S/TM-C binding interface as compared to a symmetric TM-C/TM-C interface.

3.5. Protein-Lipid Interactions

The association of the mIgM-BCR TM complex to differently ordered membrane domains was addressed by placing the BCR transmembrane domain into a membrane consisting of a three component lipid mixture composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/1,2-di-(cis-cis-9,12,15-octadecadienoyl)-sn-glycero-3-phosphocholine (DIPC)/cholesterol. This mixture is based on a three component lipid mixture of Risselada and Marrink (65) that contains a double unsaturated 1,2-di-(cis-cis-9,12-octadecadienoyl)-sn-glycero-3-phosphocholine (DUPC) as the polyunsaturated lipid and phase separates well at 295 K. In order to be able to study phase separation at 310 K, an additional C4 bead was added here mimicking an additional unsaturated bond (topology provided in the **Supplementary Material**). For each of the four obtained BCR TM domains, ten simulations of each 2 μs were performed at CG resolution starting from a randomized mixture of lipids within the bilayer.

Within hundreds of nanoseconds of simulation time, two distinguishable lipid-phases emerged: a DPPC-rich region with a high amount of cholesterol (blue/green in **Figure 8**) and a DIPC-rich phase with a significantly lower amount of cholesterol (gray/green). While the DPPC/cholesterol domain assumes a liquid-ordered, raft-like phase, a fluid-disordered phase was observed for the DIPC/cholesterol domain (65). The four tetramers showed different association preferences for the liquid-disordered and -ordered membrane phases: The BM-A-1 and BM-B-2 BCR TM structures were mainly associated with the

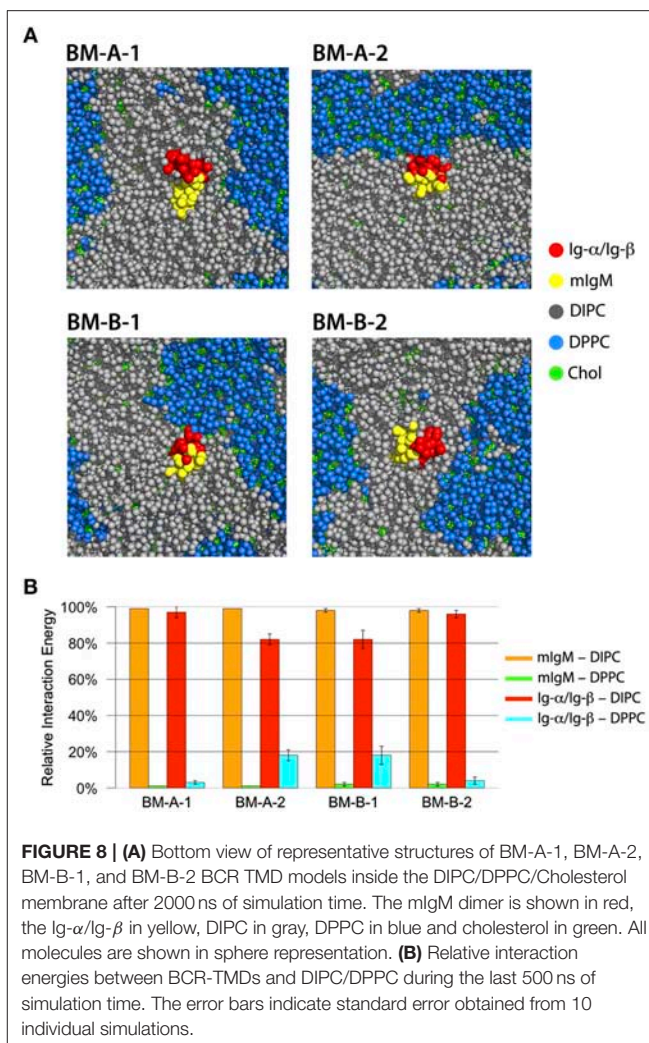


FIGURE 8 | (A) Bottom view of representative structures of BM-A-1, BM-A-2, BM-B-1, and BM-B-2 BCR TMD models inside the DIPC/DPPC/Cholesterol membrane after 2000 ns of simulation time. The mIgM dimer is shown in red, the Ig-α/Ig-β in yellow, DIPC in gray, DPPC in blue and cholesterol in green. All molecules are shown in sphere representation. **(B)** Relative interaction energies between BCR-TMDs and DIPC/DPPC during the last 500 ns of simulation time. The error bars indicate standard error obtained from 10 individual simulations.

liquid-disordered phase. Differently, the BM-A-2 and BM-B-1 conformers showed a strong preference for the domain boundaries, exposing the lipid-accessible parts of their Ig-α/Ig-β domains to the raft domains (**Figure 8**) as reflected by the relative interaction energies of the BCR TM conformers with the different lipid species (**Figure 8B**). While the lipid-accessible part of the Ig-α/Ig-β-domain is identical in BM-A-2 and BM-B-1, this part of the dimer is oriented toward mIgM in the BM-A-1 and BM-B-2 conformers (**Figure 6**). Thus, the lipid-exposed surface of the Ig-α/Ig-β dimers could be associated with the differential preference of the studied BCR TMD conformers to ordered or disordered membrane domains.

Only the BM-A-2 and BM-B-1 BCR conformers are compatible with the finding of Lillemeier and Mattila (25, 66) that active single BCRs are associated with lipid-raft-like domains. Of these, the BM-A-2 complex was unstable in atomistic simulations. Moreover, this conformer can't explain the importance of Tyr463 and Ser464 for coupling of mIgM with Ig-α/Ig-β and does not explain the 1:1 stoichiometry between mIgM and Ig-α/Ig-β. The latter experimental findings

together with our simulation results thus provide strong support for the hypothesis that the BM-B-1 conformation represents a realistic structural model for the BCR transmembrane domain.

3.6. Assembly of BM-B-1 Tetramers Into Oligomers

The association of isolated BCR TM domains to ordered lipid raft domains was seen to be driven by the lipid-exposed surface of the Ig- α /Ig- β domain (see above). In turn, BCR clusters were experimentally shown to not associate with lipid rafts (25, 66). The Ig- α /Ig- β domains thus will likely be shielded from surrounding lipids upon BCR oligomerization. As a first step of oligomerization, we here addressed the spontaneous dimerization of BCR TMDs (BM-B-1 conformation) in CG simulations. The BCR TMD monomers associated in 28 of 110 simulations during 10 μ s simulation time (Figure S10). Five preferred binding modes could be distinguished (BM-V, BM-W, BM-X, BM-Y, and BM-Z, see Figure 9). None of the obtained dimers fully blocked the lipid exposure of Ig- α /Ig- β .

However, the dimer structures allow for the construction of higher order oligomers. For example, a symmetric BCR tetramer built based on the BM-Y binding mode (see Figure 9) shields all four Ig- α /Ig- β domains from the surrounding lipid environment (Figure S11). This cluster was found to be stable on the 500 ns

timescale at atomistic resolution (Figure S12). Ig- α /Ig- β may as well be shielded in a hexameric configuration (Figure S13). This differential lipid accessibility suggested by the lipid-exposure of Ig- α /Ig- β in BCR monomers and their possible burial in higher order BCR oligomers provides a natural explanation for the observed shift in the BCR lipid environment after activation-induced BCR cluster opening (26).

4. DISCUSSION

While many antibody and antibody/antigen structures could be resolved in the past, the arrangement of antibodies within and the overall three-dimensional structure of both the cytoplasmic BCR domain and of the BCR transmembrane domain are unknown. Here, using a combination of coarse-grained and atomistic molecular dynamics simulations, we studied the spontaneous self-assembly of the helices building the transmembrane domain of IgM B-cell receptors. The obtained conformation of the BCR TMD is characterized by an asymmetric mIgM dimer (TM-C/TM-S) bound to Ig- α /Ig- β (see Figure 10). The latter contacts both mIgM molecules, a finding that is supported by previous experiments reporting that Ig- α /Ig- β only co-purified with the mIg dimer, but not with a single heavy chain/light chain pair (58). In the favored BCR TMD structure, Tyr463 of mIgM contributes significantly to the stability of the helical tetramer (see also Figure 10B). This finding is corroborated by previous mutation analysis of these sites, a double mutation (YS/VV) led to uncoupling of mIgM from Ig- α /Ig- β (10, 11, 61, 62).

The asymmetric TM-C/TM-S mIgM TM dimer (Figure 10C) is in contrast to the symmetrical TM-S/TM-S dimer proposed earlier by Reth (17). However, as outlined in the introduction, a symmetric TM-S/TM-S dimer would probably violate the 1:1 stoichiometry of IgM:Ig- α /Ig- β (7, 8). Also, complexes built using a symmetric mIgM dimer exhibited a reduced stability in atomistic MD simulations. Ig- α – Ig- β assembly resulted in a single stable dimer configuration, which experimentally has been poorly characterized so far. Its stability stems on the one hand from salt bridges between charged residues at the intra- and extracellular parts of the Ig- α and Ig- β TMDs and on the other hand from the large hydrophobic interface built by the membrane spanning regions of Ig- α and Ig- β . We could further show that the lipid accessible part of Ig- α /Ig- β within the BCR TMD likely drives the association of these complexes with ordered lipid domains (Figure 10D) (25, 66). However, it has to be noted that monomeric BCR TMDs did not fully partition to the lipid raft like domains but rather associated to the interface between ordered and disordered domains. This is possibly coupled to a recently reported enhanced enrichment of transmembrane peptides at domain interfaces in coarse-grained simulations employing the Martini forcefield (67). However, different from the latter study, we here employed different membrane phases of similar thickness. Still, a comparative partitioning analysis for various BCR TM models differing in the orientation of the Ig- α /Ig- β dimer within the BCR revealed an interface-dependent partitioning of BCR either to the disordered membrane domain (BM-A-1, BM-B-2 conformers)

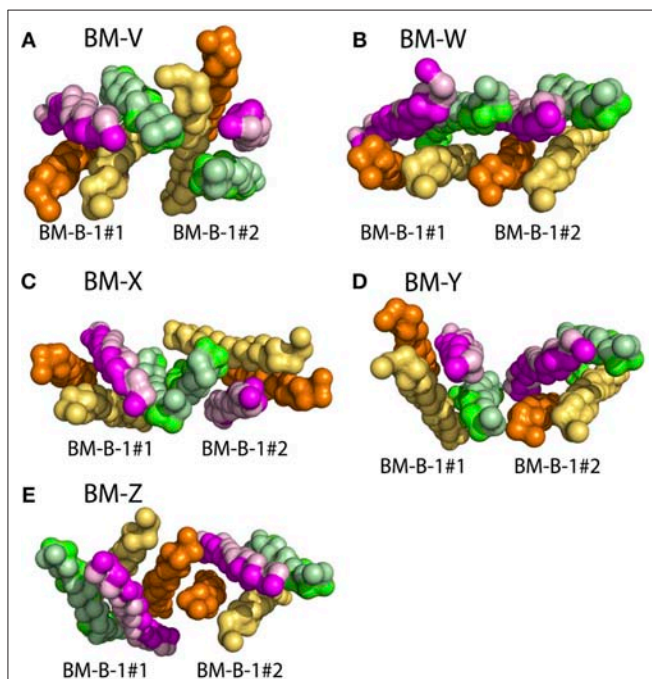
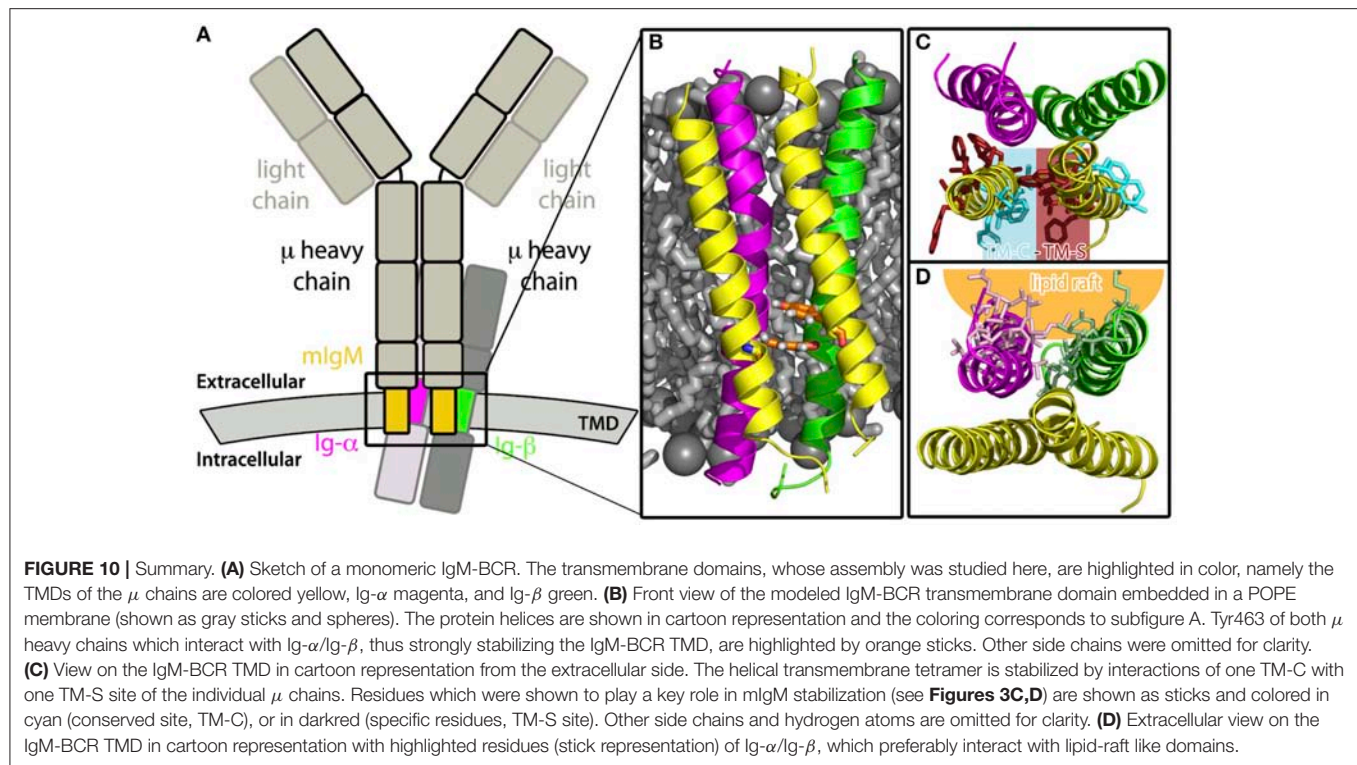


FIGURE 9 | BCR TMD dimer structures as obtained from spontaneous assembly of two BCR TMDs (BM-B-1 conformation, see Table 2, Step 6). The BCRs are colored comparable to Figure 6, however the TM-C and TM-S sites of mIgM are not highlighted here. (A) BM-V, (B) BM-W, (C) BM-X, (D) BM-Y, (E) BM-Z conformations. See Figure S10 for orientation analysis and populations of the BCR TMD dimers.



or to the ordered-disordered domain interface (BM-A-2, BM-B-1). This clearly shows a protein interface-dependent membrane partitioning within the chosen coarse-grained methodology. A more detailed analysis of the driving forces for differing membrane domain associations would require to scrutinize the underlying lipid-protein interactions in ordered, disordered, and interfacial membrane domains at atomistic resolution.

Oligomer models for the BCR TMD provide cues for the mechanisms underlying the observed translocation of BCRs upon activation from non-raft to lipid raft domains (68, 69): passive IgM-BCRs may reside as oligomers with shielded Ig- α /Ig- β interfaces within non-raft regions while the oligomers may be opened or re-organized upon activation (26, 70) resulting in release of the Ig- α /Ig- β membrane interfaces and thus changed preference for lipid raft domains.

In summary, we suggest a structural model for the transmembrane domain of IgM-BCR that is in line with the available experimental data. Monomer and oligomer structures and their differing membrane domain association provide a molecular view on the dissociation activation model, which states that activation-induced BCR cluster opening leads to a transition of single, active BCRs from fluid membranes to lipid-raft like domains (26). Similar couplings between the assembly or clustering of membrane proteins on the nanoscale and signaling were reported for a number of receptors (71), e.g., for the formation of microclusters of T-cells receptors and the linker for activation of T cells (Lat) during T cell activation (72).

Multiscale simulations, combining coarse-grained and atomistic MD simulations in a sequential manner (73) provide an exciting and promising tool in the study of the structure, the clustering and the domain preference of receptors at atomistic resolution.

AUTHOR CONTRIBUTIONS

MF and KP performed MD simulations and analysis. RB designed and supervised the study. All wrote the manuscript.

FUNDING

We acknowledge support by the DFG Research Training Group 1962, Dynamic Interactions at Biological Membranes: From Single Molecules to Tissue.

ACKNOWLEDGMENTS

We acknowledge computational support from the Computer Center of the Friedrich-Alexander University of Erlangen-Nürnberg (RRZE).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02947/full#supplementary-material>

REFERENCES

- Pieper K, Grimbacher B, Eibel H. B-cell biology and development. *J Allergy Clin Immunol.* (2013) 131:959–71. doi: 10.1016/j.jaci.2013.01.046
- Poggianiella M, Bestagno M, Burrone OR. The extracellular membrane-proximal domain of human membrane IgE controls apoptotic signaling of the B cell receptor in the mature B cell line A20. *J Immunol.* (2006) 177:3597–605. doi: 10.4049/jimmunol.177.6.3597
- Bestagno M, Vangelista L, Mandiola PA, Mukherjee S, Sepúlveda J, Burrone OR. Membrane immunoglobulins are stabilized by interchain disulfide bonds occurring within the extracellular membrane-proximal domain. *Biochemistry* (2001) 40:10686–92. doi: 10.1021/bi010506+
- Murphy K, Weaver C. *Janeway's Immunobiology*. Garland Science. 9th Edn. New York, NY; London (2017).
- Hombach J, Tsubata T, Leclercq L, Stappert H, Reth M. Molecular components of the B-cell antigen receptor complex of the IgM class. *Nature* (1990) 343:760–2. doi: 10.1038/343760a0
- Treanor B. B-cell receptor: from resting state to activate. *Immunology* (2012) 136:21–7. doi: 10.1111/j.1365-2567.2012.03564.x
- Schamel WWA, Reth M. Monomeric and oligomeric complexes of the B cell antigen receptor. *Immunity* (2000) 13:5–14. doi: 10.1016/S1074-7613(00)00003-0
- Tolar P, Sohn HW, Pierce SK. The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. *Nat Immunol.* (2005) 6:1168–76. doi: 10.1038/ni1262
- Williams GT, Venkitaraman AR, Gilmore DJ, Neuberger MS. The sequence of the μ transmembrane segment determines the tissue specificity of the transport of immunoglobulin M to the cell surface. *J Exp Med.* (1990) 171:947–52. doi: 10.1084/jem.171.3.947
- Sanchez M, Misulovin Z, Burkhardt AL, Mahajan S, Costa T, Franke R, et al. Signal transduction by immunoglobulin is mediated through Ig- α and Ig- β . *J Exp Med.* (1993) 178:1049–55. doi: 10.1084/jem.178.3.1049
- Shaw AC, Mitchell RN, Weaver YK, Campos-Torres J, Abbas AK, Leder P. Mutations of immunoglobulin transmembrane and cytoplasmic domains: effects on intracellular signaling and antigen presentation. *Cell* (1990) 63:381–92. doi: 10.1016/0092-8674(90)90171-A
- Pleiman CM, Chien NC, Cambier JC. Point mutations define a mIgM transmembrane region motif that determines intersubunit signal transduction in the antigen receptor. *J Immunol.* (1994) 152:2837–44.
- Blum JH, Stevens T, DeFranco A. Role of the mu immunoglobulin heavy chain transmembrane and cytoplasmic domains in B cell antigen receptor expression and signal transduction. *J Biol Chem.* (1993) 268:27236–45.
- Zidovetzki R, Rost B, Pecht I. Role of transmembrane domains in the functions of B- and T-cell receptors. *Immunol Lett.* (1998) 64:97–107. doi: 10.1016/S0165-2478(98)00100-X
- Stevens TL, Blum JH, Foy SP, Matsuuchi L, DeFranco AL. A mutation of the mu transmembrane that disrupts endoplasmic reticulum retention. Effects on association with accessory proteins and signal transduction. *J Immunol.* (1994) 152:4397–406.
- Radaev S, Zou Z, Tolar P, Nguyen K, Nguyen A, Krueger PD, et al. Structural and functional studies of Ig- α /Ig- β and its assembly with the B cell antigen receptor. *Structure* (2010) 18:934–43. doi: 10.1016/j.str.2010.04.019
- Reth M. Antigen receptors on B lymphocytes. *Annu Rev Immunol.* (1995) 10:97–121. doi: 10.1146/annurev.iy.10.040192.000525
- Yang J, Reth M. The dissociation activation model of B cell antigen receptor triggering. *FEBS Lett.* (2010) 584:4872–7. doi: 10.1016/j.febslet.2010.09.045
- Woodruff MFA, Reid B, James K. Effect of antilymphocytic antibody and antibody fragments on human lymphocytes *in vitro*. *Nature* (1967) 215:591–4. doi: 10.1038/215591a0
- Thyagarajan R, Arunkumar N, Song W. Polyvalent antigens stabilize B cell antigen receptor surface signaling microdomains. *J Immunol.* (2003) 170:6099–106. doi: 10.4049/jimmunol.170.12.6099
- Kim Y, Pan JY, Korbel GA, Peperzak V, Boes M, Ploegh HL. Monovalent ligation of the B cell receptor induces receptor activation but fails to promote antigen presentation. *Proc Natl Acad Sci USA.* (2006) 103:3327–32. doi: 10.1073/pnas.0511315103
- Tolar P, Hanna J, Krueger PD, Pierce SK. The constant region of the membrane immunoglobulin mediates B cell-receptor clustering and signaling in response to membrane antigens. *Immunity* (2009) 30:44–55. doi: 10.1016/j.immuni.2008.11.007
- Minguet S, Dopfer EP, Schamel WWA. Low-valency, but not monovalent, antigens trigger the B-cell antigen receptor (BCR). *Int Immunol.* (2010) 22:205–12. doi: 10.1093/intimm/dxp129
- Yang J, Reth M. Oligomeric organization of the B-cell antigen receptor on resting cells. *Nature* (2010) 467:465–9. doi: 10.1038/nature09357
- Mattila PK, Feest C, Depoil D, Treanor B, Montaner B, Otipoby KL, et al. The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling. *Immunity* (2013) 38:461–74. doi: 10.1016/j.immuni.2012.11.019
- Kläsener K, Maity PC, Hobeika E, Yang J, Reth M. B cell activation involves nanoscale receptor reorganizations and inside-out signaling by Syk. *eLife* (2014) 3:e02069. doi: 10.7554/eLife.02069
- Sharpe HJ, Stevens TJ, Munro S. A comprehensive comparison of transmembrane domains reveals organelle-specific properties. *Cell* (2010) 142:158–69. doi: 10.1016/j.cell.2010.05.037
- Maity PC, Yang J, Kläsener K, Reth M. The nanoscale organization of the B lymphocyte membrane. *BBA-Mol Cell Res.* (2015) 1853:830–40. doi: 10.1016/j.bbamcr.2014.11.010
- Bagatolli LA, Ipsen JH, Simonsen AC, Mouritsen OG. An outlook on organization of lipids in membranes: searching for a realistic connection with the organization of biological membranes. *Prog Lipid Res.* (2010) 49:378–89. doi: 10.1016/j.plipres.2010.05.001
- Pluhackova K, Gahbauer S, Kranz F, Wassenaar TA, Böckmann RA. Dynamic cholesterol-conditioned dimerization of the G protein coupled chemokine receptor type 4. *PLoS Comput Biol.* (2016) 12:e1005169. doi: 10.1371/journal.pcbi.1005169
- Wassenaar TA, Pluhackova K, Moussatova A, Sengupta D, Marrink SJ, Tieleman DP, et al. High-throughput simulations of dimer and trimer assembly of membrane proteins. The DAFT approach. *J Chem Theory Comput.* (2015) 11:2278–91. doi: 10.1021/ct5010092
- Han J, Pluhackova K, Wassenaar TA, Böckmann RA. Synaptobrevin transmembrane domain dimerization studied by multiscale molecular dynamics simulations. *Biophys J.* (2015) 109:760–71. doi: 10.1016/j.bpj.2015.06.049
- Han J, Pluhackova K, Böckmann RA. Exploring the formation and the structure of synaptobrevin oligomers in a Model Membrane. *Biophys J.* (2016) 110:2004–15. doi: 10.1016/j.bpj.2016.04.006
- Gahbauer S, Pluhackova K, Böckmann RA. Closely related, yet unique: Distinct homo- and heterodimerization patterns of G protein coupled chemokine receptors and their fine-tuning by cholesterol. *PLoS Comput Biol.* (2018) 14:e1006062. doi: 10.1371/journal.pcbi.1006062
- Pluhackova K, Wassenaar TA, Kirsch S, Böckmann RA. Spontaneous Adsorption of Coiled-Coil Model Peptides K and E to a Mixed Lipid Bilayer. *J Phys Chem B.* (2015) 119:4396–408. doi: 10.1021/acs.jpcc.5b00434
- Páll S, Abraham MJ, Kutzner C, Hess B, Lindahl E. Tackling Exascale Software Challenges in Molecular Dynamics Simulations with GROMACS. In: Markidis S, Laure E, editors. *EASC 2014*. Springer International Publishing (2014). 3–27.
- Marrink SJ, Risselada HJ, Yefimov S, Tieleman DP, De Vries AH. The MARTINI force field: coarse grained model for biomolecular simulations. *J Phys Chem B.* (2007) 111:7812–24. doi: 10.1021/jp071097f
- Monticelli L, Kandasamy SK, Periole X, Larson RG, Tieleman DP, Marrink SJ. The MARTINI coarse-grained force field: extension to proteins. *J Chem Theory Comput.* (2008) 4:819–34. doi: 10.1021/ct700324x
- Schrödinger, LLC. *The PyMOL Molecular Graphics System*, Version 1.8 (2015).
- Yesylevskyy SO, Schäfer LV, Sengupta D, Marrink SJ. Polarizable water model for the coarse-grained MARTINI force field. *PLoS Comput Biol.* (2010) 6:e1000810. doi: 10.1371/journal.pcbi.1000810
- de Jong DH, Singh G, Bennett WFD, Arnarez C, Wassenaar TA, Schäfer LV, et al. Improved parameters for the Martini coarse-grained protein force field. *J Chem Theory Comput.* (2012) 9:687–97. doi: 10.1021/ct300646g
- Wassenaar TA, Ingólfsson HI, Prieß M, Marrink SJ, Schäfer LV. Mixing MARTINI: electrostatic coupling in hybrid atomistic-coarse-grained biomolecular simulations. *J Phys Chem B.* (2013) 117:3516–30. doi: 10.1021/jp311533p

43. Bussi G, Donadio D, Parrinello M. Canonical sampling through velocity rescaling. *J Chem Phys.* (2007) 126:014101. doi: 10.1063/1.2408420
44. Berendsen HJC, van Postma JPM, van Gunsteren WF, DiNola ARHJ, Haak JR. Molecular dynamics with coupling to an external bath. *J Chem Phys.* (1984) 81:3684–90. doi: 10.1063/1.448118
45. Hess B, Bekker H, Berendsen HJC, Fraaije JGEM. LINCS: a linear constraint solver for molecular simulations. *J Comput Chem.* (1997) 18:1463–72. doi: 10.1002/(SICI)1096-987X(199709)18:12<1463::AID-JCC4>3.0.CO;2-H
46. Darden T, York D, Pedersen L. Particle mesh ewald: an $N \cdot \log(N)$ method for Ewald sums in large systems. *J Chem Phys.* (1993) 98:10089–92. doi: 10.1063/1.464397
47. Pluhackova K, Wassenaar TA, Böckmann RA. Molecular dynamics simulations of membrane proteins. *Methods Mol Biol.* (2013) 1033:85–101. doi: 10.1007/978-1-62703-487-6_6
48. Wassenaar TA, Ingólfsson HI, Böckmann RA, Tieleman DP, Marrink SJ. Computational lipidomics with INSANE: a versatile tool for generating custom membranes for molecular simulations. *J Chem Theory Comput.* (2015) 11:2144–55. doi: 10.1021/acs.jctc.5b00209
49. Wassenaar TA, Pluhackova K, Böckmann RA, Marrink SJ, Tieleman DP. Going backward: a flexible geometric approach to reverse transformation from coarse grained to atomistic models. *J Chem Theory Comput.* (2014) 10:676–90. doi: 10.1021/ct400617g
50. Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, et al. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX.* (2015) 1–2:19–25. doi: 10.1016/j.softx.2015.06.001
51. Case DA, Babin V, Berryman J, Betz RM, Cai Q, Cerutti DS, et al. Amber 14. University of California, San Francisco (2014).
52. Dickson CJ, Madej BD, Skjevik AA, Betz RM, Teigen K, Gould IR, et al. Lipid14: the amber lipid force field. *J Chem Theory Comput.* (2014) 10:865–79. doi: 10.1021/ct4010307
53. Pluhackova K, Kirsch SA, Han J, Sun L, Jiang Z, Unruh T, et al. A critical comparison of biomembrane force fields: structure and dynamics of model DMPC, POPC, and POPE bilayers. *J Phys Chem B.* (2016) 120:3888–903. doi: 10.1021/acs.jpcc.6b01870
54. Sandoval-Perez A, Pluhackova K, Böckmann RA. Critical comparison of biomembrane force fields: protein–lipid interactions at the membrane interface. *J Chem Theory Comp.* (2017) 13:2310–21. doi: 10.1021/acs.jctc.7b00001
55. Harrach MF, Drossel B. Structure and dynamics of TIP3P, TIP4P, and TIP5P water near smooth and atomistic walls of different hydroaffinity. *J Chem Phys.* (2014) 140:174501. doi: 10.1063/1.4872239
56. Vincent L, Soille P. Watersheds in digital spaces: an efficient algorithm based on immersion simulations. *IEEE Trans Patt Anal Mach Intell.* (1991) 13:583–98. doi: 10.1109/34.87344
57. White H, Wimley C. Membrane protein folding and stability: physical Principles. *Annu Rev Biophys Biomol Struct.* (1999) 28:319–65. doi: 10.1146/annurev.biophys.28.1.319
58. Benlagha K, Guglielmi P, Cooper MD, Lassoued K. Modifications of Ig α and Ig β expression as a function of B lineage differentiation. *J Biomol Chem.* (1999) 274:19389–96. doi: 10.1074/jbc.274.27.19389
59. Siegers GM, Yang J, Duerr CU, Nielsen PJ, Reth M, Schamel WWA. Identification of disulfide bonds in the Ig- α /Ig- β component of the B cell antigen receptor using the Drosophila S2 cell reconstitution system. *Int Immunol.* (2006) 18:1385–96. doi: 10.1093/intimm/dxl072
60. Reth M. Oligomeric antigen receptors: a new view on signaling for the selection of lymphocytes. *Trends Immunol.* (2001) 22:356–60. doi: 10.1016/S1471-4906(01)01964-0
61. Parikh VS, Nakai C, Yokota SJ, Bankert RB, Tucker PW. COOH terminus of membrane IgM is essential for an antigen-specific induction of some but not all early activation events in mature B cells. *J Exp Med.* (1991) 174:1103–9. doi: 10.1084/jem.174.5.1103
62. Parikh VS, Bishop GA, Liu KJ, Do BT, Ghosh MR, Kim BS, et al. Differential structure-function requirements of the transmembrane domain of the B cell antigen receptor. *J Exp Med.* (1992) 176:1025–31. doi: 10.1084/jem.176.4.1025
63. Javanainen M, Martinez-Seara H, Vattulainen I. Excessive aggregation of membrane proteins in the Martini model. *PLoS ONE* (2017) 12:187936. doi: 10.1371/journal.pone.0187936
64. Larisch N, Kirsch SA, Schambony A, Studtucker T, Böckmann RA, Dietrich P. The function of the two-pore channel TPC1 depends on dimerization of its carboxy-terminal helix. *Cell Mol Life Sci.* (2016) 73:2565–81. doi: 10.1007/s00018-016-2131-3
65. Risselada HJ, Marrink SJ. The molecular face of lipid rafts in model membranes. *Proc Natl Acad Sci USA.* (2008) 105:17367–72. doi: 10.1073/pnas.0807527105
66. Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc Natl Acad Sci USA.* (2006) 103:18992–7. doi: 10.1073/pnas.0609009103
67. Lin X, Gorfé AA, Levental I. Protein Partitioning into Ordered Membrane Domains: Insights from Simulations. *Biophys J.* (2018) 114:1936–44. doi: 10.1016/j.bpj.2018.03.020
68. Cheng PC, Dykstra ML, Mitchell RN, Pierce SK. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J Exp Med.* (1999) 190:1549–60. doi: 10.1084/jem.190.11.1549
69. Tolar P, Won Sohn H, Pierce SK. Viewing the antigen-induced initiation of B-cell activation in living cells. *Immun Rev.* (2008) 221:64–76. doi: 10.1111/j.1600-065X.2008.00583.x
70. Maity PC, Blount A, Jumaa H, Ronneberger O, Lillemeier BF, Reth M. B cell antigen receptors of the IgM and IgD classes are clustered in different protein islands that are altered during B cell activation. *Sci Signal.* (2015) 8:ra93. doi: 10.1126/scisignal.2005887
71. Wu H. Higher-order assemblies in a new paradigm of signal transduction. *Cell* (2013) 153:287–92. doi: 10.1016/j.cell.2013.03.013
72. Lillemeier BF, Mörtelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol.* (2010) 11:90–6. doi: 10.1038/ni.1832
73. Pluhackova K, Böckmann RA. Biomembranes in atomistic and coarse-grained simulations. *J Phys Condens Matter.* (2015) 27:323103. doi: 10.1088/0953-8984/27/32/323103

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tethered Signaling in Inhibitory Immune Receptors

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OPEN ACCESS

Edited by:

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United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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Specialty section:

This article was submitted to
Biomedical Physics,
a section of the journal
Frontiers in Physics

Received: 30 September 2018

Accepted: 18 December 2018

Published: 15 January 2019

Citation:

Pérez-Ferreros P, Gaus K and
Goyette J (2019) Tethered Signaling in
Inhibitory Immune Receptors.
Front. Phys. 6:158.
doi: 10.3389/fphy.2018.00158

Leukocytes play critical roles in preventing pathogenic infection and controlling transformed cells, but must remain quiescent in response to healthy tissue. To execute this function, immune cells need to integrate signals from a host of activatory, co-activatory, and co-inhibitory immune receptors. When an immune cell interacts with another cell containing ligands for these receptors, an immunological synapse is formed at the contact interface that acts as a dynamic signaling hub into which cytoplasmic enzymes are recruited and tethered. Within this interface competing tethered enzymatic activities are integrated, ultimately leading to the cellular decision to respond or remain quiescent. Here, we review recent advances in our understanding of tethered signaling reactions, focusing on proximal signaling downstream of important T cell immune receptors. We discuss how a class of co-inhibitory receptors require co-localization with activatory receptors to function, how recent evidence that T cells use microvilli to probe antigen presenting cell surfaces may be important for immune receptor function, and how co-clustering between activatory and inhibitory receptors facilitates integration of tethered reactions.

Keywords: microvilli, signal integration, PD-1, inhibitory receptors, inhibitory signaling

INTRODUCTION

T cells play a critical role in the adaptive immune system, in which they must recognize and respond to foreign antigens whilst remaining quiescent to normal tissue antigens. Upon activation T cells undergo clonal expansion, differentiation, and mediate effector functions such as cytokine production and direct target cell killing [1].

Fundamentally, T cell function is dependent on the orchestration of signals generated by receptors found in their plasma membrane. T cells are activated when T cell receptors (TCRs) on their plasma membrane interact with cognate peptide antigen-major histocompatibility complexes (pMHC) presented on the surface of antigen presenting cells (APCs). These interactions occur at the contact interface between T cells and APCs that, upon antigen recognition, matures into a structure known as the immunological synapse [2]. In addition to the TCR there are numerous co-stimulatory and co-inhibitory receptors, the signals from which must be integrated to culminate in a specific output: the cell response [3]. Due to this integration of positive and negative regulation, APCs can prevent, dampen or enhance a potential response of the T cells by the expression of ligands for co-inhibitory or co-activatory receptors.

During the process of maturation, auto-reactive T cells are eliminated in the thymus [4], however, some autoreactive T cells escape thymic selection, and migrate to the periphery. Inhibitory receptors play an important role in restoring tolerance and preventing self-reactive T cells from

initiating auto-immune reactions [4–10]. Inhibitory receptors that block the activation of T cells are also known as immune checkpoint receptors for their ability to prevent inappropriate autoreactive immune responses. Although this system is effective at protecting healthy cells from being attacked by T cells, some cancer cells and chronic viral infections take advantage of this protection mechanism, expressing high levels of the ligands for inhibitory receptors [11–13]. Due to the importance of immune checkpoint receptors, they have become increasingly important drug targets. Some of the most studied inhibitory receptors are Cytotoxic T lymphocyte-associated protein 4 (CTLA-4), Programmed death-1 (PD-1) and B and T lymphocyte attenuator (BTLA), with drugs targeting CTLA-4 and PD-1 already developed into frontline treatments for many cancers [14–17].

Here we review the mechanism of signal integration for a class of inhibitory receptors that function by recruiting cytosolic phosphatases. Specifically, we highlight how physical properties of the membrane and consequences of receptor-ligand interactions occurring at cell-cell interfaces may be important determinants of signal integration between these inhibitory receptors and the activatory signaling they modify.

INHIBITORY AND ACTIVATORY RECEPTORS NEED TO BE IN CLOSE PROXIMITY TO INTEGRATE THEIR SIGNALS

The TCR is a multi-chain complex consisting of alpha and beta subunits, responsible for binding pMHC, and CD3 chains (epsilon-delta, epsilon-gamma and zeta-zeta dimers), responsible for signaling. Upon TCR-pMHC binding, tyrosine residues within immunotyrosine-based activation motifs (ITAMs) present in the intracellular tails of the CD3 chains are phosphorylated by the Src-family kinase Lck. Phosphorylated ITAMs recruit the cytoplasmic kinase ZAP70 to the TCR complex, where the enzyme becomes activated and phosphorylates the membrane adaptor protein linker for activated T cells (LAT). Phosphorylated LAT in turn recruits other cytosolic adaptors that propagate the signal leading to full T cell activation [18, 19].

TCR mediated cellular activation can be modified by a number of co-stimulatory and co-inhibitory receptors [3]. Co-stimulatory receptors, such as CD28, can amplify T cell responses and in many contexts are essential for robust T cell mediated immune responses [20]. Similar to the TCR, when CD28 binds either of its ligands, CD80 and CD86, tyrosines on the intracellular tails are phosphorylated by Lck. These phosphorylated tyrosines recruit cytosolic mediators, such as phosphoinositide 3-kinase and Grb2, thereby augmenting T cell activation [21].

Conversely, co-inhibitory receptors, including PD-1, halt T cell activation [3, 22]. There are several mechanisms by which inhibitory receptors interfere with T cell activation. For example CTLA-4 competes with CD28 for CD80 and CD86 and thus limits co-activation via CD28. Alternatively, co-inhibitory receptors produce inhibitory intercellular signals

that by recruiting cytosolic phosphatases that are subsequently integrated into the activating signals of the TCR and co-stimulatory receptors. In this category of co-inhibitory receptors, in which PD-1 belongs, the physical principles of signal integration are key. This subset of receptors has intracellular tails that harbor immunotyrosine-based inhibitory motifs (ITIM) or immunotyrosine-based switch motifs (ITSM), which are phosphorylated by Lck when these receptors bind their cognate ligand. Once phosphorylated, the endodomains of inhibitory receptors recruit phosphatases, such as Src homology region 2 domain-containing phosphatase-1 (SHP-1) or Src homology region 2 domain-containing phosphatase-2 (SHP-2) that dephosphorylate activatory receptors, or SH2 domain-containing inositol 5'-phosphatase (SHIP) that dephosphorylates phosphatidylinositol-3,4,5-trisphosphate [23].

Cellular studies have shown that co-ligation of PD-1 with TCR and/or CD28 results in decreased phosphorylation of CD3 chains, CD28 and proteins of the downstream cascade such as Vav1, protein kinase C (PKC)- θ and extracellular signal-regulated kinase (ERK)1/2 [24–26] and *in vitro* reconstitution experiments show that SHP-2 recruited to PD-1 can directly dephosphorylate CD3 ζ and CD28 cytoplasmic tails [22]. At this point it is useful to make a distinction between receptor triggering and signal propagation. Van der Merwe and Dushek summarized it best when they defined TCR triggering as “[t]he process by which TCR binding to peptide-MHC molecules leads to biochemical changes in the cytoplasmic regions of the CD3 complex...” [27]. From this perspective, events such as ZAP70 recruitment or phosphorylation of LAT can be considered signal propagation downstream of TCR triggering. There are many competing models of TCR triggering [27, 28], but it is hard to imagine how a receptor like PD-1 might realistically interfere with any of the proposed triggering processes. Rather, the available data indicates that PD-1 intervenes post-triggering of individual TCR or CD28 receptors by decreasing the amount of phosphorylated receptor at any one time, or the lifetime of individual phosphotyrosines, on activatory receptors.

The integration of phosphatase activity tethered to co-inhibitory receptors with the kinase activity tethered to the TCR and co-inhibitory receptors can only occur if the spatial organization is such that the reach length of the phosphatase and kinase overlap. Since both signaling enzymes are tethered to the membrane via the recruitment to the respective receptors, it has become critical to examine the co-localization and co-clustering of activatory or co-activatory receptors in biological membranes. When ligands for PD-1, TCR, and CD28 are presented on the same surface the engaged receptors co-localize within clusters in the T cell [22, 26]. In an elegant series of experiments, Yokosuka et al. used PD-1 constructs with ectodomains elongated to different extents to demonstrate that the degree of mismatch between TCR/pMHC and PD-1/PD-L1 dimensions correlated inversely with co-localization and inhibitory function [26]. Elongating receptor-ligand interactions can have the additional effect of reducing the efficiency of receptor phosphorylation, which can be understood in the context of the kinetic segregation mechanism [29], and to

separate this effect the authors used PD-1 constructs with SHP-2 fused to the intracellular tail, thus circumventing the need for triggering. These experiments build on earlier work by Köhler et al. and Eriksson et al. who demonstrated a similar principle with inhibitory and activatory receptors in NK cells [30, 31]. Accordingly, it was noted that most known inhibitory and stimulatory receptors that bind surface-associated ligands form similar ligand-receptor dimensions, which are smaller than the large surface phosphatases that regulate their triggering [32], suggesting that this is a common requirement for signal integration between activatory and co-inhibitory receptors. Taken together, it seems that the degree of co-localization of some receptors is an important determinant in the integration of their function.

Before we review the complex mechanisms that underlie receptor co-localization, we would like to point out that co-localization does not seem to be a requirement for CD28-mediated co-stimulation, and is thus not a universal requirement for T cell signal integration. If ligands for CD28 are presented on separate cells from agonistic pMHC molecules, T cells still have enhanced responses, although the effect appears to be less efficient than if both ligands are presented on the same cell surface [33].

MECHANICS OF RECEPTOR-LIGAND INTERACTIONS AT CELL-CELL INTERFACES

Bonds occurring between membrane-bound receptors and ligands are subject to forces that have consequences for the organization of proteins at the surface. In this section we will review the evidence for how these forces impact on the formation of initial bonds and how they result in the size-based segregation and clustering of surface proteins.

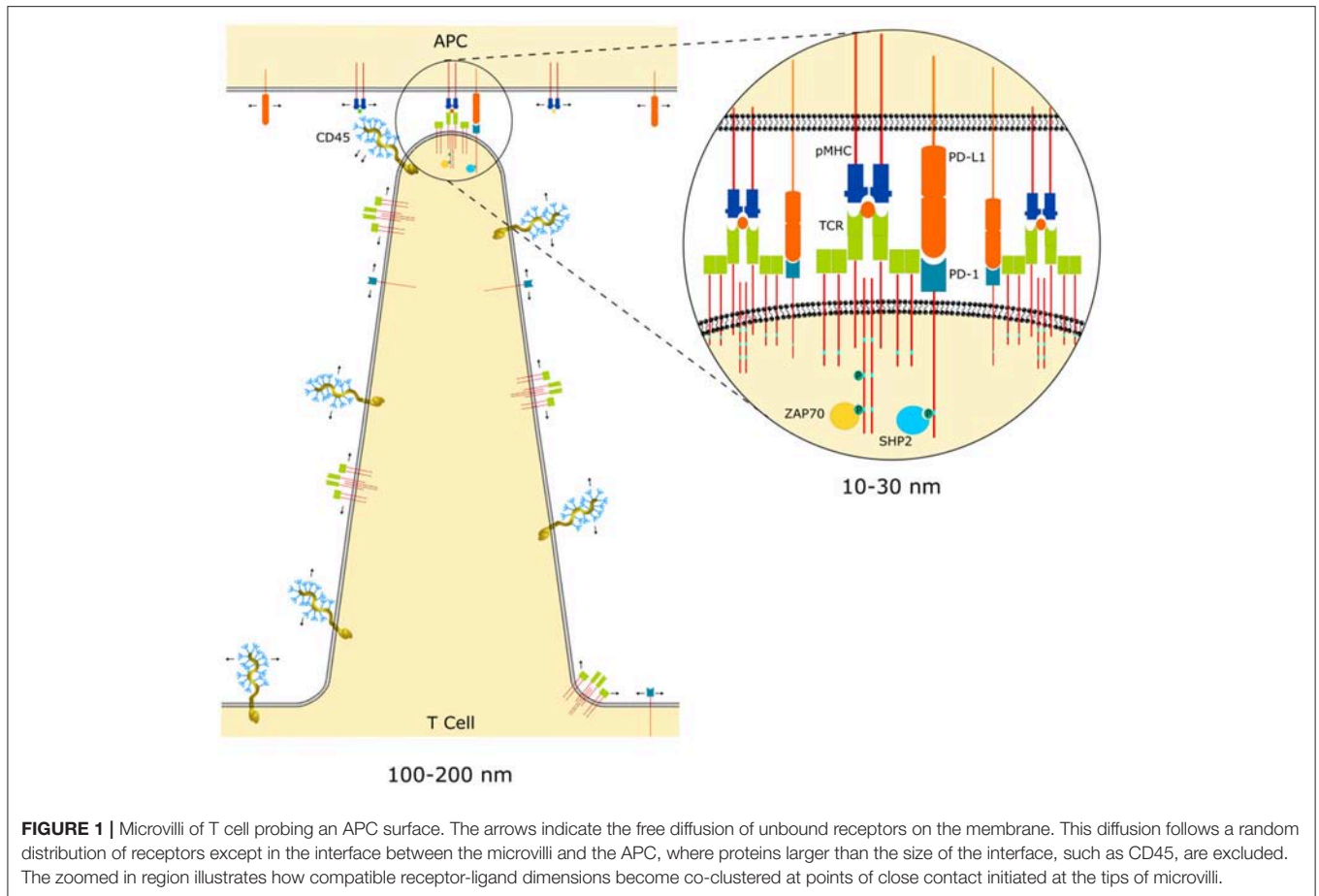
For bonds to occur between the TCR, co-inhibitory and co-activatory receptors and their respective membrane-bound ligands, the T cell and APC membranes must be the right distance apart. However, most immune receptor-ligand complexes span a short distance compared with the length of the glycocalyx. Given this size difference it is relevant to ask how bonds between smaller receptor-ligand pairs occur at all. To overcome the glycocalyx, which normally repulses the close apposition of two cell membranes, T cells actively probe APCs using thin (100–200 nm) actin-dependent finger-like membranous protrusions known as microvilli [34, 35] (**Figure 1**). These processes are highly dynamic and are continuously protruding and retracting [35]. Using a combination of super-resolution microscopy approaches Jung et al. suggested that these flexible projections are enriched with adhesion molecules and TCRs. However, it should be noted that all the molecules the authors investigated appeared to be enriched at the tip of microvilli, with the exception of CD45, which was modestly depleted [34]. The tips of microvilli were closer to the coverslip in their experiments and the authors may not have adequately corrected for the greater signal-to-noise at these points, which would be reflected in a greater probability of detection. Nevertheless, mounting

evidence suggests that T cells probe the surface of APCs with these microvilli and that these are likely the sites of the initial bonds formed between the TCR, co-inhibitory and co-activatory receptors [34, 35].

Once ligand-receptor interactions form across a cell-cell interface this has consequences for the organization of other proteins in the membrane. For example, the TCR-pMHC complex spans approximately 15 nm so that wherever these bonds exist the membranes of the two cells will be held apart at a distance equivalent to the complex dimensions (**Figure 1**). This gap will establish a size threshold that will segregate other surface proteins with an ectodomain larger than the interface height. CD45 is the best studied example of this and is excluded from points of TCR-pMHC bonds due to its large extracellular domain [36, 37]. This concept was also recently demonstrated in a reductionist system by Schmid et al. who used giant unilamellar vesicles coated with binding and non-binding fluorescent proteins of different dimensions [38]. Since this vesicular system lacked any cellular machinery the results demonstrate that segregation of proteins just 5 nm larger than the binding interface can occur purely as a result of the energy penalty caused by membrane bending to accommodate the larger protein.

Another important consequence of the uneven interface between the T cell and APC is that bonds formed between molecules on opposing membrane are much more likely to occur in regions where bonds of compatible dimensions already exist [39–41]. Thus, new ligand-receptor bonds gather around existing bonds, promoting the co-clustering of size-compatible ectodomain receptors, which is required for co-localization and signal integration between activatory and inhibitory receptors (**Figure 1**). As discussed earlier, initial interactions are likely formed at the tip of microvilli, but as the immune synapse evolves at later time points the contact interface flattens. Although, the nanoscale topology of the contact interface remains a topic of active investigation, receptor-ligand pairs with modestly different dimensions are known to segregate in the immunological synapse at late time points (several minutes after initial contact), with LFA1-ICAM-1, CD2-CD58, and TCR-pMHC forming a “bulls-eye” pattern of concentric circles in the synapse [42].

It should be noted here that TCR activation precedes full immunological synapse formation [43] and the typical “bulls-eye” pattern of the synapse (reviewed in detail elsewhere [2, 44]). It is easy to conflate events happening on the scale of the entire synapse (μm length scale, minutes time scale) with events happening at the scale of microvilli/clusters (100–200 nm length scale, 10 s of seconds time scale). This can be particularly confusing since the synapse-like patterns of integrin bonds surrounding TCR-pMHC bonds also occur in the form of micro-adhesion rings that surround TCR clusters at early timepoints [45]. However, as we will argue in the following section, signal integration between activatory and inhibitory receptors likely occurs at the scale of microvilli/clusters, and thus this distinction is important to keep in mind.



PHYSICAL PROPERTIES OF RECEPTOR TAILS ARE AN IMPORTANT DETERMINANT OF SIGNAL INTEGRATION

To understand why inhibitory receptors must co-localize with activatory receptors to function effectively we will conclude by more closely considering the molecular mechanisms of inhibitory signaling.

Proximal signaling downstream of the TCR and co-stimulatory receptor activation, initially occurs on the tails of the receptors, but eventually spreads to other proteins that are spatially separated from the receptors. In contrast, co-inhibitory receptors that recruit phosphatases do not seem to generate long-range inhibitory signals, but rather appear to function locally. The phosphatases that mediate inhibitory receptor function, SHP-1 and SHP-2, only become fully catalytically active when their SH2 domains are engaged by phosphorylated ITIM peptides [46, 47].

The concentration of substrate that SHP-1 or SHP-2 encounter when they are recruited to inhibitory receptors depends on the reach and flexibility of the cytoplasmic tail they are recruited to, the scale of co-localization with activatory receptors containing phosphorylated tyrosines, and the reach and flexibility of the cytoplasmic tail of the activatory receptors

(Figure 2). The overall reaction rate is thus dictated by the binding kinetics between the recruited enzyme and the immune receptor tail, the intrinsic catalytic rate, and the reach and mechanical properties of the immune receptor tail. There are some similarities between these membrane-tethered reactions and signaling reactions on scaffolds [48]. However, since immune receptors diffuse in the membrane, cluster and co-cluster with other immune receptors the situation is analogous but more fluid than scaffold signaling and some authors have used the term “tethered signaling” to describe proximal signaling reactions occurring on the tails of immune receptors [49].

Despite the importance of mechanics to tethered signaling reactions, the reach and flexibility of the cytoplasmic tails of activatory and inhibitory receptors remain largely unstudied. Local substrate concentration is an important consideration since tethering an enzyme to a poor substrate can override catalytic specificity and drive what would otherwise be an unfavorable reaction [50]. Mathematical modeling has been used to predict the local concentrations of enzymes tethered to substrates by unstructured linkers [51–53], which can dramatically enhance reaction rates. These studies often rely on modeling the unstructured regions of proteins the enzymes are tethered to, such as the tails of immune receptors, using the wormlike chain model. The worm-like chain model uses two

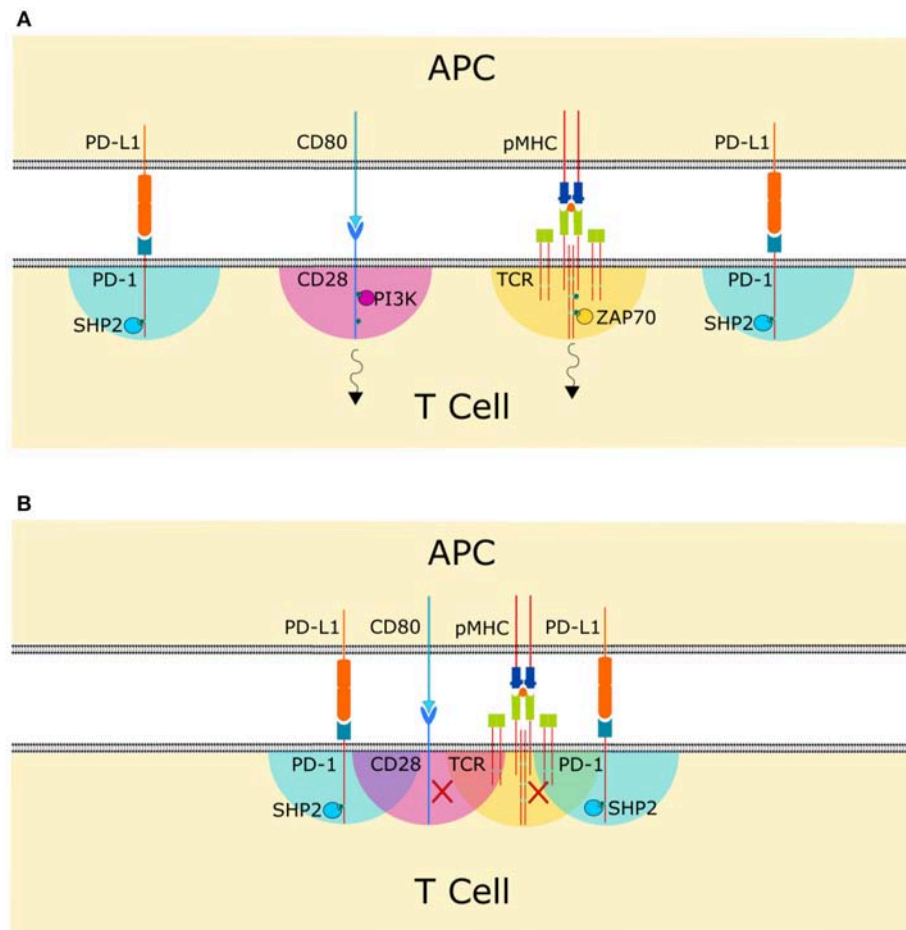


FIGURE 2 | Co-clustering of compatible size receptor-ligand bonds in the cell-cell interphase allows signal integration. The colored areas indicate the span of action of each membrane tethered enzyme. **(A)** If receptor-ligand bonds are spaced too far apart at the cell-cell interface, inhibitory receptors are not able to carry out their function and activatory receptors can initiate their signaling cascade, indicated by the black arrows. **(B)** Co-clustering of receptor-ligand bonds allows the cytoplasmic domains to overlap and for phosphatases recruited to the cytoplasmic tails of co-inhibitory receptors to reach substrate phosphotyrosines, allowing signal integration.

parameters to describe the behavior of polymers: persistence length (quantifying the stiffness of the polymer) and contour length (the end-to-end length of the polymer). Although a good estimate, the specific sequence of the unstructured region can significantly affect the behavior [54, 55] and furthermore the size and orientation of the enzyme tethered to tail is often neglected. Windisch et al. used mesoscale modeling to address this second point finding that, for a tethered reaction in a bacterial chemotaxis receptor pathway, the physical size of the rigid enzyme impeded its ability to explore a significant portion of the tether-restricted space [51]. Given the difficulties in predicting the local enzyme concentrations on membrane tethers, we found it necessary to develop an experimental system that can capture the various parameters involved in tethered signaling independently.

To directly measure important parameters describing tethered signaling reactions, Goyette et al. recently described a novel biophysical assay utilizing surface plasmon resonance (SPR) [49]. The method can be used to measure the binding, catalytic and effective reach parameters for a phosphatase interacting with

and dephosphorylating a phosphotyrosine-containing peptide immobilized on the SPR chip surface. The authors investigated the interaction of SHP-1 with an ITIM containing peptide derived from the inhibitory receptor leukocyte associated immunoglobulin like receptor 1 (LAIR1), finding that binding of the enzyme to the ITIM increased the catalytic rate 900-fold through a combination of allosteric activation and increased local substrate concentration [49]. Although the peptide used did not represent the full-length cytoplasmic tail of LAIR1 but was instead the ITIM portion with a PEG linker with shorter dimensions than the native tail, the results suggest that the enzyme itself contributed significantly to the effective reach length, which was measured to be 23 nm. Future studies using this technique with full length cytoplasmic tails of inhibitory receptors will hopefully shed some light on the effective reach and biophysical properties of tethered signaling reactions.

Based on the information with immobilized tethers, we know that the local concentration of recruited enzyme falls off rapidly with distance from the ligand-engaged receptor, highlighting the importance of close co-localization between

receptors. Although we do not know the exact physical properties of immune receptor tails, for a reasonable estimate of receptor cytoplasmic tail length, a change in co-localization distance from 5 to 50 nm can cause a 1,000-fold decrease in effective enzyme concentration [49], assuming that no lateral diffusion takes place. Beyond the maximum reach of the tether, the recruited enzyme is unable to catalyze reactions. These considerations explain the observed requirement for co-localization and compatible receptor-ligand dimensions between activating and inhibitory receptors discussed in the previous section, but also suggest a relevant scale for the co-localization (<50 nm). This scale fits well with the dimensions of the tips of microvilli (**Figure 1**), suggesting that they could be the primary sites of signal integration. It also fits well with results from Cai et al. who patterned ligands for the TCR receptor at defined intermolecular distances and found a lateral spatial threshold of <50 nm to maintain robust signaling [56]. The authors note that this distance corresponds well the predicted reach length of intracellular tails of the TCR complex, suggesting that this allows the signaling to be reinforced. We are proposing a similar mechanism, with opposite consequences, for the co-clustering of activatory receptors such as TCR or CD28, with co-inhibitory receptors, such as PD-1.

While in the SPR assay and Cai et al.'s ligand patterning, proteins are immobilized on the surface, in the plasma membrane individual receptors can diffuse laterally. The diffusion of receptor-ligand bonds on opposing membranes at a cell-cell interface however, is less clear. While there are many imaging approaches to measure membrane diffusion [57] and we recently engineered a FRET sensor that measures TCR clustering dynamics [58], it is difficult to distinguish engaged from non-engaged receptors at cell interfaces with these technologies. The problem is confounded in that only ~23% of the TCRs signaled when stimulated with excess antigen or activating antibodies [20]. Thus, the diversity of receptors—engaged vs. non-engaged, signaling vs. non-signaling receptors—that are genetically and biochemically identical makes interpretation of bulk measurements difficult. In addition, we lack a detail understanding of the spatial organization when a T cell transitions from the first contact with an APC via microvilli to a mature synapse. The best indication we have is from single molecule imaging of live cells, which show that when the TCR binds pMHC on opposing membrane (in this case a supported lipid bilayer), the resulting ligand-receptor complex does not undergo free diffusion and is localized in the same diffraction-limited (>200 nm) spot on the timescale of seconds [59]. Ultimately, over the course of tens of seconds to minutes, the complex associates with the actin cytoskeleton and is transported to the center of the synapse where internalization and signal down-regulation occurs [42]. These experiments suggest that either complex formation drastically reduces mobility of the receptor and ligand, or the complex undergoes very confined diffusion within a small close-contact region, or both occur.

Finally, even the assumption that the tip of a microvillus or the mature synapse represents a 2-dimensional zone for ligand-receptor interactions is likely too simplistic. Even when opposing membranes are held very close to the optimal distance for binding, thermal fluctuations and cortical cytoskeletal stiffness

can have large effects on binding kinetics on small scales (<100 × 100 nm) and receptor-ligand bonds can dampen thermal fluctuations, thus enhancing on-rates and reducing off-rates for compatible dimension receptors in their vicinity [60]. The theoretical upshot of this would again be distance-dependent synergy in compatible dimension receptor-ligand bond formation on a scale similar to the reach length of tethered reactions [49].

CONCLUDING REMARKS

A T cell must be able to integrate signals from inhibitory and activatory receptors. A possible mechanism is to use biophysical principles such as the spatial organization on the molecular scale to regulate biochemical reactions. This linkage occurs on two different levels: firstly, on the extracellular side, receptor engagement can lead to receptor clustering and co-localization and secondly, on the intracellular side, kinases and phosphatases can be tethered to the membrane by recruitment to phosphorylated receptor tails. The first is a result of the topology of the plasma membrane, including microvilli, together with passive mechanical forces acting to aggregate compatible dimension receptor-ligand complexes in close contact regions. Although some important specifics about the nanoscale topology of the contact interface and organization of receptors in the membrane remain uncertain, we propose a model in which these close contact points are the main site of co-inhibitory receptor signal integration. This model explains the requirement for compatible ectodomain dimensions between activatory and inhibitory ligand-receptor pairs and suggests that mechanical properties of receptor tails will have important consequences for the effectiveness of signal integration.

The relevant distances for these processes are on the molecular scale (tens of nanometres), which make them very difficult to study *in situ*. For this reason, modeling and reductionist systems have been important for highlighting the relevance of the biophysical properties of the cytoplasmic tails. With advances in microscopy techniques, and the development of complimentary biophysical methods, a clearer picture of how tethered signaling reactions occur within the complex environment of T cell-APC contact interfaces will emerge. As our understanding of signal integration processes becomes more nuanced it may become possible to manipulate and utilize them to construct more effective therapeutic strategies, such as synthetic chimeric antigen receptors [61].

AUTHOR CONTRIBUTIONS

PP-F, JG, and KG made substantial intellectual contributions to writing and editing the work.

ACKNOWLEDGMENTS

This work was supported by the Australia Research Council (CE140100011 to KG) and National Health and Medical Research Council of Australia (APP1059278 to KG and APP1163814 to JG).

REFERENCES

- Wencker M, Turchinovich G, Di Marco Barros R, Deban L, Jandke A, Cope A, et al. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat Immunol.* (2014) **15**:80–7. doi: 10.1038/ni.2773
- Alarcón B, Mestre D, Martínez-Martín N. The immunological synapse: a cause or consequence of T-cell receptor triggering? *Immunology* (2011) **133**:420–5. doi: 10.1111/j.1365-2567.2011.03458.x
- Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol.* (2013) **13**:227–42. doi: 10.1038/nri3405
- Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol.* (2005) **5**:772–82. doi: 10.1038/nri1707
- Schietinger A, Greenberg P. Tolerance and exhaustion: definin mechanisms of T cell dysfunction. *Trends Immunol.* (2014) **35**:51–60. doi: 10.1016/j.it.2013.10.001
- Hernandez J, Aung S, Redmond WL, Sherman LA. Phenotypic and functional analysis of CD8+ T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J Exp Med.* (2001) **194**:707–17. doi: 10.1084/jem.194.6.707
- Redmond WL, Marincak BC, Sherman LA. Distinct requirements for deletion versus anergy during CD8 T cell peripheral tolerance *in vivo*. *J Immunol.* (2005) **174**:2046–53. doi: 10.4049/jimmunol.174.4.2046
- Kurts C, Kosaka H, Carbone FR, Miller JFAP, Heath WR. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8+ T cells. *J Exp Med.* (1997) **186**:239–45. doi: 10.1084/jem.186.2.239
- Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol.* (2010) **11**:7–13. doi: 10.1038/ni.1818
- Schietinger A, Delrow JJ, Basom RS, Blattman JN, Greenberg PD. Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. *Science* (2013) **335**:723–7. doi: 10.1126/science.1214277
- Riley JL. PD-1 signaling in primary T cells. *Immunol Rev.* (2009) **229**:114–25. doi: 10.1111/j.1600-065X.2009.00767.x
- Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* (2006) **443**:350–4. doi: 10.1038/nature05115
- Grakoui A, John Wherry E, Hanson HL, Walker C, Ahmed R. Turning on the off switch: regulation of anti-viral T cell responses in the liver by the PD-1/PD-L1 pathway. *J Hepatol.* (2006) **45**:468–72. doi: 10.1016/j.jhep.2006.07.009
- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* (2010) **363**:711–23. doi: 10.1056/NEJMoa1003466
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med.* (2012) **366**:2443–54. doi: 10.1056/NEJMoa1200690
- Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med.* (2012) **366**:2455–65. doi: 10.1056/NEJMoa1200694
- Carosella ED, Ploussard G, LeMaout J, Desgrandchamps F. A systematic review of immunotherapy in urologic cancer: evolving roles for targeting of CTLA-4, PD-1/PD-L1, and HLA-G. *Eur Urol.* (2015) **68**:267–79. doi: 10.1016/j.eururo.2015.02.032
- Malissen B, Grégoire C, Malissen M, Roncagalli R. Integrative biology of T cell activation. *Nat Immunol.* (2014) **15**:790–7. doi: 10.1038/ni.2959
- Roncagalli R, Hauri S, Fiore F, Liang Y, Chen Z, Sansoni A, et al. Quantitative proteomics analysis of signalosome dynamics in primary T cells identifies the surface receptor CD6 as a Lat adaptor-independent TCR signaling hub. *Nat Immunol.* (2014) **15**:384–92. doi: 10.1038/ni.2843
- Pageon SV, Tabarin T, Yamamoto Y, Ma Y, Bridgeman JS, Cohnen A, et al. Functional role of T-cell receptor nanoclusters in signal initiation and antigen discrimination. *Proc Natl Acad Sci USA.* (2016) **113**:E5454–63. doi: 10.1073/pnas.1607436113
- Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol.* (2003) **3**:939–51. doi: 10.1038/nri1248
- Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) **355**:1428–33. doi: 10.1126/science.aaf1292
- Long EO, Sik Kim H, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol.* (2013) **31**:227–58. doi: 10.1146/annurev-immunol-020711-075005
- Chemnitz JM, Parry R V, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol.* (2004) **173**:945–54. doi: 10.4049/jimmunol.173.2.945
- Sheppard K-A, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett.* (2004) **574**:37–41. doi: 10.1016/j.febslet.2004.07.083
- Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med.* (2012) **209**:1201–17. doi: 10.1084/jem.20112741
- van der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. *Nat Rev Immunol.* (2011) **11**:47–55. doi: 10.1038/nri2887
- Malissen B, Bongrand P. Early T cell activation: integrating biochemical, structural, and biophysical cues. *Annu Rev Immunol.* (2015) **33**:539–61. doi: 10.1146/annurev-immunol-032414-112158
- Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol.* (2006) **7**:803–9. doi: 10.1038/ni1369
- Köhler K, Xiong S, Brzostek J, Mehrabi M, Eissmann P, Harrison A, et al. Matched sizes of activating and inhibitory receptor/ligand pairs are required for optimal signal integration by human natural killer cells. *PLoS ONE* (2010) **5**:e15374. doi: 10.1371/journal.pone.0015374
- Eriksson M, Leitz G, Fällman E, Axner O, Ryan JC, Nakamura MC, et al. Inhibitory receptors alter natural killer cell interactions with target cells yet allow simultaneous killing of susceptible targets. *J Exp Med.* (1999) **190**:1005–12.
- Dushek O, Goyette J, Van der Merwe PA. Non-catalytic tyrosine-phosphorylated receptors. *Immunol Rev.* (2012) **250**:258–76. doi: 10.1111/imr.12008
- Lim H-S, Cordoba S-P, Dushek O, Goyette J, Taylor A, Rudd CE, et al. Costimulation of IL-2 production through CD28 is dependent on the size of its ligand. *J Immunol.* (2015) **195**:5432–9. doi: 10.4049/jimmunol.1500707
- Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) **113**:E5916–24. doi: 10.1073/pnas.1605399113
- Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) **356**:eaal3118. doi: 10.1126/science.aa13118
- Chang VT, Fernandes RA, Ganzinger KA, Lee SF, Siebold C, McColl J, et al. Initiation of T cell signaling by CD45 segregation at “close contacts.” *Nat Immunol.* (2016) **17**:574–82. doi: 10.1038/ni.3392
- Cordoba S-P, Choudhuri K, Zhang H, Bridge M, Basat AB, Dustin ML, et al. The large ectodomains of CD45 and CD148 regulate their segregation from and inhibition of ligated T-cell receptor. *Blood* (2013) **121**:4295–302. doi: 10.1182/blood-2012-07-442251
- Schmid EM, Bakalar MH, Choudhuri K, Weichsel J, Ann HS, Geissler PL, et al. Size-dependent protein segregation at membrane interfaces. *Nat Phys.* (2016) **12**:704–11. doi: 10.1038/nphys3678
- Taylor MJ, Husain K, Gartner ZJ, Mayor S, Vale RD. A DNA-based T cell receptor reveals a role for receptor clustering in ligand discrimination. *Cell* (2017) **169**:108–19.e20. doi: 10.1016/j.cell.2017.03.006
- Paszek MJ, DuFort CC, Rossier O, Bainer R, Mouw JK, Godula K, et al. The cancer glycocalyx mechanically primes integrin-mediated growth and survival. *Nature* (2014) **511**:319–25. doi: 10.1038/nature13535

41. Goyette J, Gaus K. Mechanisms of protein nanoscale clustering. *Curr Opin Cell Biol.* (2017) **44**:86–92. doi: 10.1016/j.ceb.2016.09.004
42. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* (1999) **285**:221–7. doi: 10.1126/science.285.5425.221
43. Lee K-H, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS. T cell receptor signaling precedes immunological synapse formation. *Science* (2002) **295**:1539–42. doi: 10.1126/science.1067710
44. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse : a molecular machine controlling T cell activation. *Science* (1999) **285**:221–8.
45. Tane AH, Sakuma M, Ike H, Yokosuka T, Kimura Y, Ohara O, et al. Micro – adhesion rings surrounding TCR microclusters are essential for T cell activation. *J Exp Med.* (2016) **213**:1609–26. doi: 10.1084/jem.20151088
46. Hof P, Pluskey S, Dhe-paganon S, Eck MJ, Shoelson SE. Crystal structure of the tyrosine phosphatase SHP-2. (1998) **92**:441–50. doi: 10.1016/S0092-8674(00)80938-1
47. Pei D, Lorenz U, Klingmüller U, Neel BG, Walsh CT. Intramolecular regulation of protein tyrosine phosphatase SH-PTP1: a new function for Src homology 2 domains. *Biochemistry* (1994) **33**:15483–93.
48. Good MC, Zalatan JG, Lim WA. Scaffold proteins: hubs for controlling the flow of cellular information. *Science* (2011) **332**:680–6. doi: 10.1126/science.1198701
49. Goyette J, Salas CS, Gordon NC, Bridge M, Isaacson S, Allard J, et al. Biophysical assay for tethered signalling reactions reveals tether-controlled activity for the phosphatase SHP-1. *Sci Adv.* (2017) **3**:e1601692. doi: 10.1126/sciadv.1601692
50. Won AP, Garbarino JE, Lim WA. Recruitment interactions can override catalytic interactions in determining the functional identity of a protein kinase. *Proc Natl Acad Sci USA.* (2011) **108**:9809–14. doi: 10.1073/pnas.1016337108
51. Windisch B, Bray D, Duke T. Balls and chains - a mesoscopic approach to tethered protein domains. *Biophys J.* (2006) **91**:2383–92. doi: 10.1529/biophysj.105.078543
52. Krishnamurthy VM, Semetey V, Bracher PJ, Shen N, Whitesides GM. Dependence of effective molarity on linker length for an intramolecular protein-ligand system. *J Am Chem Soc.* (2007) **129**:1312–20. doi: 10.1021/ja066780e
53. Van Valen D, Haataja M, Phillips R. Biochemistry on a leash: the roles of tether length and geometry in signal integration proteins. *Biophys J.* (2009) **96**:1275–92. doi: 10.1016/j.bpj.2008.10.052
54. Marsh JA, Forman-Kay JD. Sequence determinants of compaction in intrinsically disordered proteins. *Biophys J.* (2010) **98**:2374–82. doi: 10.1016/j.bpj.2010.02.006
55. Moglich A, Joder K, Kiefhaber T. End-to-end distance distributions and intrachain diffusion constants in unfolded polypeptide chains indicate intramolecular hydrogen bond formation. *Proc Natl Acad Sci USA.* (2006) **103**:12394–9. doi: 10.1073/pnas.0604748103
56. Cai H, Muller J, Depoil D, Mayya V, Sheetz MP, Dustin ML, et al. Full control of ligand positioning reveals spatial thresholds for T cell receptor triggering. *Nat Nanotechnol.* (2018) **13**:610–7. doi: 10.1038/s41565-018-0113-3
57. Owen DM, Williamson D, Rentero C, Gaus K. Quantitative microscopy: Protein dynamics and membrane organisation. *Traffic* (2009) **10**:962–71. doi: 10.1111/j.1600-0854.2009.00908.x
58. Ma Y, Pandzic E, Nicovich PR, Yamamoto Y, Kwiatek J, Pigeon SV, et al. An intermolecular FRET sensor detects the dynamics of T cell receptor clustering. *Nat Commun.* (2017) **8**:15100. doi: 10.1038/ncomms15100
59. O'Donoghue GP, Pielak RM, Smoligovets AA, Lin JJ, Groves JT. Direct single molecule measurement of TCR triggering by agonist pMHC in living primary T cells. *Elife* (2013) **2**:e00778. doi: 10.7554/eLife.00778
60. Hu J, Lipowsky R, Weikl TR. Binding constants of membrane-anchored receptors and ligands depend strongly on the nanoscale roughness of membranes. *Proc Natl Acad Sci USA.* (2013) **110**:15283–8. doi: 10.1073/pnas.1305766110
61. Lim WA, June CH. The principles of engineering immune cells to treat cancer. *Cell* (2017) **168**:724–40. doi: 10.1016/j.cell.2017.01.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bridging the Nanoscopy-Immunology Gap

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Bridging the gap between traditional immunology and nanoscale biophysics has proved more difficult than originally thought. For cell biology applications however, super-resolution microscopy has already facilitated considerable advances. From neuronal segmentation to nuclear pores and 3D focal adhesion structure—nanoscopy has begun to illuminate links between nanoscale organization and function. With immunology, the explanation must go further, relating nanoscale biophysical phenomena to the manifestation of specific diseases, or the altered activity of specific immune cell types in a bodily compartment. What follows is a summary of how nanoscopy has elucidated single cell immunological function, and what might be achieved in the future to link quantifiable, nanoscale, biophysical phenomena with cell and whole tissue functionality. We explore where the gaps in our understanding occur, and how they might be addressed.

OPEN ACCESS

Edited by:

Jorge Bernardino de La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

Florian Baumgart,
Technische Universität Wien, Austria
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Specialty section:

This article was submitted to
Biomedical Physics,
a section of the journal
Frontiers in Physics

Received: 24 September 2018

Accepted: 18 December 2018

Published: 24 January 2019

Citation:

Shannon M and Owen DM (2019)
Bridging the Nanoscopy-Immunology
Gap. *Front. Phys.* 6:157.
doi: 10.3389/fphy.2018.00157

Keywords: T cells, immunology, SMLM, nanoscopy, super-resolution

The so called immunological synapse has proved fertile ground for the intersection of nanoscopy with immunology [1]. The word *synapse* originates from Greek *sun*—together and *haptis*—joining. Like a neuronal synapse, the immune synapse (IS) describes a communication zone at the interface between two cells. In the case of the T cell synapse, a T lymphocyte and an antigen presenting cell (APC) communicate through an intricately arranged array of receptors. Archetypal immune synapses can be recapitulated through use of supported lipid bilayers (SLBs) loaded with peptide bound major histocompatibility complex (p-MHC) [2], or more simply through the use of activating antibodies bound to glass, directed at the T cell receptor [3, 4]. In the past few years, several proteins in the T cell membrane have been found to be organized on nano-length scales, and links to cell function have begun to emerge.

In the fields of super-resolution and biophysics, the IS has had a dual function—as an area for cell biological insight in itself, and as a proving ground for super-resolution technique development. Cell biology has benefitted from the system as headway has been made in understanding the role of nanoscale molecular organization of the TCR [5–7], LFA-1 [8, 9], LAT [10, 11] and the nanoscale meshworks formed from fibers like actin [12]. Elsewhere, super-resolution imaging has been used to study nanoscale structures such as the nuclear pore complex, mechanisms of segmentation within organelles such as the mitochondria, or the axon and dendrites of neurons [13, 14]. In these cases, the use of diverse nanoscopic methodologies helped independent researchers report and verify the same structures.

The organization of the TCR has been subject to rather intense investigation over the last few years, in part because of controversy over its nanoscale organization, and in part due to artifacts emanating from the imaging. It therefore provides a useful case study of many researchers being involved in the elucidation of a phenomenon using new technology.

Bjorn Lillemeier's group were among the first to show that T cell receptor forms nanoclusters in activated cells, which group together into islands to amplify a signal, allowing T cells to

surpass a threshold for activation [6]. In a noisy, live environment, resolution was reduced, but new information afforded by this technique showed that the TCR and LAT indeed organize into nanoscale islands upon activation by a single pMHC-TCR interaction *in vivo*. This, the authors posit, is a nanoscale spatial mechanism for signal amplification—one that negates the need for 10 or more antigen peptides to be presented for full T cell activation [15].

However, the existence of TCR clusters in antigen experienced “resting” cells is less clear, and new analysis suggests that pre-clustered TCR may be an artifactual symptom of molecular overcounting [8, 16]. Alternatively, such clusters may represent ligand-independent triggering of the TCR that happens when T cells come into contact with various surfaces commonly used for *in vitro* assays, including poly-L-lysine [17]. Molecular overcounting occurs when multi-blinking fluorescent emitters are detected more than once, in slightly different places, resulting in a single molecule looking like a cluster in the output data. Varying the label density is a possible way to rule out false clusters in super resolution localization data [8]. When this technique was used, no TCR clusters were found in resting T cells. Biologically, new data suggests that the T cell receptor would seem to benefit from being a single entity in that this would speed the scanning phase in resting T cells: if TCRs are non-clustering, they have a higher probability of coming into contact with pMHC [18]. Recent work by Cai et al. supports this. Here, high resolution lattice lightsheet microscopy, along with quantification using the displacement of quantum dots showed how microvilli are used by T cells to speed the scanning process [19]. Jung et al. used variable angle TIRF and localization of fluorescently labeled TCR and Zap70 (Syk family kinase involved in TCR activation) to observe nano-clustering at the tips of the microvilli [20]. The precise characteristics of TCR and Zap70 clusters, and the function of the clustering observed at the dwell sites of microvilli requires further investigation. The Zap70 catch and release model [21] appears to match well with microvillar scanning, which might provide the amplification needed for further signaling and full activation in the presence of a strong, albeit rare signal. Together, these works provide evidence that the nanoscale spatial localization of multiple signaling molecules like this are likely to be involved in the regulation and amplification of an initial TCR-pMHC signal.

NANOSCALE T CELL MIGRATION

Intrinsically coupled to their function of antigen recognition, T cells are necessarily highly migratory. The precisely orchestrated timing of targeted T lymphocyte migration is also called “homing,” and describes a voyage through peripheral tissues, lymphoid vessels and organs, and along vasculature, during which a process of differentiation into many different T cell subtypes occurs—themselves with intrinsic migratory capacities [22]. In the lymph node, naïve T cells undertake the scanning of dendritic cells—interactions which can total 500 to 5,000 cell contacts per hour per T cell [23]. Integrin based adhesions have long been known to be important for these events, but only recently have we been able to observe their machinations on the

nanoscale. Molecular clustering is necessary for the structured formation of adhesions in T cells [24], and new nanoscopy techniques have allowed us to show that there are levels of organization beyond microscale adhesion clusters. The dynamics of such clusters likely occur across many timescales, and while live cell PALM in the T cell synapse has provided important insights into nanocluster dynamics with temporal resolution of 1–2 s [10, 11, 25–27], fast dynamics will rely on the development of much faster localization imaging.

Nanoscale T cell adhesions are short lived, small and distinct from conventional focal adhesions observed in other cell types. Recent super-resolution microscopy work has shown that T cell adhesions based around LFA-1 integrin are very small throughout the cell—on the length scale of nascent adhesions at around 100 nm in diameter [9]. Those adhesions that are anchored to ligand on the outside of the cell, and to actin on the inside remain engaged from the leading edge through the lamella, but have a total lifespan of <1 min. As the field progresses, further nanoscopic investigation may help us to answer questions about the precise composition and compartmentalization of nanoscale T cell adhesions. One possibility is that actin and the membrane work together to compartmentalize nano-adhesions, a concept pioneered by Kusumi [28, 29] and suggested for T cells by Lillemeier [6].

Recent work has linked the modulation of such nanoscale adhesions with altered migration due to the loss of function mutation of a phosphatase associated with autoimmune disease: PTPN22 [9]. Upon loss of this phosphatase, effector CD8+ and CD4+ cells migrate considerably faster and alter their nano-adhesion structure as well as their link to the actin cytoskeleton. In humans suffering from rheumatoid arthritis, PTPN22 mutation or deficiency is linked to an accumulation of such effector T cells in the joints [30]. Therefore, it is possible that phosphatases like PTPN22 act as nanoscale control switches for integrin based adhesions, and that the lack of this control causes a default fast migration phenotype, contributing to the mis-localization of cells and development of autoimmune disease conditions. The effects on the base migration phenotype and accompanying nano-adhesion architecture caused by this mutation warrant more investigation. This might be done in other fast moving cell types affected by phosphatase mutation [31], as well as in specific T cell subtypes such as T effector memory cells that migrate in the periphery or central memory T cells that migrate mainly in the secondary lymph nodes [32].

MIGRATION PLASTICITY AND NANO-ADHESION MEMORY

The base structure of nanoscale adhesions may be immunologically important in the case of memory T cells which commit themselves to special patterns of migration. Individual T cells must dynamically alter their style of migration as they move through different environments in the periphery and in the lymph nodes [33]. When inside the lymph nodes, cells use actin to push and squeeze through their surroundings [34], and LFA-1 integrin to enhance DC scanning and migration

speed [35]. Along blood vessels, migration and diapedesis relies on actin “treadmilling” and integrin based adhesions to anchor themselves and translate actin flow into cell movement [35]. In the diverse environment of the periphery, it is likely that these modes of migration are mixed and complimentary and are probably true for other exploratory immune cells. Interestingly, it is also clear that subtypes of T cells develop and maintain set migratory patterns as they differentiate. Some predominantly move around the peripheral tissues (e.g., stem memory T cells), or remain resident there (T resident memory), while others reside largely in the secondary lymph nodes, such as the central memory T cells [32, 36].

Insight into the nanoscale behavior of adhesion receptors and effectors may provide immunological understanding as to why these cells maintain such behaviors. One question might be whether cells coded for certain kinds of migration also maintain “cluster memory,” where adhesion clusters adopt specific clustering formations in the membrane based on preformed intracellular clusters. Such preformed clusters of integrins and associated intermediates/effectors have been observed in vesicles in other cells [37]. If such vesicles are preformed and stored in pools, they could be used to quickly enact specific kinds of prepackaged migration, by using spatial platforms for fast signaling in a migratory niche. The precise arrangement of molecules within vesicles, and their mechanism of formation is unknown, but may soon be accessible with the combination of new optical and analytical tools. This hypothesis of nanoscale spatial memory might extend to vesicular adhesion receptors and effectors—importantly, integrins can be pre-activated within such vesicles [37].

The optical tools to analyze such phenomena exist. Lattice light sheet microscopy (LLSM) already allows for high spatiotemporal resolution at low laser power. Whole 3D volumes can be acquired very quickly, enabling imaging of single cell dynamics with high temporal resolution. Low phototoxicity and fast 3D imaging with high signal to noise has allowed the confirmation and measurement of dynamics of the well-studied immunological synapse in a 3D matrix between a T cell and a dendritic cell [38], as well as microvilli [19]. Recent advances in adaptive optics for microscopes has increased the reach of the lattice light-sheet technique, achieving sub 100 nm lateral and axial resolution even in highly scattering tissues [39]. The technique has also been used to get single molecule localization information, and has provided impressive PAINT images in fixed cells, where the narrow and homogenous Bessel beam lightsheet allowed for superior optical sectioning [38].

While LLSM has the advantage of high speed and low dose, allowing access to fast processes that occur in immune cells, it has not yet been used for quantification of single molecules in live cells. Extending this technique to identify single molecule involved in nanoclusters in live cells might be achievable through its combination with new live cell cluster quantification techniques [40]. Instead of focusing on optics or fluorescent protein development, the algorithm relies on statistics to increase the temporal resolution, by calculating the fewest points required to reliably state the relative size and density of an under-sampled nanocluster. The algorithm works with low

molecule numbers, such that enough information is collected in only a few raw frames to extract statistically relevant nanoscale information about cluster dynamics. Through advances in such quantitation techniques, live cell microscopy techniques and reversibly switchable or replenishable fluorophore engineering [41–43], it is likely that the dynamics of individual protein clusters of signaling molecules will be investigable in live T cells. Point pattern analysis at physiologically relevant spatio-temporal resolution will provide highly quantifiable data, potentially allowing sensitive processes to be statistically analyzed.

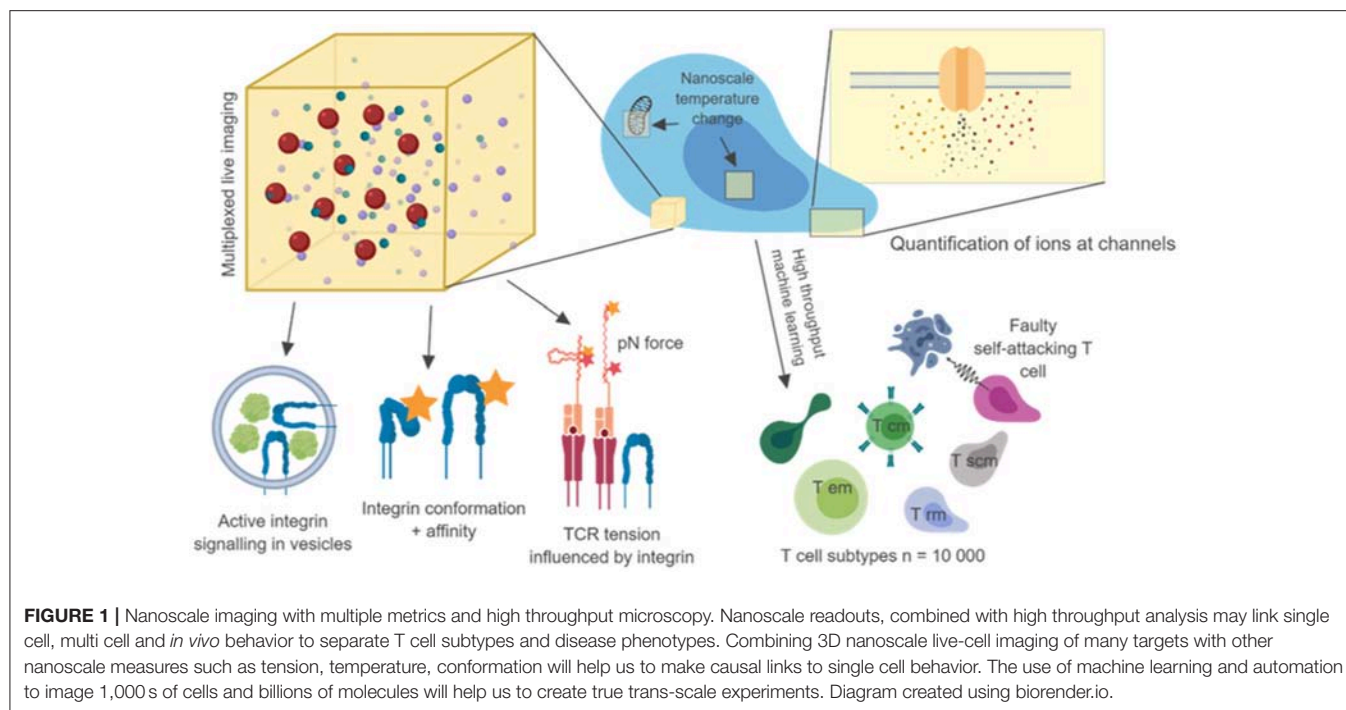
INCREASING THE SUPER-RESOLUTION SAMPLE SIZE

One concern within circles of immunologists is the relevance of tiny changes in the properties of nanoscale signaling domains to immune system function. That changes within nanoscale signaling domains are very small might seem like truism, but there is a valid point here. While researchers might use T cells sourced from several different people and observe high replicability across many samples, the nanoscale change may be tiny: cluster size changes of a few nanometers for example. Several functional changes have been linked to nanoscale alterations: the increased clustering of LAT derived from sub-cellular vesicles that correlates with T cell receptor activation and synapse formation [10], the co-clustering of Zap70 and TCR with LAT [25] as well as Lck [7, 8, 27] upon T cell activation. Recently, links to mechanism have been posited during the activation of B cells, where actin and tetraspanin reorganizes B cell receptors [44], in macrophages, where Src family kinases cause actin to rearrange FcγR1 nanoclusters [45], and in the regulation and formation of the natural killer cell lytic synapse [46].

Such studies provide important insight into phenomena in single cells. However, the low *n*-numbers lend themselves to study of the average changes within a heterogeneous dataset composed of diverse clusters, and do not allow access to population data when looking at multiple cell types in the same experiment as is common in immunology labs. This denies researchers access to possible minority phenomena—nanoscale machinations that start small, residing at the tail ends of data distributions, and result in whole cell change, as well as the effect of the change on the rest of the immune cell population in a given niche.

HIGH THROUGHPUT TRANS-SCALE MICROSCOPY

While the average super-resolution experiment relies on data from a few tens of cells, the jewel in the crown of immunology, flow cytometry, is able to analyze tens of thousands of cells in a matter of minutes. This allows for imaging and analysis of a diverse population, often giving a snapshot of immune cell populations in a given bodily niche. If super-resolution microscopy is to be used for population based imaging, the number of cells imaged, and their diversity must be increased. To achieve this, researchers have been making efforts



toward increasing the throughput of an average super-resolution experiment by automating the process [47, 48], and by using flat field illumination to increase the number of cells collectable in each image [49, 50].

Flat field super-resolution microscopy attempts to increase the illumination area to match the capabilities of scientific complementary metal oxide semiconductor (sCMOS) cameras, which offer a huge field of view [49]. sCMOS cameras used for localization microscopy also have lower readout noise and rapid readout rates, but are not as sensitive as EMCCD cameras. The latter have been the equipment of choice for most single molecule localization microscopy applications. However, while EM amplifies the signal it also amplifies the camera noise. sCMOS cameras do not use electron multiplication but have extremely low camera noise. Together, this means that contrast is higher and therefore single molecules can be localized more precisely by the latest sCMOS cameras than by EMCCD [38]. Combined with the faster readout rate and large field of view, as well as potential for better quantum yields in the future, sCMOS will likely supersede EMCCD cameras for localization microscopy.

Most super-resolution microscopes illuminate the sample such that there is a relatively narrow Gaussian distribution of intensity from the center of the field of view, which is made narrower in some cases by the use of a condenser lens to increase excitation intensity. Fluorophores near the edge of this illumination zone are less strongly excited than those in the center, and as a result are less precisely localized. To address this, Suliana Manley's group set up a flat field microscope, which relies on an inexpensive microlens array to image 100 μm^2 regions with super-resolution, more than quadrupling the current limits [49]. This could well be applied to immunology where samples

of a mixed population of T cells must be imaged all at once, identified separately and characterized on the nanoscale by super-resolution.

Automated super-resolution imaging and single particle tracking has been performed by Masato Yasui of the Ueda lab in Osaka [47]. His system uses fast focusing based on pixel intensity at the iris to select the correct focal plane, then machine learning to find and image cells with a given morphology and fluorescent signal. Combined with automated addition of chemicals or desired treatments using robotics, the system has the capacity to image several 100 cells per day. Single particle tracking or super-resolution localization microscopy can then be carried out in multiple conditions, resulting in hundreds of cells per condition, and millions of analyzable molecules. In conjunction are servers that can handle the localization of huge numbers of molecules, and localization and analysis algorithms that make most efficient use of the CPU or GPU of the systems involved. As an automated process, new ways to validate the quality of super-resolution data [51, 52], could be built into the process of screening before image processing.

MINORITY BIOLOGY

Increasing the *n* number from an average of 10 cells to 1,000 cells may also allow us access to new biological phenomena, undetectable when comparing the means of two conditions. Heterogeneity of clustering in cells is clear—usually the distributions display a wide range of cluster shapes, densities and colocalizations with other clustered/non-clustered molecules. Data comprised of many cells and many thousands more single molecule detections has the potential to

be mined in various ways to reveal minority or rare nanoscale phenomena.

Minority phenomena represent putative events that have a lot of power for change, but occur at the fringes of distributions. One might imagine a molecular cluster exhibiting divergent behavior. Such a cluster might be made of precisely organized layers of kinases, phosphatases and mechanical intermediates [such as a focal adhesion [53]] which at a given quorum of molecular participants [molecular conformations and phosphorylation states considered] initiates a signal cascade affecting neighboring clusters. This seed cluster could build up a level of signaling that then reorganizes actin [through the local action of Rho-GTPases for example [54]] or the strength of an adhesion [through modulation of vinculin binding proteins [55]] to change the direction of cell migration or to initiate the formation of a full synapse after initial microvillus based scanning.

Such a cluster would certainly be overlooked by conventional comparison of mean values between two separate distributions representing the characteristics of thousands of clusters. The kind of N -numbers available by increasing the throughput of super-resolution microscopy may allow for statistical analysis of segregated minority events at the tail ends of the population distribution on the nanoscale but also at single cell or cell population level. The transition from “scanning” to “swarming” mode of neutrophils in the lymph node is a good example of a single cell instigating a change in the behavior of the population [56]. High throughput, trans-scale type experiments may aid us to investigate such phenomena, allowing elucidation of the link between initial nanoscale behavior and cell to population wide functional outcomes.

FUNCTIONAL SUPER-RESOLUTION IMAGING

Nanoscale colocalization of transmembrane proteins with intracellular signaling intermediates with well-characterized functions can provide some level of insight into functional output. Such “proxy” measures are likely to lead to important discoveries, and with sub 10 nm resolution can complement FRET techniques. However, clear links between nanoscale molecular arrangements and their mechanisms will require the combination of super-resolution microscopy with direct functional readouts, which together might provide clearer answers about the importance of nanoscale events and how they relate to cell function, cell population function and immune system function (**Figure 1**).

One example of linking function to super resolved coordinate data is Travis Moore's work linking LFA-1 integrin conformation with its affinity for ligand [57]. By labeling the head group of the integrin and the inner leaflet of the plasma membrane, Moore and the AIC at Janelia research institute used interferometric PALM to directly detect conformational change in the protein by measuring the distance between the two [57]. The structure of LFA-1 integrin relates to its affinity, therefore it is important that the researchers confirmed that the integrin structures predicted from x ray crystallography matched those in cells.

DNA tension sensors represent another way single molecule data could be enriched, and have successfully been used to investigate the force applied by TCR when it binds to pMHC [58]. Here, the authors describe a positive relationship between the pulling force of TCR upon peptide-MHC and full T cell activation. LFA-1 binding to its ligand ICAM-1 augmented TCR/pMHC tension, implying crosstalk between the two pathways. Such an interaction represents a catch bond—one that becomes stronger as the force across it is increased. Other molecules that undergo catch bond behavior have been investigated by optical traps, such as vinculin [59]: an intracellular actin/integrin linker that reveals cryptic binding sites as it is stretched. Many techniques can now be utilized on a single cell or single molecule level to map forces in cells [59]. If such techniques can be multiplexed with super resolution microscopy, this may allow for matching tension with the spatio-temporal arrangement of molecules on the nanoscale. Adding layers of information to localization data could provide a route to discover whether specific structures or cluster types (with regards molecular content, density, size, and location) link to the mechanical tension they exert on their surroundings. This might also be used to find out whether clusters of molecules such as integrins are homogenous in their mechanical tension within single clusters. By imaging cells live, we may be able to discern whether these characteristics play a role in the basis of many immune cell functions, such as the formation of microvilli, of migration in response to chemokine, or of immune synapse formation. Temperature sensors represent another avenue of development which may be combined with super-resolution imaging. Recently, it was reported that mitochondria are maintained at a temperature of 50 degrees celcius, far above that of the cytoplasm [60] using a mito-targeted organic probe [61]. ERthermAC, a thermosensitive organic dye, has been used to detect large temperature changes in the endoplasmic reticulum of adipocytes [62]. It should not be discounted therefore that these temperature changes and others might occur in immune cells.

Finally, the imaging of ions at super-resolution would be useful for many areas related to immune cell behavior. Changes in ion flow through single channels are good candidates for minority events that may cause cell wide nanoscale changes that translate to function. Calcium ions are highly relevant to T cell activation, delivered through store operated calcium channels (SOC) [63] but also T-type channels [64] upon activation of the T cell receptor. Potassium ions are shown to be inhibitory to T cells, particular in the tumor microenvironment [65]. In addition, potassium efflux may be involved in chronic maintenance of T_{EM} cells, but several different potassium channels have compensatory roles, and the dynamics of ion flow at the level of single receptors is unknown [65]. The investigation of ion flux at super-resolution is therefore an area ripe for study in the context of peripheral T cell migration, interaction with target cells and during a failed response to cancer. Imaging ions, their respective channels/pumps and intermediates on the nanoscale has the potential to reveal more about how T cells respond quickly to different environments, and may be

achievable soon through the development of a raft of ion sensing probes [61].

CONCLUSION

Through the combination of cell friendly single molecule imaging, artifact controls, high throughput microscopy and multiple metrics on single molecules, trans-scale experiments can be undertaken. Such techniques may hold the key to many immunological phenomena that start on the nanoscale, representing the natural next step for immune function basic research. They may help us to understand how immune cells can be used for cancer immunotherapy [66], how the immune system co-develops with the body's bacterial population [67], and how immune cells malfunction and attack our own cells, causing autoimmune disease. The nano-scale is now truly accessible

to immunologists, and the right combinations of techniques will soon help us link that nanoscale world to holistic, causal outcomes.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by ERC Starter Grant #337187. MS was supported by the King's Bioscience Institute and the Guy's and St Thomas' Charity Prize Ph.D. Programme in Biomedical and Translational Science.

REFERENCES

- Dustin ML. The immunological synapse. *Cancer Immunol Res.* (2014) 2:1023–33. doi: 10.1158/2326-6066.CIR-14-0161
- Valvo S, Mayya V, Seraia E, Afrose J, Novak-Kotzer H, Ebner D, et al. Comprehensive analysis of immunological synapse phenotypes using supported lipid bilayers. *Methods Mol Biol.* (2017) 1584:423–41. doi: 10.1007/978-1-4939-6881-7_26
- Smoligovets AA, Smith AW, Wu HJ, Petit RS, Groves JT. Characterization of dynamic actin associations with T-cell receptor microclusters in primary T cells. *J. Cell Sci.* (2012) 125:735–42. doi: 10.1242/jcs.092825
- Kumari S, Curado S, Mayya V, Dustin ML. T cell antigen receptor activation and actin cytoskeleton remodeling. *Biochim Biophys Acta Biomembr.* (2014) 1838:546–56. doi: 10.1016/j.bbamem.2013.05.004
- Sherman E, Barr V, Samelson LE. Super-resolution characterization of TCR-dependent signaling clusters. *Immunol Rev.* (2013) 251:21–35. doi: 10.1111/imr.12010
- Hu YS, Cang H, Lillemeier BF. Superresolution imaging reveals nanometer- and micrometer-scale spatial distributions of T-cell receptors in lymph nodes. *Proc Natl Acad Sci USA.* (2016) 113:7201–6. doi: 10.1073/pnas.1512331113
- Owen DM, Rentero C, Rossy J, Magenau A, Williamson D, Rodriguez M, et al. PALM imaging and cluster analysis of protein heterogeneity at the cell surface. *J Biophotonics* (2010) 3:446–54. doi: 10.1002/jbio.200900089
- Baumgart F, Arnold AM, Leskova K, Staszek K, Fölser M, Weghuber J, et al. Varying label density allows artifact-free analysis of membrane-protein nanoclusters. *Nat Methods* (2016) 13:661–4. doi: 10.1038/nmeth.13897
- Burn GL, Cornish GH, Potrzebowski K, Samuelsson M, Griffié J, Minoughan S, et al. Superresolution imaging of the cytoplasmic phosphatase PTPN22 links integrin-mediated T cell adhesion with autoimmunity. *Sci Signal.* (2016) 9:ra99. doi: 10.1126/scisignal.aaf2195
- Williamson DJ, Owen DM, Rossy J, Magenau A, Wehrmann M, Gooding JJ, et al. Pre-existing clusters of the adaptor Lat do not participate in early T cell signaling events. *Nat Immunol.* (2011) 12:655–62. doi: 10.1038/ni.2049
- Griffié J, Shlomovich L, Williamson DJ, Shannon M, Aaron J, Khuon S, et al. 3D Bayesian cluster analysis of super-resolution data reveals LAT recruitment to the T cell synapse. *Sci Rep.* (2017) 7:4077. doi: 10.1038/s41598-017-04450-w
- Ashdown GW, Cope A, Wiseman PW, Owen DM. Molecular flow quantified beyond the diffraction limit by spatiotemporal image correlation of structured illumination microscopy data. *Biophys J.* (2014) 107:L21–3. doi: 10.1016/j.bpj.2014.09.018
- Sahl SJ, Hell SW, Jakobs S. Fluorescence nanoscopy in cell biology. *Nat Rev Mol Cell Biol.* (2017) 18:685–701. doi: 10.1038/nrm.2017.71
- Baddeley D, Bewersdorf J. Biological insight from super-resolution microscopy: what we can learn from localization-based images. *Annu. Rev. Biochem.* (2018) 87:965–89. doi: 10.1146/annurev-biochem-060815-014801
- Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature* (2002) 419:845–9. doi: 10.1038/nature01076
- Baumgart F, Arnold AM, Rossboth BK, Brameshuber M, Schütz GJ. What we talk about when we talk about nanoclusters. *Methods Appl. Fluoresc.* (2018) 7:013001. doi: 10.1088/2050-6120/aed0f
- Santos AM, Ponjavic A, Fritzsche M, Fernandes RA, de la Serna JB, Wilcock MJ, et al. Capturing resting T cells: the perils of PLL. *Nat Immunol.* (2018) 19:203–5. doi: 10.1038/s41590-018-0048-8
- Rossboth B, Arnold AM, Ta H, Platzer R, Kellner F, Huppa JB, et al. TCRs are randomly distributed on the plasma membrane of resting antigen-experienced T cells. *Nat Immunol.* (2018) 19:821–7. doi: 10.1038/s41590-018-0162-7
- Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) 356:eaal3118. doi: 10.1126/science.aa13118
- Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) 113:E5916–24. doi: 10.1073/pnas.1605399113
- Katz ZB, Novotná L, Blount A, Lillemeier BF. A cycle of Zap70 kinase activation and release from the TCR amplifies and disperses antigenic stimuli. *Nat Immunol.* (2017) 18:86–95. doi: 10.1038/ni.3631
- Chang JT, Wherry EJ, Goldrath AW. Molecular regulation of effector and memory T cell differentiation. *Nat Immunol.* (2014) 15:1104–15. doi: 10.1038/ni.3031
- Miller MJ, Hejazi AS, Wei SH, Cahalan MD, Parker I. T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. *Proc Natl Acad Sci USA.* (2004) 101:998–1003. doi: 10.1073/pnas.0306407101
- Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. *J Cell Sci.* (2003) 116:4695–705. doi: 10.1242/jcs.00876
- Sherman E, Barr V, Manley S, Patterson G, Balagopalan L, Akpan I, et al. Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity* (2011) 35:705–20. doi: 10.1016/j.immuni.2011.10.004

26. Lillemeier BF, Mörtelmaier MA, Forstner MB, Huppa JB, Groves JT, et al. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol.* (2010) **11**:90–6. doi: 10.1038/ni.1832
27. Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat Immunol.* (2013) **14**:82–9. doi: 10.1038/ni.2488
28. Shibata AC, Fujiwara TK, Chen L, Suzuki KG, Ishikawa Y, Nemoto YL, et al. Archipelago architecture of the focal adhesion: membrane molecules freely enter and exit from the focal adhesion zone. *Cytoskeleton* (2012) **69**:380–92. doi: 10.1002/cm.21032
29. Fujiwara T, Ritchie K, Murakoshi H, Jacobson K, Kusumi A. Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J Cell Biol.* (2002) **157**:1071–82. doi: 10.1083/jcb.200202050
30. Burn GL, Svensson L, Sanchez-Blanco C, Saini M, Cope AP. Why is PTPN22 a good candidate susceptibility gene for autoimmune disease? *FEBS Lett.* (2011) **585**:3689–98. doi: 10.1016/j.febslet.2011.04.032
31. Bottini N, Peterson EJ, Bottini N, Peterson EJ. Tyrosine phosphatase PTPN22: multifunctional regulator of immune signaling, development, and disease. *Annu Rev Immunol.* (2014) **32**:83–119. doi: 10.1146/annurev-immunol-032713-120249
32. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol.* (2013) **31**:137–61. doi: 10.1146/annurev-immunol-032712-095954
33. Krummel MF, Friedman RS, Jacobelli J. Modes and mechanisms of T cell motility: roles for confinement and Myosin-IIA. *Curr Opin Cell Biol.* (2013) **30**:9–16. doi: 10.1016/j.ccb.2014.05.003
34. Hons M, Kopf A, Hauschild R, Leithner A, Gaertner F, Abe J, et al. Chemokines and integrins independently tune actin flow and substrate friction during intranodal migration of T cells. *Nat Immunol.* (2018) **19**:606–16. doi: 10.1038/s41590-018-0109-z
35. Teixeira A, Hunter MC, Russo E, Proulx ST, Frei T, Debes GF, et al. T cell migration from inflamed skin to draining lymph nodes requires intralymphatic crawling supported by ICAM-1/LFA-1 Interactions. *Cell Rep.* (2017) **18**:857–65. doi: 10.1016/j.celrep.2016.12.078
36. Buchholz VR, Schumacher TN, Busch DH. T cell fate at the single-cell level. *Annu Rev Immunol.* (2016) **34**:65–92. doi: 10.1146/annurev-immunol-032414-112014
37. Alanko J, Ivaska J. Endosomes: emerging platforms for integrin-mediated FAK signalling. *Trends Cell Biol.* (2016) **26**:391–8. doi: 10.1016/j.tcb.2016.02.001
38. Chen BC, Legant WR, Wang K, Shao L, Milkie DE, Davidson MW, et al. Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science* (2014) **346**:1257998. doi: 10.1126/science.1257998
39. Liu TL, Upadhyayula S, Milkie DE, Singh V, Wang K, Swinburne IA, et al. Observing the cell in its native state: imaging subcellular dynamics in multicellular organisms. *Science* (2018) **360**:eaq1392. doi: 10.1126/science.aaq1392
40. Griffie J, Owen D, Rubin-Delanchy P, Burn G. Quantitative analysis of membrane protein clustering from live-cell, single-molecule super-resolution microscopy data. *Biophys J.* (2017) **112**:144a–5a. doi: 10.1016/j.bpj.2016.11.794
41. Kotera I, Iwasaki T, Imamura H, Noji H, Nagai T. Reversible dimerization of *Aequorea victoria* fluorescent proteins increases the dynamic range of FRET-based indicators. *ACS Chem Biol.* (2010) **5**:215–22. doi: 10.1021/cb900263z
42. Arai Y, Takauchi H, Ogami Y, Fujiwara S, Nakano M, Matsuda T, et al. A spontaneously blinking fluorescent protein for simple single laser super-resolution live cell imaging. *bioRxiv* (2017) 200014. doi: 10.1101/200014
43. Tiwari DK, Arai Y, Yamanaka M, Matsuda T, Agetsuma M, Nakano M, et al. A fast- and positively photoswitchable fluorescent protein for ultralow-laser-power RESOLFT nanoscopy. *Nat Methods* (2015) **12**:515–8. doi: 10.1038/nmeth.3362
44. Mattila PK, Feest C, Depoil D, Treanor B, Montaner B, Otipoby KL, et al. The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling. *Immunity* (2013) **38**:461–74. doi: 10.1016/j.immuni.2012.11.019
45. Lopes FB, Bálint Š, Valvo S, Felce JH, Hessel EM, Dustin ML, et al. Membrane nanoclusters of FcγRI segregate from inhibitory SIRPα upon activation of human macrophages. *J Cell Biol.* (2017) **216**:1123–41. doi: 10.1083/jcb.201608094
46. Mace EM, Orange JS. High- and super-resolution microscopy imaging of the NK cell immunological synapse. *Methods Mol Biol.* (2016) **1441**:141–50. doi: 10.1007/978-1-4939-3684-7_12
47. Yasui M, Hiroshima M, Kozuka J, Sako Y, Ueda M. Automated single-molecule imaging in living cells. *Nat Commun.* (2018) **9**:3061. doi: 10.1038/s41467-018-05524-7
48. Holden SJ, Pengo T, Meibom KL, Fernandez Fernandez C, Collier J, Manley S. High throughput 3D super-resolution microscopy reveals *Caulobacter crescentus* in vivo Z-ring organization. *Proc Natl Acad Sci USA.* (2014) **111**:4566–71. doi: 10.1073/pnas.1313368111
49. Douglass KM, Sieben C, Archetti A, Lambert A, Manley S. Super-resolution imaging of multiple cells by optimized flat-field epi-illumination. *Nat Photonics* (2016) **10**:705–8. doi: 10.1038/nphoton.2016.200
50. Rowlands CJ, Ströhl F, Ramirez PPV, Scherer KM, Kaminski CF. Flat-field super-resolution localization microscopy with a low-cost refractive beam-shaping element. *Sci Rep.* (2018) **8**:5630. doi: 10.1038/s41598-018-24052-4
51. Culley S, Albrecht D, Jacobs C, Pereira PM, Leterrier C, Mercer J, et al. NanoJ-SQUIRREL: quantitative mapping and minimisation of super-resolution optical imaging artefacts. *bioRxiv* (2017) 158279. doi: 10.1101/158279
52. Fox-Roberts P, Marsh R, Pfisterer K, Jayo A, Parsons M, Cox S. Local dimensionality determines imaging speed in localization microscopy. *Nat Commun.* (2017) **8**:13558. doi: 10.1038/ncomms13558
53. Kanchanawong P, Shtengel G, Pasapera AM, Ramko EB, Davidson MW, Hess HF, et al. Nanoscale architecture of integrin-based cell adhesions. *Nature* (2010) **468**:580–4. doi: 10.1038/nature09621
54. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. *Science* (2003) **302**:1704–9. doi: 10.1126/science.1092053
55. Goult BT, Zacharchenko T, Bate N, Tsang R, Hey F, Gingras AR, et al. RIAM and vinculin binding to talin are mutually exclusive and regulate adhesion assembly and turnover. *J Biol Chem.* (2013) **288**:8238–49. doi: 10.1074/jbc.M112.438119
56. Lämmermann T. In the eye of the neutrophil swarm-navigation signals that bring neutrophils together in inflamed and infected tissues. *J Leukoc Biol.* (2016) **100**:55–63. doi: 10.1189/jlb.1MR0915-403
57. Moore TI, Aaron J, Chew TL, Springer TA. Measuring integrin conformational change on the cell surface with super-resolution microscopy. *Cell Rep.* (2018) **22**:1903–2. doi: 10.1016/j.celrep.2018.01.062
58. Liu Y, Blanchfield L, Ma VP, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci USA.* (2016) **113**:5610–5. doi: 10.1073/pnas.1600163113
59. Huang DL, Bax NA, Buckley CD, Weis WI, Dunn AR. Vinculin forms a directionally asymmetric catch bond with F-actin. *Science* (2017) **357**:703–6. doi: 10.1126/science.aan2556
60. Chrétien D, Béné P, Ha HH, Keipert S, El-Khoury R, Chang YT, et al. Mitochondria are physiologically maintained at close to 50°C. *PLOS Biol.* (2018) **16**:e2003992. doi: 10.1371/journal.pbio.2003992
61. Arai S, Suzuki M, Park SJ, Yoo JS, Wang L, Kang NY, et al. Mitochondria-targeted fluorescent thermometer monitors intracellular temperature gradient. *Chem Commun.* (2015) **51**:8044–7. doi: 10.1039/C5CC01088H
62. Arai S, Lee SC, Zhai D, Suzuki M, Chang YT. A molecular fluorescent probe for targeted visualization of temperature at the endoplasmic reticulum. *Sci Rep.* (2015) **4**:6701. doi: 10.1038/srep06701
63. Zhang Y, Ge C, Zhu C, Salaita K. DNA-based digital tension probes reveal integrin forces during early cell adhesion. *Nat Commun.* (2014) **5**:5167. doi: 10.1038/ncomms6167
64. Lacinova L, Weiss N. It takes two T to shape immunity: emerging role for T-type calcium channels in immune cells. *Gen Physiol Biophys* (2016) **35**:393–6. doi: 10.4149/gpb_2016034

65. Eil R, Vodnala SK, Clever D, Klebanoff CA, Sukumar M, Pan JH, et al. Ionic immune suppression within the tumour microenvironment limits T cell effector function. *Nature* (2016) **537**:539–43. doi: 10.1038/nature19364
66. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, et al. Systemic immunity is required for effective cancer immunotherapy. *Cell* (2017) **168**:487–502.e15. doi: 10.1016/j.cell.2016.12.022
67. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* (2012) **336**:1268–73. doi: 10.1126/science.1223490

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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How T Cells Do the “Search for the Needle in the Haystack”

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OPEN ACCESS

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Specialty section:

This article was submitted to
Biomedical Physics,
a section of the journal
Frontiers in Physics

Received: 29 September 2018

Accepted: 17 January 2019

Published: 11 February 2019

Citation:

Baumgart F, Schneider M and
Schütz GJ (2019) How T Cells Do the
“Search for the Needle in the
Haystack”. *Front. Phys.* 7:11.
doi: 10.3389/fphy.2019.00011

In the body, a T cell is confronted with millions of antigen-presenting cells (APCs) in the search for potentially harmful antigen. To elicit an appropriate immune response, this search has to be performed as fast and as precise as possible. These two requirements, however, are at odds with each other: fast searches lack accuracy, whereas high fidelity decisions are typically time-consuming. Here, we use the archetypical *search for the needle in the haystack* as an analogy for the T cell's search problem. We provide a statistical framework to quantitatively estimate the constraints of search strategies for rare instances. Particularly, we propose a solution for balancing the demand for high speed with low error rates. It takes advantage of a two-phase search process, which combines a first rapid scan with a second high-fidelity check. Finally, we provide arguments that support a two-phase search model for identification of antigen-positive APCs by T cells.

Keywords: T cells, antigen-presenting cells, T cell antigen recognition, adaptive immune response, search for rare events, error rates, data mining, information retrieval

INTRODUCTION

T cells have evolved to launch specific immune responses when they detect peptides derived from potentially harmful intruders. Such peptides are displayed to T cells on the surface of antigen-presenting cells (APCs) via the major histocompatibility complex (MHC) [1]. A particular cognate peptide-loaded MHC (pMHC) might be present on only very few APCs out of tens of millions in the whole body [2]; conversely only very few down to even single T cells specific for a particular cognate antigen are present in an individual [3, 4]. T cells have thus the need (i) to detect very rare instances against immense background, and (ii) to scan as many APCs as possible in a short time, so that the immune system can react to the intruder as fast as possible.

In other words, T cells are challenged with the classical search problem of a rare event, often referred to as the *search for the needle in the haystack*. What is more, they have to do their job fast enough to avoid that potentially harmful intruders inflict significant damage on the body.

On the molecular level, a specific T cell receptor (TCR), which is expressed exclusively on the surface of one particular T cell clone, has to bind a given pMHC in order to elicit a T cell response. Binding is then translated into biochemical events in the T cells and eventually into an immune response [1]. Notably, only a small fraction of pMHC molecules—as few as 1 to 5 single molecules—on the surface of APCs actually contains peptides from dangerous intruders; the vast majority of peptides—as many as 200,000 [5, 6]—stems from endogenous components that have been captured and processed by APCs along with foreign ones. Identifying cognate antigen at high fidelity hence imposes a huge challenge: if this process was performed at high speed, it could lead to spurious decisions; on the other hand, if it was performed at high precision it would take a long time.

Here, we want to elaborate on some of the conceptual constraints of this statistical search problem. We show that the search for the needle in the haystack massively benefits from a two-phase search approach, which combines a first rapid scan with a second high-fidelity check. This sheds some light on why certain observed features of the T cell's search for antigen might have evolved in particular ways.

FORMULATING THE STOCHASTIC PROBLEM

In analogy to the *search for the needle in the haystack*, let us suppose a barn full of hay, and the task is to find the proverbial needle. People may come up with different strategies to perform this search: on one end of the spectrum, there are the structured personalities, who start on one corner and search their way through the whole haystack; on the other end, we have the intuitive searchers who randomly start somewhere, without any clear search strategy. But is there an optimum search strategy? This depends on (i) the search question, (ii) the cost function, and (iii) whether there is prior information available, e.g., on the position of the needle.

i) The search question

Before starting the search, the underlying search question has to be specified. As a matter of fact, there is a variety of questions which may be asked in the context of the needle in the haystack problem. Examples include:

- Is there a needle in the haystack?
- How many needles are in the haystack?
- Is there a specific spatial arrangement of needles within the haystack?

The type of question affects the search strategy: for example, it does not make sense to continue the search after finding the needle, if the first question should be answered. In case of T cell activation, we are just beginning to understand how to phrase the search question: while it is undoubted that T cells integrate signals over time and modulate their response accordingly, the underlying mechanisms are not well-understood [7]. Also the recruitment of APCs into the lymph node [8] as well as potential clustering of pMHC at the cell surface [9] will affect a T cell's search process. In this paper, we restrict ourselves to the analysis of the question about the presence of APCs presenting cognate antigen.

ii) The cost function

Optimization problems can be reduced to the task of minimizing or maximizing the cost function. In our case, there is a variety of cost functions which may be considered:

- The total search time
- The rate of false positives or false negatives
- The precision of the counting result

The search problem gets more complicated, if two or more cost functions should be optimized at the same time. In such a

multi-objective optimization problem, there is typically no single solution that simultaneously optimizes all cost functions. In other words, the different cost functions may be conflicting. In many cases, however, it is not important to find the global minima of all cost functions; a value below a certain threshold may be sufficient. This multi-objective optimization problem becomes important below, where we discuss the T cell's challenge to harmonize the conflicting needs for both specific and fast identification of antigen.

iii) Additional information

Prior information helps to focus a search. For example, we may ask the owner of the barn about the number of needles in the haystack, their position, how they are spatially distributed, etc. Also during the search, we may learn more about the organization of the haystack, which allows for updating the prior information and modify our search strategy accordingly. Finally, it could be the case that—during our search—additional needles are thrown into the haystack, making the problem

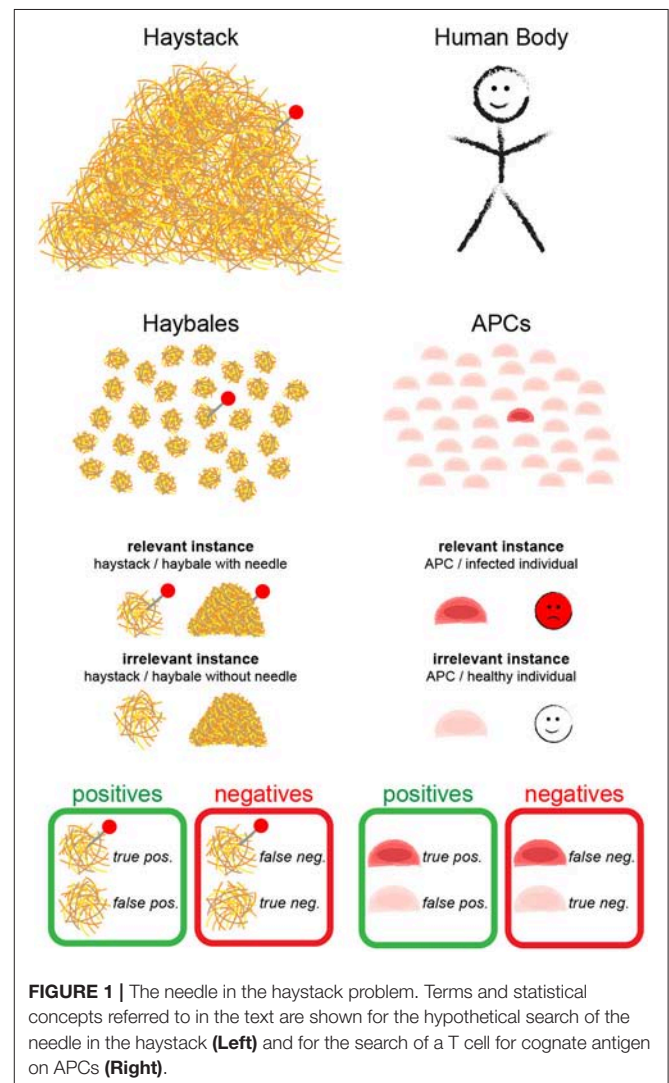


TABLE 1 | Statistical parameters.

GENERAL PARAMETERS	
p ...	probability of an individual haybale to contain a needle (i.e., to be a relevant instance)
n ...	number of checked haybales
k ...	number of detected relevant instances
PROBABILITY FOR A SINGLE HAYBALE TO CONTAIN A NEEDLE	
$p = \frac{M}{N}$	The probability of an individual haybale to be a relevant instance is given by the number of bales M that contain a needle divided by the total number of bales N .
GLOBAL AND LOCAL TRUE POSITIVE RATES (TPR , tpr)	
$TPR = \frac{TP}{Rel.} = \frac{TP}{TP+FN}$	The global true positive rate (TPR) is the probability of correctly identifying a haystack that contains a needle as a relevant case. It is given by the number of true positives (TP) divided by the number of all relevant instances, which is equal to the sum of true positives and false negatives ($TP + FN$).
$tpr = \frac{tp}{rel.} = \frac{tp}{tp+fn}$	The local true positive rate (tpr) is the probability of correctly identifying an individual haybale containing a needle as a relevant case. It is calculated analogously to TPR .
GLOBAL AND LOCAL FALSE POSITIVE RATES (FPR , fpr)	
$FPR = \frac{FP}{irr.} = \frac{FP}{TN+FP}$	The global false positive rate (FPR) is the probability of incorrectly classifying a haystack that does not contain any needle as a relevant case. It is given by the number of false positives (FP) divided by the number of all irrelevant instances, which is equal to the sum of true negatives and false positives ($TN + FP$).
$fpr = \frac{fp}{irr.} = \frac{fp}{tn+tp}$	The local false positive rate (fpr) is the probability of incorrectly classifying an individual haybale that does not contain any needle as a relevant case. It is calculated analogously to FPR .
GLOBAL AND LOCAL TRUE NEGATIVE RATES (TNR , tnr)	
$TNR = \frac{TN}{irr.} = \frac{TN}{TN+FP}$	The global true negative rate (TNR) is the probability of correctly identifying a haystack that contains no needle as an irrelevant case. It is given by the number of true negatives (TN) divided by the number of all irrelevant instances, which is equal to the sum of true negatives and false positives ($TN + FP$).
$tnr = \frac{tn}{irr.} = \frac{tn}{tn+tp}$	The local true negative rate (tnr) is the probability of correctly identifying an individual haybale containing no needle as an irrelevant case. It is calculated analogously to TPR .
GLOBAL AND LOCAL FALSE NEGATIVE RATES (FNR , fnr)	
$FNR = \frac{FN}{Rel.} = \frac{FN}{FN+TP}$	The global false negative rate (FNR) is the probability of incorrectly classifying a haystack that contains a needle as an irrelevant case. It is given by the number of false negatives (FN) divided by the number of all relevant instances, which is equal to the sum of false negatives and true positives ($FN + TP$).
$fnr = \frac{fn}{rel.} = \frac{fn}{fn+tp}$	The local false positive rate (fpr) is the probability of incorrectly classifying an individual haybale that contains a needle as an irrelevant case. It is calculated analogously to FPR .

dynamic. For the T cells' search problem, priors such as co-stimulating and co-inhibiting signals [10], or cytokines [11] convey additional information about the nature of potential antigens. This modulates the functional outcome of TCR signaling. In addition, the architecture of lymph nodes and T cell migratory patterns have evolved in a way that maximizes the chances of antigen encounter [4, 12]. Despite their importance in the real live scenario, we will neglect such priors in our discussion, as they would further complicate the line of argumentation without affecting the principal arguments.

DIFFERENT SEARCH PROBLEMS

Following our analogy further, the T cell would correspond to the person, who is searching for the needle—i.e., the cognate antigen—and all APCs together make up the haystack; the body corresponds to the barn (Figure 1). As APCs are separate entities, however, we can modify our search problem by splitting the haystack into smaller haybales, each corresponding to a single APC. The task is now to move through the barn, and to test—bale by bale—whether it contains a needle. The average probability

of finding an APC bearing agonist pMHC is given by $p = \frac{M}{N}$, with M the number of APCs bearing agonist pMHC and N the total number of APCs. It should be mentioned here that different mechanisms exist that direct T cells and APCs to lymph nodes during infections [4]; i.e., T cells in lymph nodes have to deal only with a subsample of all APCs in the body. However, since T cells roam multiple lymph nodes during their search for antigen and still interact with a great number of APCs, our assumption appears realistic.

In the following, we formulate a statistical approach to solve the search for the needle in the haystack. In the main text we confine ourselves to a discussion of the main results; derivations of the formulas are provided as **Supplementary Information**. During the text, we introduce statistical parameters that are important to quantify the different problems (see Table 1 for an overview). We call a “relevant instance” a scenario which would be detected by an ideal test, and “irrelevant instances” all other cases. Depending on the task, a single haybale—but also the whole haystack—carrying a needle could be a relevant instance.

Let us consider the search question, whether the barn contains haybales with needles. Let the null hypothesis be that there are no haybales with needles; this null hypothesis shall be tested. A straight-forward approach would be an unbiased random

search, in which the haybales are classified as needle-positive or -negative. The procedure is stopped after n haybales; if we find $k \geq 1$ relevant instances, we reject the null hypothesis, otherwise we keep it.

In order to provide statistically quantifiable arguments, multiple search experiments of this kind shall be performed: we assume many (ideally infinite amounts of) haystacks, some containing haybales with needles, others do not; this experiment allows to determine the four key figures of merit (**Figure 1**):

- True positives (TP): relevant instances that were correctly detected
- False negatives (FN): relevant instances that were erroneously rejected
- False positives (FP): irrelevant instances that were erroneously detected
- True negatives (TN): irrelevant instances that were correctly rejected

Since no errors for the classification step of single haybales were included up to now, this approach cannot yield any false positive results: if there are no needles, no needles will be detected. In contrast, we might miss haybales containing needles, in particular if they are rare and we do not search long enough. An appropriate measure of the search fidelity is the true positive rate (TPR), also called sensitivity or recall. Maximizing TPR is equivalent to minimizing false negatives, with maximum sensitivity being given by zero false negatives. In other words, at maximum TPR we will find the needle without doubt.

In **Figure 2** we show the true positive rate as a function of the number of checked haybales, n , for different values of the probability of finding a relevant instance, p . We neglected here correlations between the haybales, which allowed us to assume a Binomial distribution of events. Naturally, TPR increases with increasing n . Detection becomes unlikely for $p \ll 1$, in which case we can approximate $TPR \approx n \cdot p$. In other words, for rare relevant instances one needs more checks n , and—hence—longer time for the overall search to reach a similar value of the TPR .

Up to now the search problem was unrealistically simple: difficulties arising from the classification of each haybale were not considered so far. In our T cell activation scenario, however, it may well occur that a T cell erroneously classifies irrelevant APCs as relevant or *vice versa*. To consider such local mistakes, the model gets slightly more complicated: every haybale itself may belong to the group of true positives (it contains a needle, and was correctly detected), false negatives (it contains a needle, but was erroneously rejected), false positives (it does not contain a needle, but was erroneously detected), and true negatives (it does not contain a needle and was correctly rejected). To avoid confusion, we denote with upper case letters the figures of merit of the global search problem (the haystack), with lower case letters the figures of merit of the local search problem (the haybales).

Let us consider the situation, where there are needles present in the haystack. A true positive result of the global search problem is defined as the correct identification of such a haystack, and the true positive rate as the probability to correctly detect such haystacks as being relevant (i.e., containing needles). We can improve the global TPR by increasing the number of local true

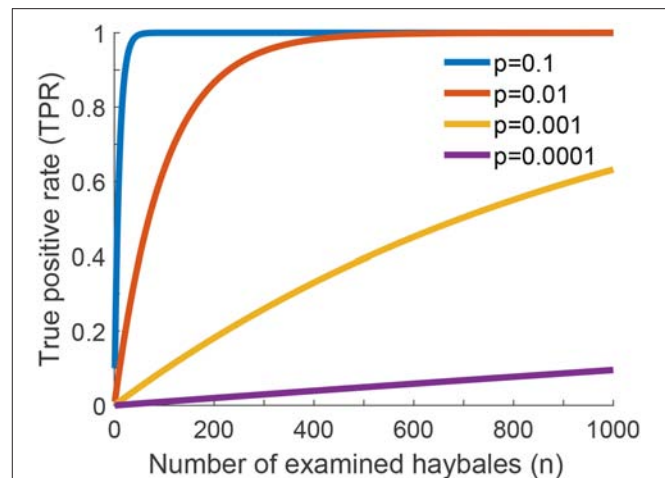


FIGURE 2 | True positive rate (TPR) for one-phase search strategy assuming no errors in the classification of individual haybales. The global true positive rate (TPR) of the search problem as a function of the number of examined haybales for different values of p is shown (see Equation 2 of **Supplementary Information**). p is the fraction of relevant instances present in the haystack.

positives: high numbers of haybales containing needles, and a high yield of correctly detecting them would suffice. On the other hand, however, we can also improve the global TPR by increasing local false positives: the more erroneous detections of needles, the more likely is a positive result of the global search. Let us look at this seemingly contradictory statement in more detail: Apparently, TPR is not the only parameter, which has to be considered when assessing the quality of our search process. For illustrating the diagnostic properties of a binary classifier system, statistics often uses receiver operating characteristic (ROC) curves. For this, a second figure of merit, the false positive rate, is calculated. It captures the likelihood of a global positive detection for the irrelevant case (no needles in the haystack). The idea of a good classifier system is achieving reasonably high TPR , while keeping FPR reasonably low.

In a ROC plot, TPR is plotted vs. FPR for various parameters of the test. In principle, if a test has no discriminative power, the curve follows the diagonal (also called the line of no discrimination). The better the test gets, the further the points locate in the upper half of the plot, with the point ($TPR = 1, FPR = 0$) representing perfect classification. In contrast, points below the diagonal correspond to classifications worse than random. The ROC curve for our problem is shown in **Figure 3A**, where we used the number of checked haybales n as parameter. Curves are shown for $p = 10^{-4}$ and various values of the local false positive rate fpr . For $fpr = 10^{-4}$ the classifier is doing reasonably well, with all data being above the diagonal (blue line). If the local false positive rate is increased, the performance of the classifier declines and the test loses its discriminative power (red line). In contrast, the test performs better when fpr is further reduced. Ultimately, if there were no local false assignments at all ($fpr = 0$), the curve would follow

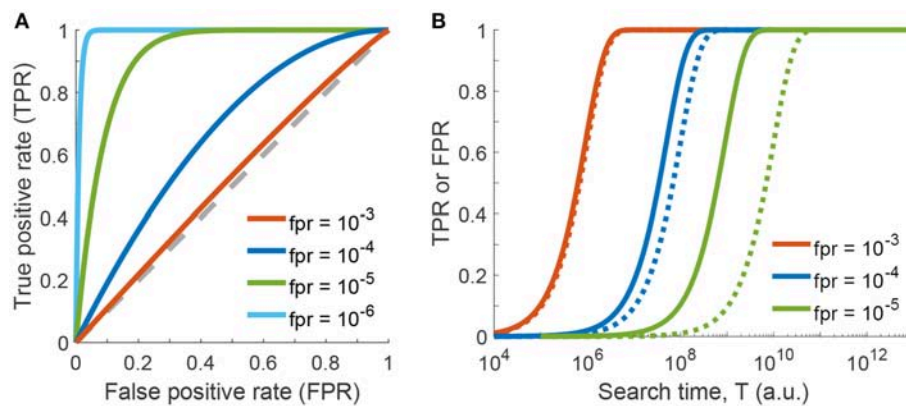


FIGURE 3 | One-phase search strategy, considering errors in classification of individual haybales. **(A)** Receiver operating characteristic (ROC) curve showing the global true positive rate TPR as a function of the global false positive rate FPR (see Equations 5 and 8 of **Supplementary Information**). Curves were calculated for $p = 10^{-4}$ and various values of local false positive rates fpr by varying the parameter n (the number of checked haybales). The line of no discrimination, i.e., where the test has no discriminative power, is shown as dashed gray line. **(B)** Global true or false positive rates as a function of search time T (see Equation 16 of **Supplementary Information**, $\Delta t = 1$) are shown for various values of local false positive rates fpr . Global true positive rates (TPR) are shown as solid lines, global false positive rates (FPR) as dotted lines.

the y-axis, which would be identical to the case discussed above in the context of **Figure 2**.

Taken together, an appropriate search strategy keeps the local false positive rates low while checking as many haybales as possible. While this strategy appears reasonable, it is not realistic. High global true positive rates do not come for free: we need a considerable number of tested haybales n and hence a rather long overall search time to achieve an appropriate TPR . Even more so, keeping fpr low is not simple in practice: too fast local searches will likely increase fpr .

In the final step of our statistical model, we will now include the search time in our considerations. For the sake of simplicity, let us assume that the time for testing a single haybale scales with fpr^{-1} . This reflects the idea that more time allows for more TCR-pMHC encounters, which improves the fidelity of the decision. Hence, the total time for testing n haybales is given by $T = n \cdot \Delta t / fpr$, where Δt denotes the time needed to check a single haybale. **Figure 3B** shows the resulting global true and false positive rates as a function of time T for different values of the local fpr . On the one extreme, for $fpr = 10^{-3}$ (red curve) the TPR rapidly approaches 1, however, there is no discriminative power against FPR . In other words, the test cannot discriminate between true and false positive events and is therefore useless. On the other end of the spectrum, for $fpr = 10^{-5}$ (green curves) discrimination works very well. However, we need more than 5 orders of magnitude longer to achieve the same TPR .

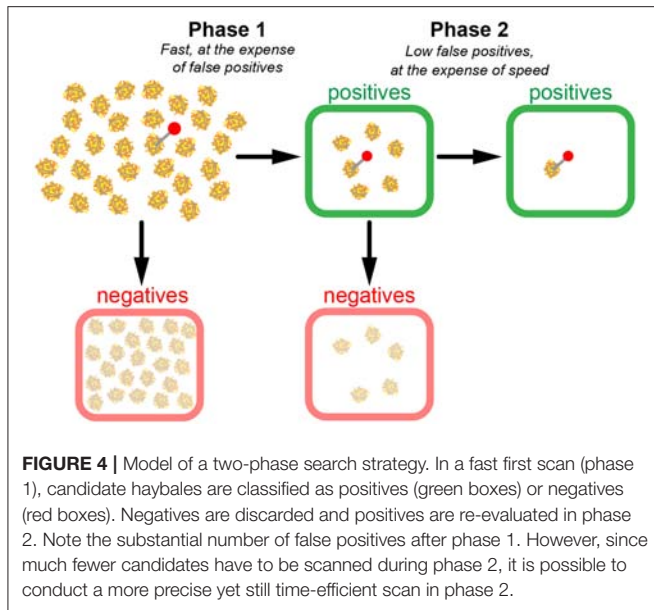
From these considerations, it becomes apparent that in case of extremely low relevant instances, a two-phase search strategy could make sense (**Figure 4**). In a first quick test phase, TPR is optimized without investing time to keep fpr low. In our picture, n_1 haybales are first superficially screened; the negatives are disregarded, whereas the positives are kept for a second phase, in which the hits of the first phase are scrutinized. The first phase, hence, contains all instances, the second phase only the positives of the first phase.

The huge difference to the situation before, however, is the altered total time required for coming up with a decision. The duration of the first phase is calculated in the same way as before, $T_1 = n_1 \cdot \Delta t / fpr_1$, however, it is performed now at much higher false positive rate and hence carried out much faster. For phase 2, more time has to be invested for keeping the false positive rate low, however, only the positives of phase 1 have to be considered. The two-phase search approach is thus massively faster than the one phase search approach, if we compare the time required for achieving the same figures of merit for TPR and FPR (**Figure 5**). As an example, we compared the search times of the one-phase and two-phase approaches using the parameters $p = 10^{-4}$, $fpr_1 = 10^{-2}$, and $fpr_2 = 10^{-4}$ (two-phase approach), which corresponds to $fpr = fpr_1 \cdot fpr_2 = 10^{-6}$ for the one-phase approach. The time improvement is an impressive factor of $\sim 5,000$ (see Equations 22 and 23 of **Supplementary Information**).

Note that one can further improve the speed of the search by stopping, whenever a relevant case was detected in phase 1, and immediately proceed with phase 2. If phase 2 does not confirm the result of phase 1, one continues with phase 1 on the next haybale. While this analogy is closer to the real situation a T cell experiences, there is no substantial difference in the argumentation: The two-phase approach still outperforms the one-phase approach by orders of magnitude.

FINDING COGNATE ANTIGENS ON APCs INSTEAD OF NEEDLES IN HAYSTACKS

Let us now put our calculations into context with what we know from experiments on the search of T cells for antigen presented by APCs. A human body carries approximately 50 million dendritic cells [2], of which around 10^3 to 10^5 accumulate per lymph node upon infection [13]. On the other hand, a given



T cell is specific for only one antigen-bearing APC out of $\sim 10^5$ – 10^6 [12]. Together, there is a need to identify approximately one relevant APC out of 10^4 , as we assumed in our calculations by setting $p = 10^{-4}$. Also our assumption of a random search appears justified, as T cell migratory behavior within lymph nodes is essentially random [14].

In the lymph node, the typical dwell time of a T cell on a dendritic cell is on the order of 1 min, if no cognate antigen is presented on a dendritic cell [15, 16]. In our terminology, this would correspond to phase 1, where TPR is maximized and fpr is neglected. This process can be achieved at rather high speeds. In practice, for a single T cell-APC encounter we should consider three contributions to this time: (i) the time needed for the T cell to move to the next dendritic cell, which is less than a second [17] and can hence be neglected here. (ii) The time for the TCR to encounter cognate pMHC, which can be of the order of seconds to minutes, depending on TCR and pMHC concentrations and diffusion coefficients. Note that the average encounter time is lowest for purely random TCR distributions. (iii) The time required for reaching a preliminary decision during phase 1. In the model formulation, we assumed this time to be given by $\Delta t / fpr_1$.

It is interesting to discuss which approach is used by T cells to make the initial search (phase 1 in our model) most efficient and fast. A number of models are currently discussed, which address how T cells come up with a first decision during phase 1: they include cooperative effects [18, 19], conformational changes of TCR subunits [20, 21], kinetic segregation of the phosphatase CD45 [22, 23], kinetic proofreading [24, 25], and pulling forces [26–28]. In addition, the Krümmel lab recently proposed that microvilli on the T cell palpate an APC in search of cognate pMHC [15]. The dynamic nature of these microvilli would facilitate 98% coverage of the APC surface within 1 min. Such a palpatory search strategy would be beneficial if pMHC was quite immobile. Of course, the spatial distribution of the

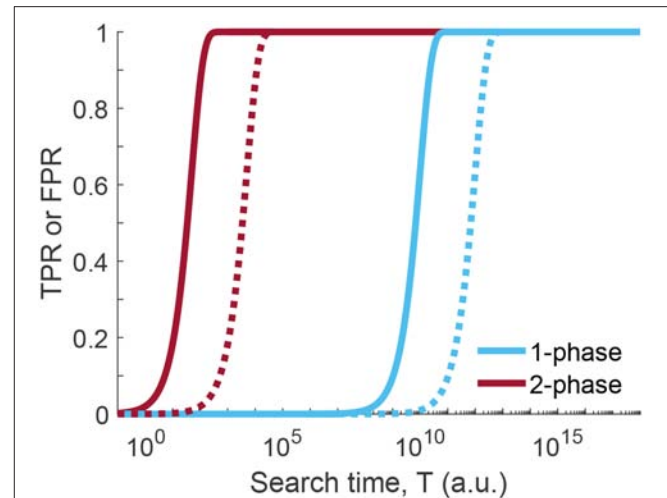


FIGURE 5 | Time comparison of one-phase and two-phase search strategy. True positive rate (TPR , solid line) or false positive rate (FPR , dotted line) for one-phase and two-phase search strategy as a function of the search time T (see Equations 16 and 21 of the one-phase and two-phase search, respectively; $\Delta t = 1$). Curves were calculated for $p = 10^{-4}$, local false positive rates were set to $fpr_1 = 10^{-2}$ and $fpr_2 = 10^{-4}$ for the two-phase search and $fpr = fpr_1 \cdot fpr_2 = 10^{-6}$ for the one-phase search, yielding the same figures of merit for TPR and FPR . For the calculation of the total search time T we set $\Delta t = 1$. Clearly, less time is needed in case of the two-phase search for the same level of discrimination between TPR and FPR .

TCR also plays an important role in making phase 1 as fast as possible. It was hypothesized for some time that the TCR could be organized in nanoscopic clusters in resting T cells [29–32]. This would lead to cooperativity between TCRs upon ligand binding and/or enhanced rebinding of cognate pMHC within a cluster. However, such a clustered organization would decrease the on-rate of TCR to cognate pMHC and hence the speed to find potentially harmful antigen. Clustering of the TCR is therefore unexpected considering that fast scanning of an APC is one of the main tasks of a T cell. In this respect, we recently provided evidence that the TCR is essentially distributed as monomers and at random on the plasma membrane of T cells interacting with model surfaces [33, 34]. Such a configuration would increase the probability and speed to find cognate pMHC on APCs and thus enhance phase 1. TCR enrichment at the tips of microvilli as the sites of first contact with APCs would further improve recognition at the onset of synapse formation [35].

One of the remaining questions is how phase 2, i.e., scrutinizing the first decision, is brought about. As soon as the T cell discovers cognate antigen on a dendritic cell, massive rearrangements of the surface molecules take place, leading to the formation of the immunological synapse [36], and the cell to cell contact becomes prolonged to hours [37–39]. According to our terminology, we have now entered phase 2: The T cell has encountered antigen of interest, which shall be further scrutinized. Microclusters containing TCR and signaling molecules are formed now [40, 41], which are believed to constitute the basic signaling unit in T cells [42]. They are densely packed with a diverse set of proteins, including, besides

the TCR, Lck, the costimulatory molecule CD28, and ZAP-70. According to our model, T cells may occasionally enter phase 2 erroneously after classifying APCs as positive, although they actually lack cognate antigenic peptide; in other words, we would expect non-zero false positive rates. Recent literature indicates that such scenarios indeed happen: TCR microcluster formation was also observed under non-activating conditions [43], and premature interruption of the T cell-APC contact might lead to reversal and/or alteration of T cell commitment to differentiation [7, 44, 45]. Notably, parts of these processes likely start already during phase 1 and acquire additional fidelity via multiple rounds of checks and rechecks during phase 2.

Taken together, considering both the principal search requirements and experimental data it appears likely that T cells do not do the search for the needle in the haystack in a single phase, but rather in several phases. One consequence of our model is the occurrence of false positive classifications at early stages of T cell signaling, which do not progress to full activation. In principle, this could be tested experimentally in single-T cell activation measurements, where T cells are allowed to screen mixtures of APCs with very few relevant and a huge excess of irrelevant APCs. The idea would be to compare the read-out of proximal T cell signaling such as phosphorylated ITAMs (e.g., via quantifying ZAP-70 recruitment to the plasma membrane), TCR or LAT microcluster formation or the presence of Ca^{2+} signals with later signaling events such as transcription factor translocation, cytokine production (e.g. IL-2) [39] and the expression of CD69 [46] or CD25 [47]. According to our model, we would expect T cells to react occasionally with the onset of proximal signaling events, even if an irrelevant APC

was contacted. This would allow to determine the local true and false positive rates of the first phase, tpr_1 and fpr_1 . The global true and false positive rates TPR and FPR could be determined from the late activation markers. If our proposed model was correct, improved ROC plots for the global rates compared to the local rates should be obtained; particularly, we would expect $FPR \ll fpr_1$. In conclusion, the proposed two-phase search approach offers a plausible framework to conceptualize the search for antigen by T cells. Experiments such as the one described above should provide the means to test the proposed hypothesis and possibly help to understand the molecular mechanisms underlying the T cell's search problem.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by the Austrian Science Fund (FWF) projects P 26337-B21, F 6809-N36 (GS), P27941-B28 (FB), and by the Vienna Science and Technology Fund (WWTF) project LS13-030 (GS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphy.2019.00011/full#supplementary-material>

REFERENCES

- Huppa JB, Davis MM. T-cell-antigen recognition and the immunological synapse. *Nat Rev Immunol.* (2003) 3:973–83. doi: 10.1038/nri1245
- Fearnley DB, Whyte LF, Carnoutsos SA, Cook AH, Hart DNJ. Monitoring human blood dendritic cell numbers in normal individuals and in stem cell transplantation. *Blood* (1999) 93:728–36.
- Jenkins MK, Chu HH, McLachlan JB, Moon JJ. On the composition of the preimmune repertoire of T cells specific for peptide-major histocompatibility complex ligands. *Ann Rev Immunol.* (2010) 28:275–94. doi: 10.1146/annurev-immunol-030409-101253
- Bajénoff M, Egen JG, Qi H, Huang AYC, Castellino F, Germain RN. Highways, byways and breadcrumbs: directing lymphocyte traffic in the lymph node. *Trend Immunol.* (2007) 28:346–52. doi: 10.1016/j.it.2007.06.005
- Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature* (2002) 419:845–9. doi: 10.1038/nature01076
- Harding CV, Unanue ER. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* (1990) 346:574. doi: 10.1038/346574a0
- Mayya V, Dustin ML. What scales the T cell response? *Trends Immunol.* (2016) 37:513–22. doi: 10.1016/j.it.2016.06.005
- Girard JP, Moussion C, Förster R. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat Rev Immunol.* (2012) 12:762. doi: 10.1038/nri3298
- Jenei A, Varga S, Bene L, Matyus L, Bodnar A, Bacso Z, et al. HLA class I and II antigens are partially co-clustered in the plasma membrane of human lymphoblastoid cells. *Proc Natl Acad Sci USA.* (1997) 94:7269–74. doi: 10.1073/pnas.94.14.7269
- Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol.* (2013) 13:227–42. doi: 10.1038/nri3405
- Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol.* (2010) 22:333–40. doi: 10.1016/j.coi.2010.02.013
- Krummel MF, Bartumeus F, Gérard A. T cell migration, search strategies and mechanisms. *Nat Rev Immunol.* (2016) 16:193. doi: 10.1038/nri.2015.16
- Usherwood EJ, Hogg TL, Woodland DL. Enumeration of antigen-presenting cells in mice infected with sendai virus. *J Immunol.* (1999) 162:3350–5.
- Miller MJ, Wei SH, Cahalan MD, Parker I. Autonomous T cell trafficking examined *in vivo* with intravital two-photon microscopy. *Proc Natl Acad Sci USA.* (2003) 100:2604–9. doi: 10.1073/pnas.2628040100
- Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) 356:eaa13118. doi: 10.1126/science.aal3118
- Gérard A, Patino-Lopez G, Beemiller P, Nambiar R, Ben-Aissa K, Liu Y, et al. Detection of rare antigen-presenting cells through t cell-intrinsic meandering motility, mediated by myo1g. *Cell* (2014) 158:492–505. doi: 10.1016/j.cell.2014.05.044
- Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair D, Waite J, et al. Functional anatomy of T cell activation and synapse formation. *Ann Rev Immunol.* (2010) 28:79–105. doi: 10.1146/annurev-immunol-030409-101308
- Su X, Ditlev JA, Hui E, Xing W, Banjade S, Okrut J, et al. Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* (2016) 352:595–9. doi: 10.1126/science.aad9964
- Huang WYC, Yan Q, Lin WC, Chung JK, Hansen SD, Christensen SM, et al. Phosphotyrosine-mediated LAT assembly on membranes drives kinetic bifurcation in recruitment dynamics of the Ras activator SOS. *Proc Natl Acad Sci USA.* (2016) 113:8218–23. doi: 10.1073/pnas.1602602113

20. Swamy M, Beck-Garcia K, Beck-Garcia E, Frederike Hartl A, Morath A, Yousefi OS, et al. A cholesterol-based allosteric model of T cell receptor phosphorylation. *Immunity* (2016) **44**:1091–101. doi: 10.1016/j.immuni.2016.04.011
21. Xu C, Gagnon E, Call ME, Schnell JR, Schwieters CD, Carman CV, et al. Regulation of T cell receptor activation by dynamic membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif. *Cell* (2008) **135**:702–13. doi: 10.1016/j.cell.2008.09.044
22. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol.* (2006) **7**:803–9. doi: 10.1038/ni1369
23. Chang VT, Fernandes RA, Ganzinger KA, Lee SF, Siebold C, McColl J, et al. Initiation of T cell signaling by CD45 segregation at 'close contacts.' *Nat Immunol.* (2016) **17**:574–82. doi: 10.1038/ni.3392
24. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci USA.* (1995) **92**:5042–6. doi: 10.1073/pnas.92.1.5042
25. Lever M, Maini PK, van der Merwe PA, Dushek O. Phenotypic models of T cell activation. *Nat Rev Immunol.* (2014) **14**:619–29. doi: 10.1038/nri3728
26. Feng Y, Brazil KN, Kobayashi E, Mallis RJ, Reinherz EL, Lang MJ. Mechanosensing drives acuity of $\alpha\beta$ T-cell recognition. *Proc Natl Acad Sci USA.* (2017) **114**:E820–13. doi: 10.1073/pnas.1703559114
27. Liu B, Chen W, Brian Evavold D, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* (2014) **157**:357–68. doi: 10.1016/j.cell.2014.02.053
28. Klotzsch E, Gerhard Schütz J. Improved ligand discrimination by force-induced unbinding of the T cell receptor from peptide-MHC. *Biophys J.* (2013) **104**:1670–5. doi: 10.1016/j.bpj.2013.03.023
29. Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol.* (2010) **11**:90–6. doi: 10.1038/ni.1832
30. Schamel WW, Arechaga I, Risueno RM, van Santen HM, Cabezas P, Risco C, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J Exp Med.* (2005) **202**:493–503. doi: 10.1084/jem.20042155
31. Kumar R, Ferez M, Swamy M, Arechaga I, Maria Rejas T, Jose Valpuesta M, et al. Increased sensitivity of antigen-experienced T cells through the enrichment of oligomeric T cell receptor complexes. *Immunity* (2011) **35**:375–87. doi: 10.1016/j.immuni.2011.08.010
32. Pagon SV, Tabarin T, Yamamoto Y, Ma Y, Bridgeman JS, Cohnen A, et al. Functional role of T-cell receptor nanoclusters in signal initiation and antigen discrimination. *Proc Natl Acad Sci USA.* (2016) **113**:E5454–63. doi: 10.1073/pnas.1607436113
33. Rossboth B, Arnold AM, Ta H, Platzter R, Kellner F, Huppa JB, et al. TCRs are randomly distributed on the plasma membrane of resting antigen-experienced T cells. *Nat Immunol.* (2018) **19**:821–7. doi: 10.1038/s41590-018-0162-7
34. Brameshuber M, Kellner F, Rossboth BK, Ta H, Alge K, Sevcik E, et al. Monomeric TCRs drive T cell antigen recognition. *Nat Immunol.* (2018) **19**:487–96. doi: 10.1038/s41590-018-0092-4
35. Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) **113**:E5916–24. doi: 10.1073/pnas.1605399113
36. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) **395**:82–6. doi: 10.1038/25764
37. Bousso P, Robey E. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol.* (2003) **4**:579. doi: 10.1038/ni928
38. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* (2004) **427**:154–9. doi: 10.1038/nature02238
39. Huang J, Brameshuber M, Zeng X, Xie J, Li QJ, Chien YH, et al. A single peptide-major histocompatibility complex ligand triggers digital cytokine secretion in CD4+ T cells. *Immunity* (2013) **39**:1–12. doi: 10.1016/j.immuni.2013.08.036
40. Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol.* (2005) **6**:1253–62. doi: 10.1038/ni1272
41. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med.* (2005) **202**:1031–6. doi: 10.1084/jem.20051182
42. Hashimoto-Tane A, Saito T. Dynamic regulation of TCR-microclusters and the microsynapse for T cell activation. *Front Immunol.* (2016) **7**:255. doi: 10.3389/fimmu.2016.00255
43. Crites TJ, Padhan K, Muller J, Krogsgaard M, Gudla PR, Lockett SJ, et al. TCR microclusters pre-exist and contain molecules necessary for TCR signal transduction. *J Immunol.* (2014) **193**:56–67. doi: 10.4049/jimmunol.1400315
44. Marangoni F, Murooka TT, Manzo T, Kim EY, Carrizosa E, Elpek NM, et al. The transcription factor nfat exhibits signal memory during serial T cell interactions with antigen presenting cells. *Immunity* (2013) **38**:237–49. doi: 10.1016/j.immuni.2012.09.012
45. Das J, Ho M, Zikherman J, Govern C, Yang M, Weiss A, et al. Digital signaling and hysteresis characterize Ras activation in lymphoid cells. *Cell* (2009) **136**:337–51. doi: 10.1016/j.cell.2008.11.051
46. Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clin Diagn Lab Immunol.* (1996) **3**:301–4.
47. Shatrova AN, Mityushova EV, Vassilieva IO, Aksenov ND, Zenin VV, Nikolsky NN, et al. Time-dependent regulation of IL-2R α -chain (CD25) expression by TCR signal strength and IL-2-induced STAT5 signaling in activated human blood T lymphocytes. *PLoS ONE* (2016) **11**:e0167215. doi: 10.1371/journal.pone.0167215

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mechanical Proofreading: A General Mechanism to Enhance the Fidelity of Information Transfer Between Cells

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
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Innovation, United Kingdom

Reviewed by:

Pavel Tolar,
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United Kingdom
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Columbia University, United States

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Specialty section:

This article was submitted to
Biophysics,
a section of the journal
Frontiers in Physics

Received: 08 November 2018

Accepted: 23 January 2019

Published: 19 February 2019

Citation:

Brockman JM and Salaita K (2019)
Mechanical Proofreading: A General
Mechanism to Enhance the Fidelity of
Information Transfer Between Cells.
Front. Phys. 7:14.
doi: 10.3389/fphy.2019.00014

The cells and receptors of the immune system are mechanically active. Single molecule force spectroscopy, traction force microscopy, and molecular tension probe measurements all point to the importance of piconewton (pN) molecular forces in immune function. For example, forces enhance the ability of a T-cell to discriminate between nearly identical antigens. The role of molecular forces at these critical immune recognition junctions is puzzling as mechanical forces generally facilitate bond dissociation, potentially increasing the difficulty for a receptor to recognize its cognate antigen. The advantage that molecular forces confer in the process of immune recognition is not clear. Why would cells expend energy to exert force on the critical, but tenuous bonds that mediate immune surveillance? Do molecular forces provide some advantage to the immune system? The premise of this review is that molecular forces provide a specificity advantage to immune cells. Inspired by the recent discovery that receptor forces regulate immune signaling in T-cell and B-cells, we dub this notion “mechanical proofreading,” akin to more classic kinetic proofreading models. During the process of mechanical proofreading, cells exert pN receptor forces on receptor-ligand interactions, deliberately increasing the energy cost of the immune recognition process in exchange for increased specificity of signaling. Here, we review the role of molecular forces in the immune system and suggest how these forces may facilitate mechanical proofreading to increase the specificity of the immune response.

Keywords: mechanical proofreading, molecular forces, mechanobiology, immune recognition, T-cell activation, mechanotransduction

INTRODUCTION

Immune cells must detect and respond to rare traces of malignancies or infection. Accordingly, the immune response must display extraordinary sensitivity and specificity. The requirements of specificity and sensitivity are often mutually exclusive: for example, if the signaling threshold required to initiate an immune response is set very high, the immune system is unlikely to make a mistake, but also more likely to miss an infection.

T-cell antigen recognition is a striking example of a vital immune recognition event that must balance both extreme sensitivity and specificity. The T-cell receptor (TCR) physically engages with peptide antigens bound to the major histocompatibility complex (pMHC). Virtually all nucleated cells present fragments of their proteome on the MHC for TCR inspection. When a TCR recognizes an antigen, TCR-pMHC binding triggers biochemical signaling leading to T-cell activation [1]. However, the origins of T-cell triggering in response to antigen binding are the

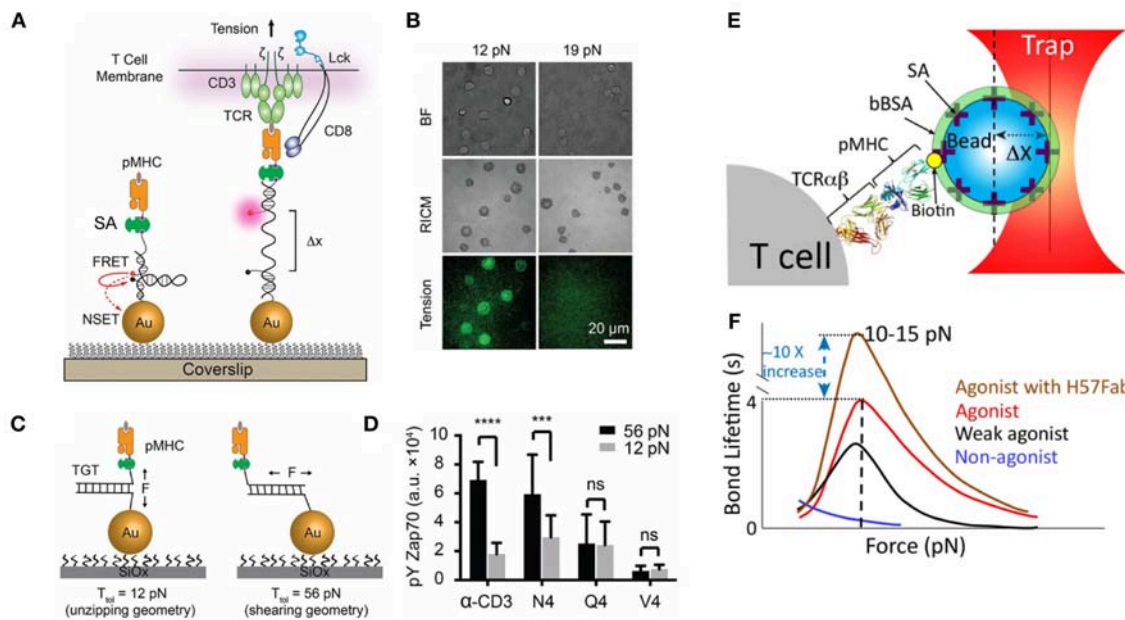
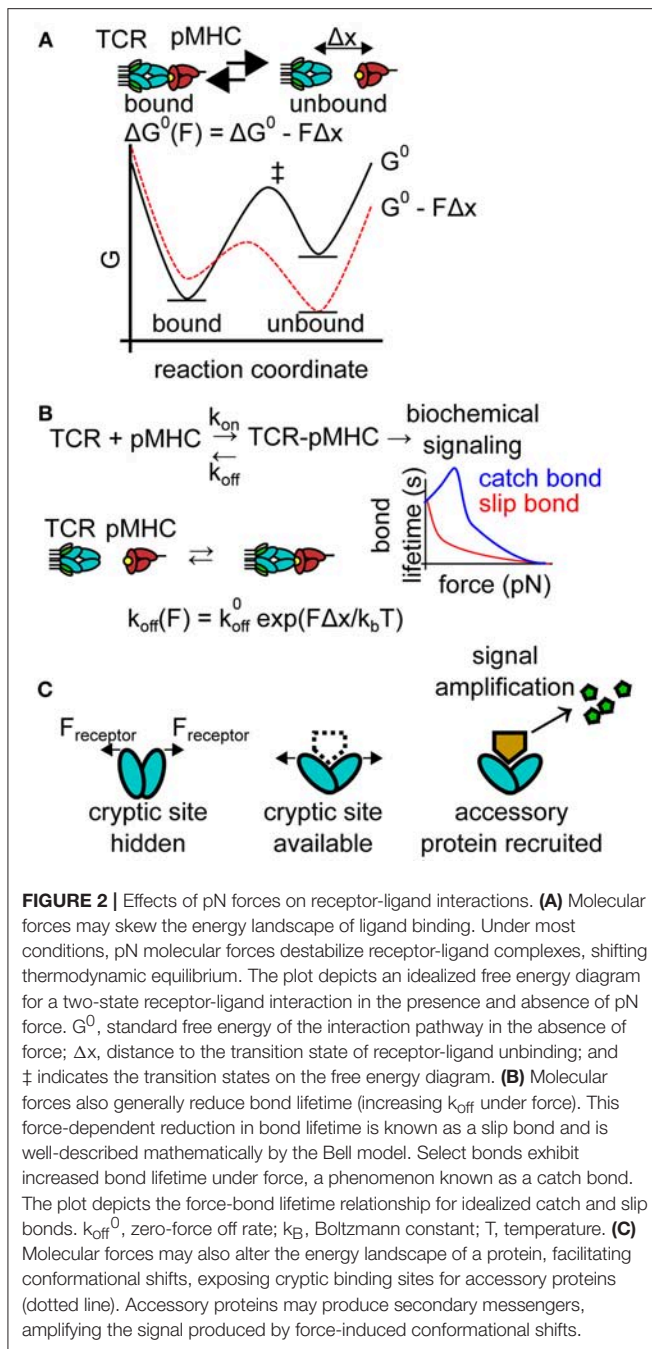


FIGURE 1 | Piconewton (pN) molecular forces are important to TCR antigen specificity. **(A)** Molecular tension probes are comprised of a fluorophore-quencher pair flanking a force-extensible domain. Molecular forces extend the probe, separating the fluorophore-quencher pair. DNA-based molecular tension probes, highlighted here, produce up to a 100x increase in fluorescence under the influence of cellular forces. **(B)** Using DNA molecular tension probes, OT-1 CD8⁺ T-cells were found to exert defined (12–19 pN) forces through the TCR. **(C)** Tension gauge tethers rupture irreversibly when receptor forces reach a threshold, capping the maximum tension a cell can exert through a receptor. **(D)** OT-1 CD8⁺ T-cells exhibit more potent activation when the pMHC-TCR complex is more mechanically stable. **(E)** Optical tweezers, formed by trapping a bead near the focus of a tightly focused laser, enable precise control over the movement of the bead and are useful for exerting pN forces on receptor-ligand interactions. **(F)** Optical tweezer manipulation of pMHC-presenting beads enabled the application of pN forces to TCR-pMHC complexes. TCR-pMHC bond lifetime increases for agonist pMHC (catch bond behavior) but decreases for non-agonist pMHC (slip bond behavior). The TCR FG loop is thought to elongate under cellular force and to be responsible for catch bond behavior. Stabilizing the FG loop with the H57Fab frag further enhances bond lifetime. **(A–D)** Are adapted from with permission from Liu et al. [20] while panels **(E,F)** are adapted from Feng et al. [21] licensed under Creative Commons (CC BY 4.0). NSET, near surface energy transfer; BF, brightfield; RCM, reflection interference contrast microscopy which displays cell-surface contact area; TGT, tension gauge tether; T_{tol} , the tension tolerance of a TGT, the force at which the TGT ruptures within 2 s; bBSA, biotinylated bovine serum albumin; SA, streptavidin. *** Indicates $p < 0.001$ and **** indicates $p < 0.0001$.

subject of intense debate [2]. For example, TCR-pMHC affinity (as measured by 3D techniques such as surface plasmon resonance) is low, with dissociation constants in the range of 1–100 μ M [3], one of the weakest measured affinities for receptor-ligand binding. Somehow, despite the poor TCR-pMHC affinity, single amino acid alterations in the peptide antigen can produce a 10,000-fold difference in T-cell activation [4] and some reports suggest that the TCR possesses single-molecule antigen sensitivity [5, 6]. TCR binding affinity (K_D), off rates (k_{off}), and on rates (k_{on}) sometimes, but not always correlate with T-cell activity [7–9]. Additionally, a comparison of crystal structures of TCR-pMHC bound and unliganded TCRs, reveal only minor conformational shifts upon TCR-pMHC binding [10]. The mechanism through which TCR-pMHC binding produces a high-fidelity signal to trigger T-cell activation remains a mystery [2, 11].

Further complicating the issue of T-cell antigen recognition is the observation that T-cells are mechanically active. The pMHC-TCR interaction forms only when a T-cell physically touches target cells; thus, it is likely that the TCR-pMHC complex experiences force. In support of this notion, soluble, monovalent pMHC can bind to the TCR but fails to activate

T-cells [12, 13], while pMHC attached to a planar lipid bilayer does activate T-cells [14]. Collectively, these results suggest that mechanical forces may regulate TCR triggering. A seminal optical tweezer study by Reinherz et al. demonstrated that T-cells trigger in response to forces exerted on the TCR-pMHC complex, positioning the TCR as a mechanosensor [15]. Furthermore, the TCR is not a passive recipient of external forces. Our group pioneered the development of molecular probes to map pN forces applied by cells [16–19]. In particular, DNA-based probes revealed that the TCR transmits defined piconewton forces to the pMHC, and that these forces facilitate TCR antigen discrimination (**Figures 1A–D**) [20, 22]. Traction force microscopy and micropillar measurements also demonstrate that T-cells transmit forces through the TCR-pMHC interaction and through the CD3 complex [23, 24]. Additionally, biomembrane force probe and optical tweezer measurements have revealed that TCR-pMHC bond lifetimes increase under force (catch bond behavior) for agonists, but decrease under force for non-agonist pMHC [21, 25–27] (**Figures 1E,F**). Additional studies have revealed the role molecular forces play in regulating the pore-forming capability of cytotoxic T-cells [28] and mediating the antigen recognition by CD8⁺ T-cells [20]. The many contexts



in which receptor forces regulate immune processes have been reviewed elsewhere [29, 30]. Many mechanisms to explain T-cell antigen recognition have been proposed [2], but vital questions remain unanswered. Why would a T-cell expend energy to exert force on an already tenuous, low affinity interaction? How do molecular forces aid in immune recognition?

Receptor forces have many potential effects on receptor-ligand binding which have been excellently reviewed elsewhere [31]. Many effects of molecular forces might hinder antigen recognition (**Figure 2**). For example, receptor forces alter equilibrium by shifting the energy landscape of bound and

unbound receptor-ligand pairs (**Figure 2A**). Cell-generated forces may therefore destabilize receptor-ligand complexes critical to immune recognition events, potentially dampening cellular activation. Additionally, as predicted by the Bell model, tensile forces on receptor ligand interactions typically increase the bond dissociation rate in a force-dependent manner [32], a phenomenon termed a slip-bond (**Figure 2B**). The increased dissociation rate reduces the number of bound receptors and is expected to dampen signaling. Individual slip bonds do not provide a specificity benefit under constant forces for pure affinity-based discrimination. For example, if a cognate antigen displays a $k_{\text{off,cognate}} = 1 \text{ s}^{-1}$ while a non-cognate antigen has $k_{\text{off,non-cognate}} = 10 \text{ s}^{-1}$ then the binding error rate (assuming identical on rates) is approximately the ratio of the off rates, $k_{\text{off,cognate}}/k_{\text{off,non-cognate}} = 1/10$. The Bell model (see equation, **Figure 2B**) dictates that force alters the off rate of both the non-cognate and cognate antigen by the same factor of $\exp(F\Delta x/k_B T)$, leaving the ratio $k_{\text{off,cognate}}$ and $k_{\text{off,non-cognate}}$ unchanged when both antigens experience the same cellular force and assuming Δx is similar for both antigens. This simple analysis is valid for constant forces, but dynamic forces [33] will have more complex effects that are difficult to treat in the context of this review. In rare cases, forces increase the lifetime of receptor-ligand interactions under force, a phenomenon known as a catch bond [34, 35]. Catch bonds could conceivably stabilize receptor-ligand recognition under force, facilitating signaling. Finally, pN forces alter protein energy landscapes, which may facilitate conformational shifts (**Figure 2C**). Force-induced conformational changes may lead to the recruitment of accessory proteins to mechanically strained receptors, amplifying the signal of mechanically strained receptor-ligand complexes. Given the many possible effects of molecular forces, it is crucial to define the precise role of pN molecular forces in the immune system.

The purpose of this review is to suggest that pN molecular forces provide a benefit to receptor-ligand interactions by increasing the specificity of signaling. In effect, cells expend energy in the form of pN receptor forces in exchange for increased specificity. With kinetic proofreading as a hallmark example, literature precedent exists for biological systems expending energy to purchase specificity. DNA replication and protein synthesis both leverage kinetic proofreading, increasing fidelity by using far-from-equilibrium intermediate states driven by triphosphate hydrolysis. These irreversible intermediate steps enable discrimination based on the off rates of “correct” vs. “incorrect” substrates [36]. In addition to kinetic proofreading, the serial engagement model has also been widely discussed as a mechanism to facilitate TCR antigen discrimination [37, 38]. These models have been reviewed elsewhere [2, 39], and are not the focus of this review. Instead, we focus on how molecular forces open many new possibilities to explain the specificity of immune recognition events.

Here, we review evidence for the importance of molecular forces in the immune system. Inspired by the work of Tolar [40], Zhu [25], Reinherz et al. [15], Lang et al. [21], and by our recent work [20], we interpret how forces might enable a cell to expend mechanical work to increase signaling accuracy. In an analogy to kinetic proofreading, we term the concept that cells

harness mechanical work to increase the specificity of signaling “mechanical proofreading.”

Mechanisms of Mechanical Proofreading

We highlighted three potential mechanisms for mechanical proofreading. First, bond lifetime may be altered by molecular forces to facilitate mechanical proofreading. Most biological bonds decrease in duration under force (slip bonds), but a subset of bonds exhibit an increased lifetime under force, a phenomenon known as catch bonds [34, 35]. The change in bond lifetime due to molecular forces may provide a potent means of mechanical proofreading (Figures 2B, 3A). Through the alteration of the bond lifetime, mechanical proofreading may enable immune receptors to select for the rare ligands that produce catch bond behavior under force, increasing the fidelity of signaling. Second, mechanical proofreading could occur via a “stress test.” Stress is defined as force per unit area. Analogously, cells may apply forces too great for a single receptor-ligand interaction to withstand. Only clusters of proteins sharing cellular forces among many bonds reduce the applied stress below a threshold level, enabling longer, more stable bonds (Figure 3B). The mechanical proofreading stress test selects for multivalent interactions and does not require catch bonds to function. A third mechanism of mechanical proofreading is a “strain test.” Strain is defined as the change in length of a material due to mechanical stress divided by the original length of the material. Analogously, if a receptor-ligand bond can withstand forces applied to it, the force may produce a conformational change (loosely analogous to strain) in one or both binding partners. This conformational change could result in adaptor protein recruitment, or exposure of cryptic binding sites, producing downstream signaling (Figure 3C). The mechanical proofreading strain test selects for, and subsequently amplifies, the signal from mechanically stable individual receptor-ligand bonds that can survive mechanical forces. While the strain test mechanism may contribute to the formation of catch bonds, the

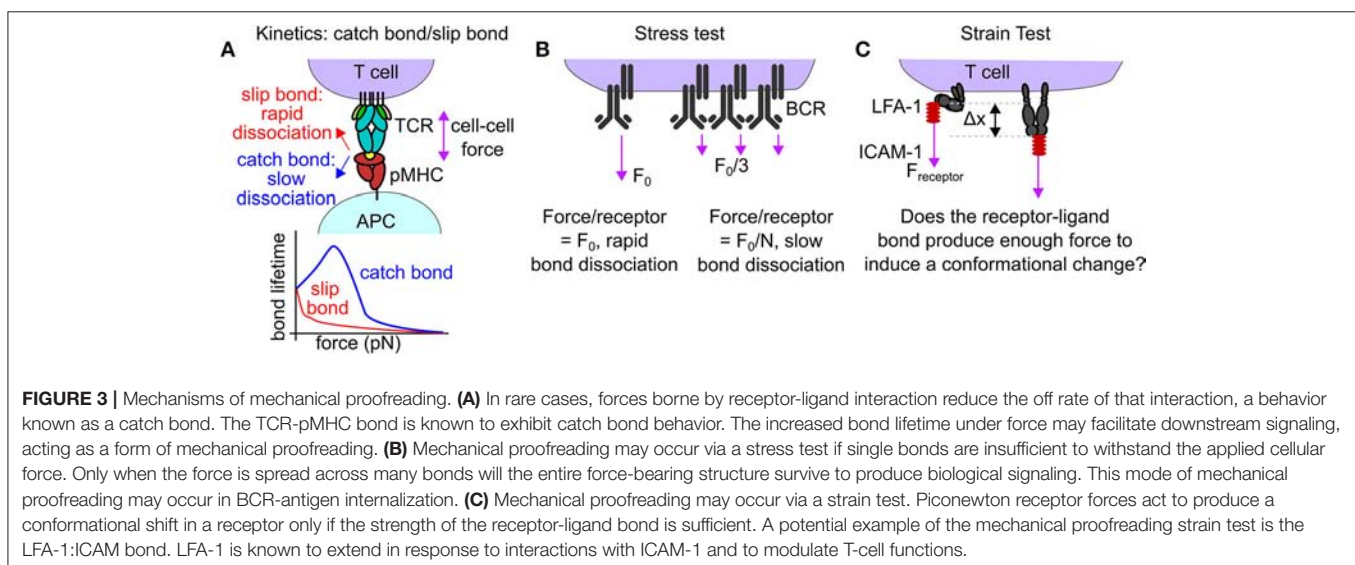
signaling outcome of the strain model is not the increased bond lifetime, but rather the activation of a mechanosensitive switch that enhances the fidelity of receptor-ligand binding.

MODE 1: ALTERATION OF BOND LIFETIME

Receptor-ligand interactions at cell-cell or cell-extracellular matrix junctions frequently experience force. These pN forces alter receptor-ligand bond kinetics (Figures 2B, 3A). Most frequently, receptor-ligand forces produce reduced bond lifetime (slip bonds) [32]. In special cases, bond lifetime increases under force, a phenomenon known as a catch bond [34]. AFM measurements provided the first direct evidence for a catch bond between P-selectin and P-selectin glycoprotein ligand-1 [35]. Since this initial observation, catch bonds have been demonstrated in selectins, integrins, the platelet glycoprotein GPIIb/IIIa, E-cadherin, and recently in the TCR-pMHC interaction [25, 35, 41–43].

Qualitatively, catch bonds provide a means to spend mechanical energy (from cytoskeletal and motor protein forces) in exchange for specificity. Most molecular bonds exhibit slip bond behavior; therefore, if a cell expends mechanical energy via applying force to a receptor-ligand bond and bond lifetime increases, the interaction is very likely to be “correct.” This enhancement of bond lifetime under force may amplify the downstream effects of cognate ligand binding events relative to the shorter bond lifetime (under force) of non-cognate ligands through the classic kinetic proof reading model. Catch bonds thus complement the kinetic proofreading model and offer a potent means for mechanical proofreading.

T-cells are mechanosensitive, transducing TCR forces into biochemical signals, such as calcium flux [15]. Additionally, the TCR transmits piconewton pN forces to the pMHC, and these forces are important to T-cell antigen recognition [20]. 2D affinity measurement of the TCR-pMHC bond demonstrated



that agonist pMHC actually has a shorter bond lifetime than weak agonist pMHC [8]. Conversely, biomembrane force probe measurements revealed that the TCR-pMHC interaction exhibits catch bond behavior, but only for agonist antigen [25] (**Figure 3A**). The catch bond behavior of the TCR-pMHC bond was also confirmed by optical tweezer measurements [26]. Sibner and colleagues recently interrogated TCRs that bind pMHC but are not activated. When agonist peptides to the formerly non-responsive TCRs were generated, catch bonds that correlated with CD 45 exclusion from the T-cell antigen presenting the cell contact area were identified in the new, activating interactions [44]. This finding further emphasizes the importance of catch bonds, and thus mechanical proofreading, in T-cell antigen discrimination. The accumulation of TCR-pMHC bond lifetimes may link TCR forces with the serial-engagement model of T-cell activation [38], allowing the catch bond behavior of many TCR-pMHC interactions to cumulatively trigger sustained calcium signaling during T-cell activation [25, 45]. The increase in bond lifetime for agonist pMHC could serve as an extraordinarily specific indicator that a T-cell has found agonist antigen, specificity purchased at the cost of mechanical work.

Catch bonds are not limited to the TCR-pMHC interaction. A catch bond has been demonstrated in the bond between lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) [41]. Combined with the observation that dendritic cells (DC) immobilize ICAM-1 on their surface in response to inflammatory signals [46], these results raise the possibility that the LFA-1-ICAM-1 catch bond may help T-cells recognize stimulated DCs.

Finally, catch bonds likely mediate mechanical proofreading in non-immune biological systems. For example, the platelet glycoprotein GPIIb α exhibits catch bond behavior when interacting with Von Willebrand factor [42]. Bacterial adhesion under flow is regulated by a catch bond between FimH and mannose [47]. Leukocyte adhesion against the flow of blood depends on a catch bond [35]. It is likely that catch bonds are a biologically general mechanism for mechanical proofreading, ensuring that biological processes occur only at specific, desirable interfaces via extremely specific mechanically stable receptor-ligand bonds.

MODE 2: STRESS TEST

Most receptor-ligand interactions are not catch bonds; however, slip bonds in groups of mechanically strained receptors can also facilitate mechanical proofreading. Receptor clustering is crucial to many biological signaling pathways and may also provide a mechanical advantage to force-bearing receptors. When force-bearing receptors cluster together, the stress (defined as force per unit area) applied at a cell-cell junction may be reduced because the force is distributed over many bonds. Receptor clustering therefore offers an opportunity for a mechanical proofreading “stress test,” which selects for cellular structures composed of many force-bearing proteins. Collectively, clustered proteins may withstand forces that would rupture any single bond. Note that for the stress test mechanism to function, the cellular force-generating machinery must be connected to groups of receptors (e.g., one actin stress fiber transmitting force to many integrins).

Consider a cluster of N receptors withstanding a total force F at a cell-cell junction. Force balance dictates that each receptor is bearing a force of approximately F/N . Clustering leads to an N -fold decrease in the force experienced per ligand-receptor pair. Based on the Bell model [32], the reduction in force on each receptor-ligand pair will lead to a significant enhancement in the bond lifetime, increasing the probability that the cellular structure will survive long enough to initiate biochemical signaling. To achieve the desired specificity and sensitivity, biological systems can alter both F and N . Increasing F produces shortened bond lifetime, decreasing sensitivity. Conversely, increasing N reduces the per-receptor force, facilitating signaling under force, potentially decreasing specificity. High F and N may achieve both high sensitivity and specificity by enabling only highly multivalent interactions to initiate biochemical signaling.

B cell receptor (BCR) antigen internalization is an example of a mechanical proofreading stress test. BCR signaling is intricately related to the B cell cytoskeleton [48]. Clusters of BCRs are thought to use mechanical force to internalize antigen. Natkanski et al. demonstrated that B cells internalize plasma membrane sheet bound antigen in a myosin IIA dependent manner, and that clathrin-coated structures seem to be associated with antigen internalization [40]. Interestingly, BCR microclusters were observed to resist contractile forces for 20–30 s; however, AFM measurements demonstrated that single BCR-antigen bonds do not endure long enough for the observed membrane invagination to occur [40]. The authors hypothesize that forces exerted by B cells shorten the BCR-antigen bond lifetime, ensuring that only multivalent, high-affinity antigens which collectively withstand the cellular force are internalized (**Figure 3B**) [40]. These findings are supported by the observation that B cells exert measurable traction forces through the B cell receptor, and that the magnitude of traction force scales with the number of clustered BCRs involved in force transmission [49]. Additionally, DNA-based molecular tension sensor measurements demonstrated that the BCR utilizes molecular forces to extract antigen from follicular dendritic cells, and that stiff follicular dendritic cells produce stronger BCR forces and more stringent antigen affinity discrimination [50]. Collectively, this evidence points toward a mechanical stress test facilitating BCR selection for high-affinity antigen.

A mechanical stress test may also be important to TCR signaling. The TCR is known to exert pN forces on the TCR-pMHC bond and also on TCR-pMHC clusters [20, 22], but the importance of nanoclusters in TCR force transmission is not known. Super-resolution imaging has suggested that TCRs form nanoclusters, and that clustered TCRs are more likely to be phosphorylated and to participate in downstream signaling [51]. TCRs are known to cluster at the surface of microvilli on the T-cell surface [52] and TCR-bearing microvilli are selectively stabilized at the T-cell antigen presenting cell interface [53]. Collectively, this evidence raises the possibility that TCR nanoclusters, and potentially microvilli, may facilitate a mechanical stress test.

Mechanical stress within other supramolecular complexes may also be important. For example, the focal adhesion has been proposed to behave like a molecular clutch, with mechanical unfolding of adaptor proteins serving to recruit more integrins

to the adhesion site to share the applied load [54]. Thus, focal adhesions may sense and respond to substrate stiffness via a mechanical proofreading stress test, where specific combinations of force and adhesion site density produce focal adhesion growth and maturation.

MODE 3: STRAIN TEST

Another mode of mechanical proofreading is a “strain test.” Strain is defined as the change in length of an object relative to its original length. Analogously, many receptors undergo conformational changes due to ligand or allosteric interactions. Some conformational shifts are facilitated by pN scale forces [55, 56]. Conformational shifts may expose cryptic binding sites, leading to protein recruitment or to phosphorylation of a previously inaccessible site. Force-reconfigurable proteins therefore offer a third potential mode of mechanical proofreading. In the mechanical proofreading strain test, a receptor-ligand bond must withstand a threshold force to produce a conformational shift before eliciting biochemical signaling. Note that for the strain test to function, a large energy threshold must prevent the receptor from spontaneously experiencing the conformational shift in the absence of a large input of mechanical work by a cell.

Integrins are well-studied examples of the mechanical proofreading strain test. They exhibit profound conformational shifts, existing in a low-affinity, bent conformation at rest, but able to adopt a high affinity extended state [57]. Integrins may be an ultrasensitive molecular switch, able to extend in response to pN forces [55].

In the context of lymphocytes, the integrin $\alpha_L\beta_2$, also known as LFA-1, binds to ICAM, and is important to immune cell-cell adhesion and to T-cell function [58]. LFA-1 undergoes outside-in activation in response to surface bound, but not soluble ICAM [59], and molecular forces increase the rate of LFA-1 extension and slow the rate of LFA-1 bending (**Figure 3C**) [56]. It is believed that actin forces transmitted through LFA-1 to immobilized ICAM-1 induce extension of LFA-1 and that actin is critical to the formation and maintenance of the immunological synapse [60]. Additionally, recent interference photoactivation localization microscopy (iPALM) measurements, which resolve the location of fluorophores along the microscope optical axis with ~ 10 nm resolution, have measured the extension of LFA-1 at a T-cell surface interface [61]. Outside-in activation of LFA-1 has functional consequences; for example, severing ICAM's cytoskeletal anchorage prevented natural killer cells from forming junctions with target cells and disrupted granule polarization [62]. Additionally, when DCs are induced to mature via treatment with lipopolysaccharide, they utilize their actin cytoskeleton to restrict ICAM-1 surface mobility. T-cells sense the change in ICAM-1 mobility, producing more extended LFA-1 on the T-cells surface [46]. Collectively, these findings demonstrate that LFA-1 exhibits force-sensitive outside-in activation, positioning LFA-1 as a potential mechanical proofreading strain sensor.

The T-cell receptor (TCR) may also utilize a mechanical strain test during T-cell antigen recognition. Optical tweezer

measurements demonstrated a force dependent extension in the FG loop of the TCR [26]. The FG loop extends 8–15 nm under the influence of pN forces. Deleting the FG loop of the TCR removed the force-dependent extension behavior and reduced the ability of T-cells to respond to antigen as assessed by IL-2 production [26]. The FG loop extension of the TCR therefore presents another example of a potential mechanical proofreading strain test. Because TCR forces are between 12 and 19 pN [20], and because the FG loop extension is known [26], the height of the mechanical proofreading energy barrier that TCR forces must surmount has been calculated as $\sim 37 k_B T$ (assuming 10 nm displacement and 15 pN forces), or almost twice the energy of one ATP hydrolysis. This energy of discrimination could explain the remarkable specificity of T-cell antigen recognition [21]. The mechanical proofreading strain test is therefore capable of extreme specificity.

The strain test mode of mechanical proofreading is not limited to the immune system. For example, platelets action must be tightly regulated to prevent erroneous clot formation. The platelet integrin $\alpha_{IIb}\beta_3$ is anisotropically mechanosensitive, requiring lateral forces to undergo outside in activation to enable platelet spreading and activation on a surface [63, 64] which may explain why platelets ignore soluble fibrinogen in the blood, only binding to fibrinogen attached to other activated platelets. Additionally, the platelet glycoprotein complex GPIb-IX-V, has a mechanosensitive domain that extends several nanometers under force [65], making it another likely candidate for a strain test for mechanical proofreading.

COMBINATIONS OF MECHANICAL PROOFREADING MODES

The modes of mechanical proofreading may also work together to produce increased specificity. For example, the GP1b-IX-V: von-Willebrand factor bond is a catch bond [42]. The prolonged bond lifetime under force may facilitate the opening of the mechanosensitive domain of GP1b-IX-V [66]. Similarly, the TCR exhibits catch bond behavior, an extension of its FG loop, and receptor clustering [25, 26, 51]. Various mechanisms invoked to explain the extreme sensitivity and specificity of the TCR-pMHC bond involve each of the proposed modes of mechanical proofreading [2, 11]. The molecular clutch model of the focal adhesion [54, 67] argues that talin unfolding under force (a strain test) regulates integrin recruitment to the focal adhesion (regulating stress), thus these two models may work cooperatively in focal adhesions. Elucidating which mechanisms are critical to regulating cellular decision-making will require careful experimentation to isolate the effects of each of the modes of mechanical proofreading.

CONCLUSION

Here, we have focused on applications of mechanical proofreading within the immune system, but mechanical proofreading is likely a biologically general mechanism. Because mechanical forces generally disrupt receptor-ligand interactions,

as described by the Bell model [32], careful examination of force-exerting systems is required to determine what functional benefit is derived by expending cellular energy on force generation. Mechanical forces may be vital to biological differentiation between soluble and a surface bound ligand. Soluble ligands are not capable of resisting mechanical force. Indeed, it is conceivable that from a mechanistic point of view, mechanical forces are the principle difference between cell-cell interfaces and receptors interacting with soluble ligands.

Mechanical proofreading likely has great physiological relevance. Zhu et al. found that during thymic selection, negative selection ligands produced cooperative trimolecular catch bonds (TCR-pMHC-CD8) while positive selection ligands formed slip bonds [68]. This result strongly suggests that mechanical proofreading, by means of catch bonds, assists in the elimination of strongly self-reactive thymocytes during negative selection. Additionally, Huse et al. recently found that the exertion of force by T-cells is spatially and temporally linked to the release of cytotoxin release and target cell killing [28]. Thus, mechanical proofreading is also implicated in the killing of target cells by cytotoxic T-cells. Finally, T-cells have been demonstrated to respond to increased stiffness of a substrate presenting activating antibodies to CD3 and CD28 with increased IL-2 secretion [69]. Importantly, human primary immune cells, including macrophages and dendritic cells, modulate their stiffness in response to inflammatory signals [70]. Collectively, the ability of antigen-presenting cells to modulate their stiffness in response to inflammatory signals combined with the stiffness sensitivity of T-cells suggests that mechanical proofreading may play a role in enabling T-cells to respond appropriately to complex environmental signals.

Additionally, mechanical proofreading has the potential to resolve longstanding debates in immunology. For example, mechanical proofreading may provide insight into how adaptive immune responses are initiated. TCR binding affinity for pMHC does not always correlate with T-cell activity [7–9]. The ability of a pMHC to bind to the TCR does not guarantee that the TCR will activate [71]. Garcia et al. recently interrogated TCRs that bind pMHC but are not activated. When agonist peptides were produced to the formerly non-responsive TCRs, these new agonist pMHC-TCR bonds exhibited catch bond behavior [44]. This finding further emphasizes the importance of mechanical forces, catch bonds, and mechanical proofreading in T-cell antigen discrimination.

Finally, mechanical proofreading may have utility for cell-based immunotherapies. Kam et al. have recently demonstrated the use of polydimethylsiloxane microbeads coated in activating antibodies to CD3 and CD28 to enhance the *ex vivo* activation

and proliferation of both CD4⁺ and CD8⁺ T-cells for immunotherapy [72]. Intriguingly, T-cell expansion was found to be enhanced in soft beads, implicating a role for mechanical forces in *ex vivo* T-cell expansion. It is also possible that some variety of mechanical proofreading may operate in chimeric antigen receptor (CAR) T-cell recognition of antigen. Chen and colleagues recently rewired CAR-T-cells to recognize soluble antigen [73]. However, CAR signaling in these cells required ligand-mediated CAR dimerization, which the authors believe implicates the role of mechanotransduction in CAR signaling. Interestingly, the chimeric antigen-receptor is comprised of an antibody-derived fragment for ligand binding. Antibodies exhibit a slip bond character under force [74]. Mechanotransduction during CAR signaling likely proceeds via a different mechanism than the catch bond that influences TCR-pMHC interactions.

Experiments designed to test the models outlined here may be critical in determining the mechanical origins of cellular decision-making. For example, deletion of mechanically sensitive domains in the TCR reduced IL-2 production [26]. Likewise, the mechanosensitivity of platelets has been partially explained via a mechanosensitive domain discovered in the platelet glycoprotein GpIb α [65]. Deletion of a portion of the mechanosensitive domain produces constitutive GpIb α signaling [75]. These experiments may be facilitated by the recent development of powerful molecular tools capable of measuring both the magnitude and orientation of molecular forces [18, 20, 22, 64, 76]. We anticipate that understanding the precise role of molecular forces in receptor-ligand interactions will provide fundamental insight into the exquisite specificity of cellular decision-making.

AUTHOR CONTRIBUTIONS

JB and KS conceived the idea of mechanical proofreading and wrote the manuscript.

FUNDING

This work was supported through NIGMS R01 GM124472 (KS), NSF 1350829 (KS), NSF GRFP 1444932 (JB), and NCI-F99CA234959 (JB). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Institute of Health or of the National Science Foundation.

ACKNOWLEDGMENTS

We thank Victor Pui-Yan Ma for comments on the manuscript and for helpful discussion.

REFERENCES

- van der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. *Nat Rev Immunol.* (2011) 11:47–55. doi: 10.1038/nri2887
- Chakraborty AK, Weiss A. Insights into the initiation of TCR signaling. *Nat Immunol.* (2014) 15:798–807. doi: 10.1038/ni.2940
- Matsui K, Boniface JJ, Steffner P, Reay PA, Davis MM. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci USA.* (1994) 91:12862–6.
- Sasada T, Ghendler Y, Wang JH, Reinherz EL. Thymic selection is influenced by subtle structural variation involving the p4 residue of an MHC class I-bound peptide. *Eur J Immunol.* (2000) 30:1281–9. doi: 10.1002/(SICI)1521-4141(200005)30:5<1281::AID-IMMU1281>3.0.CO;2-D

5. Sykulev Y, Joo M, Vturina I, Tsomides TJ, Eisen HN. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity* (1996) 4:565–71.
6. Irvine DJ, Purbhoo MA, Krosgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature* (2002) 419:845–9. doi: 10.1038/nature01076
7. Stone JD, Chervin AS, Kranz DM. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* (2009) 126:165–76. doi: 10.1111/j.1365-2567.2008.03015.x
8. Huang J, Zarnitsyna VI, Liu B, Edwards LJ, Jiang N, Evavold BD, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature* (2010) 464:932–6. doi: 10.1038/Nature08944
9. Lyons DS, Lieberman SA, Hampl J, Boniface JJ, Chien Y, Berg LJ, et al. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* (1996) 5:53–61.
10. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol.* (2006) 24:419–66. doi: 10.1146/annurev.immunol.23.021704.115658
11. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annu Rev Immunol* (2009) 27:591–619. doi: 10.1146/annurev.immunol.021908.132706
12. Boniface JJ, Rabinowitz JD, Wülfing C, Hampl J, Reich Z, Altman JD, et al. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands [corrected]. *Immunity* (1998) 9:459–66
13. Cochran JR, Cameron TO, Stern LJ. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* (2000) 12:241–50. doi: 10.1016/S1074-7613(00)80177-6
14. Watts TH, McConnell HM. High-affinity fluorescent peptide binding to I-Ad in lipid membranes. *Proc Natl Acad Sci USA.* (1986) 83:9660–4.
15. Kim ST, Takeuchi K, Sun ZY, Touma M, Castro CE, Fahmy A, et al. The alphabeta T cell receptor is an anisotropic mechanosensor. *J Biol Chem.* (2009) 284:31028–37. doi: 10.1074/jbc.M109.052712
16. Stabley DR, Jurchenko C, Marshall SS, Salaita KS. Visualizing mechanical tension across membrane receptors with a fluorescent sensor. *Nat Methods* (2012) 9:64–7. doi: 10.1038/Nmeth.1747
17. Jurchenko C, Salaita KS. Lighting up the force: investigating mechanisms of mechanotransduction using fluorescent tension probes. *Mol Cell Biol.* (2015) 35:2570–82. doi: 10.1128/MCB.00195-15
18. Liu Y, Galior K, Ma VP, Salaita K. Molecular tension probes for imaging forces at the cell surface. *Acc Chem Res.* (2017) 50:2915–24. doi: 10.1021/acs.accounts.7b00305
19. Zhang Y, Ge C, Zhu C, Salaita K. DNA-based digital tension probes reveal integrin forces during early cell adhesion. *Nat Commun.* (2014) 5:5167. doi: 10.1038/ncomms6167
20. Liu Y, Blanchfield L, Ma VP, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci USA.* (2016) 113:5610–5. doi: 10.1073/pnas.1600163113
21. Feng Y, Reinherz EL, Lang MJ. $\alpha\beta$ T Cell Receptor Mechanosensing Forces out Serial Engagement. *Trends Immunol.* (2018) 39:596–609. doi: 10.1016/j.it.2018.05.005
22. Ma VP, Liu Y, Blanchfield L, Su H, Evavold BD, Salaita K. Ratiometric tension probes for mapping receptor forces and clustering at intermembrane junctions. *Nano Lett.* (2016) 16:4552–9. doi: 10.1021/acs.nanolett.6b01817
23. Bashour KT, Gondarenko A, Chen H, Shen K, Liu X, Huse M, et al. CD28 and CD3 have complementary roles in T-cell traction forces. *Proc Natl Acad Sci USA.* (2014) 111:2241–6. doi: 10.1073/pnas.1315606111
24. Hui KL, Balagopalan L, Samelson LE, Upadhyaya A. Cytoskeletal forces during signaling activation in Jurkat T-cells. *Mol Biol Cell* (2015) 26:685–95. doi: 10.1091/mbc.E14-03-0830
25. Liu B, Chen W, Evavold BD, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* (2014) 157:357–68. doi: 10.1016/j.cell.2014.02.053
26. Das DK, Feng Y, Mallis RJ, Li X, Keskin DB, Hussey RE, et al. Force-dependent transition in the T-cell receptor beta-subunit allosterically regulates peptide discrimination and pMHC bond lifetime. *Proc Natl Acad Sci USA.* (2015) 112:1517–22. doi: 10.1073/pnas.1424829112
27. Feng Y, Brazin KN, Kobayashi E, Mallis RJ, Reinherz EL, Lang MJ. Mechanosensing drives acuity of alphabeta T-cell recognition. *Proc Natl Acad Sci USA.* (2017) 114:E8204–13. doi: 10.1073/pnas.1703559114
28. Basu R, Whitlock BM, Husson J, Le Floch A, Jin W, Oyler-Yaniv A, et al. Cytotoxic T cells use mechanical force to potentiate target cell killing. *Cell* (2016) 165:100–110. doi: 10.1016/j.cell.2016.01.021
29. Huse M. Mechanical forces in the immune system. *Nat Rev Immunol.* (2017) 17:679–90. doi: 10.1038/nri.2017.74
30. Chen W, Zhu C. Mechanical regulation of T-cell functions. *Immunol Rev.* (2013) 256:160–76. doi: 10.1111/imr.12122
31. Chen Y, Ju L, Rushdi M, Ge C, Zhu C. Receptor-mediated cell mechanosensing. *Mol Biol Cell* (2017) 28:3134–55. doi: 10.1091/mbc.E17-04-0228
32. Bell GI. Models for the specific adhesion of cells to cells. *Science* (1978) 200:618–27.
33. Plotnikov SV, Pasapera AM, Sabass B, Waterman CM. Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. *Cell* (2012) 151:1513–27. doi: 10.1016/j.cell.2012.11.034
34. Dembo M, Torney DC, Saxman K, Hammer D. The reaction-limited kinetics of membrane-to-surface adhesion and detachment. *Proc R Soc Lond B Biol Sci.* (1988) 234:55–83.
35. Marshall BT, Long M, Piper JW, Yago T, McEver RP, Zhu C. Direct observation of catch bonds involving cell-adhesion molecules. *Nature* (2003) 423:190–3. doi: 10.1038/nature01605
36. Hopfield JJ. Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specificity. *Proc Natl Acad Sci USA.* (1974) 71:4135–9.
37. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci USA.* (1995) 92:5042–46.
38. Valitutti S, Müller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* (1995) 375:148–51. doi: 10.1038/375148a0
39. Valitutti S. The serial engagement model 17 years after: from TCR triggering to immunotherapy. *Front Immunol.* (2012) 3:272. doi: 10.3389/fimmu.2012.00272
40. Natkanski E, Lee WY, Mistry B, Casal A, Molloy JE, Tolar P. B cells use mechanical energy to discriminate antigen affinities. *Science* (2013) 340:1587–90. doi: 10.1126/science.1237572
41. Chen W, Lou J, Zhu C. Forcing switch from short- to intermediate- and long-lived states of the alphaA domain generates LFA-1/ICAM-1 catch bonds. *J Biol Chem.* (2010) 285:35967–78. doi: 10.1074/jbc.M110.155770
42. Yago T, Lou J, Wu T, Yang J, Miner JJ, Coburn L, et al. Platelet glycoprotein Ib/alpha forms catch bonds with human WT vWF but not with type 2B von Willebrand disease vWF. *J Clin Invest.* (2008) 118:3195–207. doi: 10.1172/JCI35754
43. Rakshit S, Zhang Y, Manibog K, Shafraz O, Sivasankar S. Ideal, catch, and slip bonds in cadherin adhesion. *Proc Natl Acad Sci USA.* (2012) 109:18815–20. doi: 10.1073/pnas.1208349109
44. Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell* (2018) 174:672.e27–87. doi: 10.1016/j.cell.2018.06.017
45. Pryschep S, Zarnitsyna VI, Hong J, Evavold BD, Zhu C. Accumulation of serial forces on TCR and CD8 frequently applied by agonist antigenic peptides embedded in MHC molecules triggers calcium in T cells. *J Immunol.* (2014) 193:68–76. doi: 10.4049/jimmunol.1303436
46. Comrie WA, Li S, Boyle S, Burkhardt JK. The dendritic cell cytoskeleton promotes T cell adhesion and activation by constraining ICAM-1 mobility. *J Cell Biol.* (2015) 208:457–73. doi: 10.1083/jcb.201406120
47. Yakovenko O, Sharma S, Forero M, Tchesnokova V, Aprikian P, Kidd B, et al. FimH forms catch bonds that are enhanced by mechanical force due to allosteric regulation. *J Biol Chem.* (2008) 283:11596–605. doi: 10.1074/jbc.M707815200
48. Tolar P. Cytoskeletal control of B cell responses to antigens. *Nat Rev Immunol.* (2017) 17:621–34. doi: 10.1038/nri.2017.67
49. Wang J, Lin F, Wan Z, Sun X, Lu Y, Huang J, et al. Profiling the origin, dynamics, and function of traction force in B cell activation. *Sci Signal.* (2018) 11:eaai9192. doi: 10.1126/scisignal.aai9192

50. Spillane KM, Tolar P. B cell antigen extraction is regulated by physical properties of antigen-presenting cells. *J Cell Biol.* (2017) **216**:217–30. doi: 10.1083/jcb.201607064
51. Paeon SV, Tabarin T, Yamamoto Y, Ma Y, Nicovich PR, Bridgeman JS, et al. Functional role of T-cell receptor nanoclusters in signal initiation and antigen discrimination. *Proc Natl Acad Sci USA.* (2016) **113**:E5454–63. doi: 10.1073/pnas.1607436113
52. Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) **113**:E5916–24. doi: 10.1073/pnas.1605399113
53. Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science* (2017) **356**:eaal3118. doi: 10.1126/science.aal3118
54. Oria R, Wiegand T, Escribano J, Elosegui-Artola A, Uriarte JJ, Moreno-Pulido C, et al. Force loading explains spatial sensing of ligands by cells. *Nature* (2017) **552**:219–24. doi: 10.1038/nature24662
55. Li J, Springer TA. Integrin extension enables ultrasensitive regulation by cytoskeletal force. *Proc Natl Acad Sci USA.* (2017) **114**:4685–90. doi: 10.1073/pnas.1704171114
56. Chen W, Lou J, Evans EA, Zhu C. Observing force-regulated conformational changes and ligand dissociation from a single integrin on cells. *J Cell Biol.* (2012) **199**:497–512. doi: 10.1083/jcb.201201091
57. Li J, Su Y, Xia W, Qin Y, Humphries MJ, Vestweber D, et al. Conformational equilibria and intrinsic affinities define integrin activation. *EMBO J.* (2017) **36**:629–45. doi: 10.15252/embj.201695803
58. Hogg N, Patzak I, Willenbrock F. The insider's guide to leukocyte integrin signalling and function. *Nat Rev Immunol.* (2011) **11**:416–26. doi: 10.1038/nri2986
59. Feigelson SW, Pasvolksky R, Cemurski S, Shulman Z, Grabovsky V, Ilani T, et al. Occupancy of lymphocyte LFA-1 by surface-immobilized ICAM-1 is critical for TCR- but not for chemokine-triggered LFA-1 conversion to an open headpiece high-affinity state. *J Immunol.* (2010) **185**:7394–404. doi: 10.4049/jimmunol.1002246
60. Comrie WA, Babich A, Burkhardt JK. F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *J Cell Biol.* (2015) **208**:475–91. doi: 10.1083/jcb.201406121
61. Moore TI, Aaron J, Chew TL, Springer TA. Measuring integrin conformational change on the cell surface with super-resolution microscopy. *Cell Rep.* (2018) **22**:1903–12. doi: 10.1016/j.celrep.2018.01.062
62. Gross CC, Brzustowski JA, Liu D, Long EO. Tethering of intercellular adhesion molecule on target cells is required for LFA-1-dependent NK cell adhesion and granule polarization. *J Immunol.* (2010) **185**:2918–26. doi: 10.4049/jimmunol.1000761
63. Zhang Y, Qiu Y, Blanchard AT, Chang Y, Brockman JM, Ma VP, et al. Platelet integrins exhibit anisotropic mechanosensing and harness pN forces to mediate platelet aggregation. *Proc Natl Acad Sci USA.* (2017) **115**:325–30. doi: 10.1073/pnas.1710828115
64. Brockman JM, Blanchard AT, Pui-Yan V, Derricotte WD, Zhang Y, Fay ME, et al. Mapping the 3D orientation of piconewton integrin traction forces. *Nat Methods* (2018) **15**:115–8. doi: 10.1038/nmeth.4536
65. Zhang W, Deng W, Zhou L, Xu Y, Yang W, Liang X, et al. Identification of a juxtamembrane mechanosensitive domain in the platelet mechanosensor glycoprotein Ib-IX complex. *Blood* (2015) **125**:562–9. doi: 10.1182/blood-2014-07-589507
66. Cheng Z, Huang M, Chen G, Yang G, Zhou X, Chen C, et al. Cell-based assays in combination with ultra-high performance liquid chromatography-quadrupole time of flight tandem mass spectrometry for screening bioactive capilliposide C metabolites generated by rat intestinal microflora. *J Pharm Biomed Anal.* (2016) **119**:130–8. doi: 10.1016/j.jpba.2015.11.029
67. Elosegui-Artola A, Oria R, Chen Y, Kosmalska A, Pérez-González C, Castro N, et al. Mechanical regulation of a molecular clutch defines force transmission and transduction in response to matrix rigidity. *Nat Cell Biol.* (2016) **18**:540–8. doi: 10.1038/ncb3336
68. Hong J, Ge C, Jothikumar P, Yuan Z, Liu B, Bai K, et al. A TCR mechanotransduction signaling loop induces negative selection in the thymus. *Nat Immunol.* (2018) **19**:1379–90. doi: 10.1038/s41590-018-0259-z
69. Judokusumo E, Tabdanov E, Kumari S, Dustin ML, Kam LC. Mechanosensing in T lymphocyte activation. *Biophys J.* (2012) **102**:L5–7. doi: 10.1016/j.bpj.2011.12.011
70. Bufi N, Saitakis M, Dogniaux S, Buschinger O, Bohineust A, Richert A, et al. Human Primary Immune Cells Exhibit Distinct Mechanical Properties that Are Modified by Inflammation. *Biophys J.* (2015) **108**:2181–90. doi: 10.1016/j.bpj.2015.03.047
71. Adams JJ, Narayanan S, Liu B, Birnbaum ME, Kruse AC, Bowerman NA, et al. T cell receptor signaling is limited by docking geometry to peptide-major histocompatibility complex. *Immunity* (2011) **35**:681–93. doi: 10.1016/j.immuni.2011.09.013
72. Lambert LH, Goebrecht GK, De Leo SE, O'Connor RS, Nunez-Cruz S, Li TD, et al. Improving T cell expansion with a soft touch. *Nano Lett.* (2017) **17**:821–6. doi: 10.1021/acs.nanolett.6b04071
73. Chang ZL, Lorenzini MH, Chen X, Tran U, Bangayan NJ, Chen YY. Rewiring T-cell responses to soluble factors with chimeric antigen receptors. *Nat Chem Biol.* (2018) **14**:317–24. doi: 10.1038/nchembio.2565
74. Schwesinger F, Ros R, Strunz T, Anselmetti D, Güntherodt HJ, Honegger A, et al. Unbinding forces of single antibody-antigen complexes correlate with their thermal dissociation rates. *Proc Natl Acad Sci USA.* (2000) **97**:9972–7. doi: 10.1073/pnas.97.18.9972
75. Deng W, Xu Y, Chen W, Paul DS, Syed AK, Dragovich MA, et al. Platelet clearance via shear-induced unfolding of a membrane mechanoreceptor. *Nat Commun.* (2016) **7**:12863. doi: 10.1038/ncomms12863
76. Polacheck WJ, Chen CS. Measuring cell-generated forces: a guide to the available tools. *Nat Methods* (2016) **13**:415–23. doi: 10.1038/nmeth.3834

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Light-Controlled Affinity Purification of Protein Complexes Exemplified by the Resting ZAP70 Interactome

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 21 December 2018

Accepted: 28 January 2019

Published: 26 February 2019

Citation:

Hörner M, Eble J, Yousefi OS,
Schwarz J, Warscheid B, Weber W
and Schamel WWA (2019)
Light-Controlled Affinity Purification of
Protein Complexes Exemplified by the
Resting ZAP70 Interactome.
Front. Immunol. 10:226.
doi: 10.3389/fimmu.2019.00226

Multiprotein complexes control the behavior of cells, such as of lymphocytes of the immune system. Methods to affinity purify protein complexes and to determine their interactome by mass spectrometry are thus widely used. One drawback of these methods is the presence of false positives. In fact, the elution of the protein of interest (POI) is achieved by changing the biochemical properties of the buffer, so that unspecifically bound proteins (the false positives) may also elute. Here, we developed an optogenetics-derived and light-controlled affinity purification method based on the light-regulated reversible protein interaction between phytochrome B (PhyB) and its phytochrome interacting factor 6 (PIF6). We engineered a truncated variant of PIF6 comprising only 22 amino acids that can be genetically fused to the POI as an affinity tag. Thereby the POI can be purified with PhyB-functionalized resin material using 660 nm light for binding and washing, and 740 nm light for elution. Far-red light-induced elution is effective but very mild as the same buffer is used for the wash and elution. As proof-of-concept, we expressed PIF-tagged variants of the tyrosine kinase ZAP70 in ZAP70-deficient Jurkat T cells, purified ZAP70 and associating proteins using our light-controlled system, and identified the interaction partners by quantitative mass spectrometry. Using unstimulated T cells, we were able to detect the known interaction partners, and could filter out all other proteins.

Keywords: optogenetics, affinity purification, phytochrome, ZAP70, protein-protein interaction, mass spectrometry

INTRODUCTION

Most, if not all, biochemical processes in cells, such as signal transduction, rely on protein-protein-interactions (1, 2). In the cells of the immune system for example, signalosomes change in their composition upon stimulation of cell surface receptors (3, 4). A well-studied example is the binding of the T cell antigen receptor (TCR) complex to the tyrosine kinase ZAP70 in resting T cells (5–8). Upon ligand binding to the TCR, ZAP70 gets activated by phosphorylation and detaches from the TCR (9) leading to the generation of new intracellular interactions and the remodeling of signalosomes (10). As those protein-protein-interactions control cell behavior, their investigation is of key interest in immunological and biological research.

Interaction partners of proteins are usually identified by purification of a protein of interest (POI) and analyzing the co-purified proteins by mass spectrometry (11, 12). Besides the use of POI-specific antibodies, one common approach is the purification of the POI via an affinity tag. There, the POI is expressed as a fusion protein with an affinity tag that binds specifically to a resin material. Then the cells expressing the fusion protein are lysed with a detergent, insoluble material is removed by centrifugation, and the lysate is added to the resin. Subsequently, the POI together with its interacting proteins binds to the resin. After washing, the POI together with its interaction partners can be eluted from the resin, e.g., by a change in the pH (FLAG tag, HA tag), ion concentration (Poly-Arg tag), temperature (protein G tag), or by addition of metal chelators (CBP tag) or molecules that compete with the binding of the affinity tag to the resin (Strep tag, SBP tag, Poly-His tag) (13, 14). Alternatively, the affinity tag is linked to the POI using a sequence that can be cleaved by a protease, such as Tobacco Etch Virus (TEV) protease (15). Hence, elution is achieved by adding the protease, cleaving the POI off the resin. Importantly, all the different approaches have in common that the changed biochemical environment in the elution step can also result in the release of unspecifically bound proteins from the resin material resulting in false positive hits in the following analysis. One approach to reduce the number of contaminants in the eluate is the sequential usage of two different affinity tags, named tandem affinity purification (TAP) (16). However, due to the thereby prolonged time needed for purification, more transient and weak interactors may be lost during this procedure.

In this study, we address this problem by developing an optogenetics-based purification approach allowing elution of tagged proteins by simply changing the wavelength of light with which the resin is illuminated. Thus, all biochemical parameters, such as the ones mentioned above, stay constant, minimizing the elution of proteins that were bound to the resin and not to the POI. We made use of the light-dependent protein-protein interaction between phytochrome B (PhyB) and its phytochrome interacting factor 6 (PIF6) both from *Arabidopsis thaliana* (17). Upon illumination with 660 nm red light PhyB switches to its Pfr conformational state (PhyB far-red absorbing state) in which it interacts with PIF6 with a nanomolar affinity (18). With 740 nm far-red light PhyB undergoes a conformational transition to the Pr state (PhyB red absorbing state) preventing binding to PIF6. This light-dependent protein-protein interaction was applied for several optogenetic applications (19), such as the control of protein or organelle localization (18, 20), signaling (21), nuclear transport of proteins (22), or gene expression (23).

Here, we make the red light-dependent interaction between PhyB and PIF6 applicable to the affinity purification of protein complexes. To this end, we identified a truncated variant of PIF6 comprising only 22 amino acids that reversibly interacts with PhyB and therefore can be used as an affinity tag for the POI. After characterization of the key parameters of our light-controlled affinity purification approach, we applied our method for the identification of interaction partners of ZAP70 in resting T cells by quantitative mass spectrometry.

RESULTS AND DISCUSSION

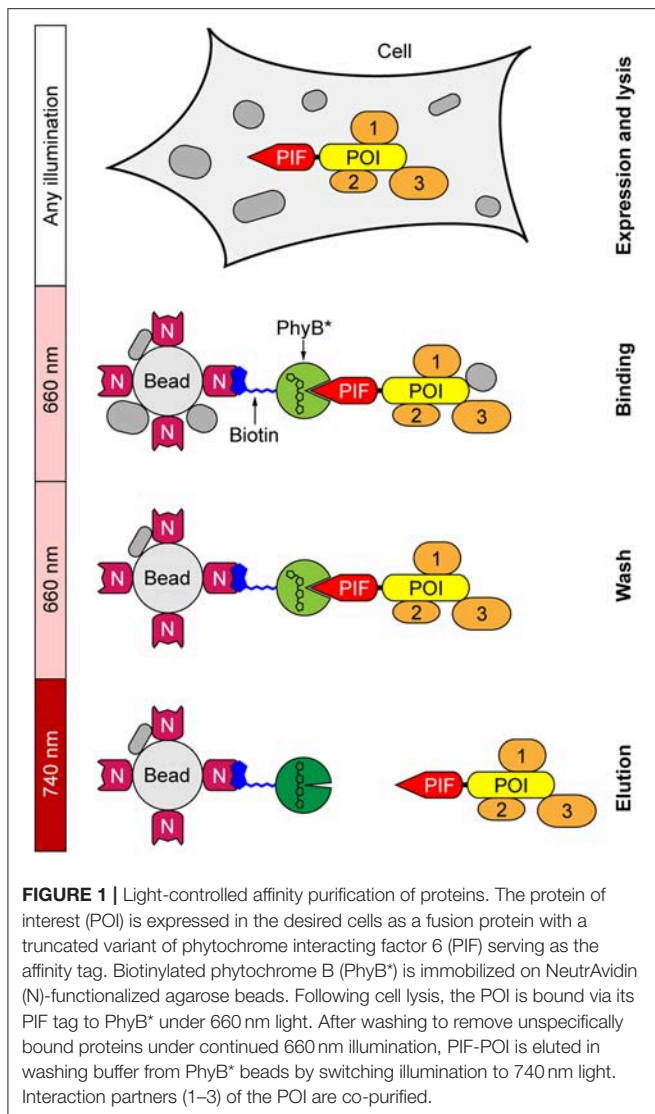
In our new light-controlled affinity purification approach, a fusion protein between the POI and a truncated version of PIF6 is expressed in the desired cells (**Figure 1**). After cell lysis, the lysate is loaded under 660 nm light illumination onto agarose beads that have been functionalized with PhyB. Illumination with 660 nm light switches PhyB into the Pfr state, thus immobilizing the PIF-POI fusion protein and potential interaction partners to the PhyB beads. Afterwards the beads are washed under continued 660 nm illumination for removal of unspecific bound proteins. Finally, PIF-POI and its binding partners are eluted in the same buffer as used in the washing steps by simply changing illumination to 740 nm light, as light of this wavelength switches PhyB into the Pr state that terminates the interaction with PIF.

PhyB*-Functionalized Resin Material

In order to functionalize agarose beads with PhyB, we used a biotinylated and hexahistidine-tagged variant of PhyB comprising amino acids 1-651 (24). This protein was produced together with the enzymes for the biosynthesis of the required chromophore phycocyanobilin (PCB) in *E. coli*. We purified this PhyB variant (designated as PhyB*) via immobilized metal ion affinity chromatography and quantified its biotinylation by binding of the protein to NeutrAvidin-functionalized agarose beads. Using an excess of beads, we observed that ~98% of PhyB* was biotinylated (**Supplementary Figure 1**). To determine the PhyB* binding capacity of the NeutrAvidin agarose beads, we incubated a fixed amount of beads with increasing amounts of PhyB* and monitored a decrease in the ratio of beads-bound PhyB* (**Supplementary Figure 1**). Saturation of the beads was reached with approximately 0.2 nmol PhyB* per 1 μ l beads. In the following, 1 μ l NeutrAvidin beads were always incubated with ~0.27 nmol PhyB* resulting in a coupling of ~0.24 nmol PhyB* per 1 μ l of beads (~89% coupling efficiency, designated as PhyB* beads).

Truncation of the PIF6 Tag

It was shown that the N-terminal 100 amino acids of *A. thaliana* PIF6 [PIF6(1-100)] are sufficient for the reversible and light-dependent interaction with PhyB (17, 18). It is desirable to minimize the size of an affinity tag in order to disturb the fused POI as minimally as possible and to reduce undesired protein binding to the tag. Therefore, we aimed to further truncate PIF6(1-100) while maintaining its light-dependent interaction properties with PhyB. To this end, we performed a sequence alignment of different PIF variants from several plants to identify a region within the N-terminal 100 amino acids that is well-conserved and hence should constitute the core domain of PIF6 responsible for the light-dependent interaction with PhyB (**Supplementary Figure 2**). We found a conserved region between amino acids 15 and 36. Thus, we tested four different truncated PIF6 variants containing different amino acids within the 15–36 region and compared them to PIF6(1-100) (**Figure 2A**). To this end, we expressed the green fluorescent protein (GFP) fused to the selected PIF6 variants together with the untagged red fluorescent protein mCherry



in human embryonic kidney (HEK)-293T cells. Subsequently, PIF-tagged GFP was purified from the cell lysates with the PhyB*-functionalized beads and spin columns. During the purification process, we monitored the fluorescence of mCherry as a background control and of GFP as our POI (**Figure 2B**). We observed that all tested PIF tags allowed a light-controlled purification of the fused GFP. Remarkably, PIF6(15-33) and PIF6(15-36) showed a similar efficiency compared to PIF6(1-100). The amount of mCherry in the eluates was at least 4,000-fold reduced compared to the lysates, demonstrating the specificity of our purification protocol. Next, we compared our light-controlled purification approach with established affinity chromatography methods. These were based on the streptavidin binding peptide [SBP tag (25)] or on the TEV mediated cleavage of the POI from protein A [TEV_{CS}-ProteinA tag (26)]. We observed a similar recovery (70–80%, **Supplementary Figure 3**) and purity of the eluates (**Figure 2C**).

Characterization of the Purification Process

We characterized the purification process of GFP-PIF6(1-100) and GFP-PIF6(15-36) with PhyB* beads in more detail. We determined that within a binding period of 2 h at 660 nm illumination both PIF-tagged proteins were binding equally well to the PhyB* beads with a binding efficacy of ~85% (using an excess of PhyB* beads, **Figure 3A**). When the PIF-tagged proteins were in excess compared to PhyB*, ~0.21 nmol of GFP-PIF6(1-100) or GFP-PIF6(15-36) were binding per 1 μ l of the PhyB* beads. We further observed that GFP-PIF6(1-100) was binding faster to the PhyB* beads under 660 nm light than GFP-PIF6(15-36) with 50% bound protein within 0.25 min compared to 15 min, respectively (**Figure 3B**). In contrast, GFP-PIF6(1-100) eluted slower from the PhyB* beads under 740 nm illumination than GFP-PIF6(15-36) with 80% released protein within 180 s compared to 16 s, respectively (**Figure 3C**).

Generation of ZAP70-PIF6 Tag Cell Lines

We tested our light-controlled purification approach for the identification of interaction partners of the kinase ZAP70 by quantitative mass spectrometry. ZAP70 is a central kinase involved in TCR signaling, which interacts with the components of the TCR such as CD3 δ , CD3 ϵ , CD3 γ , or CD247 (5, 6, 10). We transduced the ZAP70-deficient Jurkat cell line P116 (27) with constructs expressing PIF6(1-100)- or PIF6(15-36)-tagged human ZAP70 and the fluorescent protein ZsGreen1 (**Figure 4A**). Based on the fluorescence of the marker ZsGreen1, we sorted transduced cells with low ZsGreen1 expression levels using flow cytometry to avoid overexpression of our ZAP70 fusion proteins. As expected, most of the sorted cells stably expressed ZsGreen1 as shown by flow cytometry (**Figure 4B**). Expression and correct size of the fusion proteins were confirmed by SDS-PAGE and Western Blotting using an anti-ZAP70 antibody (**Figure 4C**). Both tagged ZAP70 proteins were expressed to lower levels than endogenous ZAP70 in the Jurkat cell line. ZAP70 is directly downstream of the TCR, transducing TCR signals into the cell, such as Ca²⁺ influx into the cytosol. Indeed, when stimulating the TCR with anti-CD3 antibodies we detected a Ca²⁺ response in Jurkat cells, but not in P116 cells (**Figure 4D**). Moreover, the restored anti-CD3 induced Ca²⁺ influx in our stable cell lines showed that the fusion of the PIF tags to ZAP70 did not impair the functionality of ZAP70 (**Figure 4D**). The reduced Ca²⁺-flux in comparison to Jurkat cells can likely be attributed to the reduced expression level of our PIF-ZAP70 fusion constructs in comparison to the endogenous ZAP70 level of Jurkat cells (**Figures 4C,D**).

Identification of ZAP70 Interaction Partners by Mass Spectrometry

To test our new purification approach, we used both P116-derived stable cell lines in resting state, i.e., unstimulated. We purified PIF-tagged ZAP70 from these cell lines using our PhyB* beads and analyzed the cell lysates, the flow-throughs of the last washing step and the eluates by Western blotting against ZAP70 and GAPDH (**Figure 5A**). As a negative control, we performed

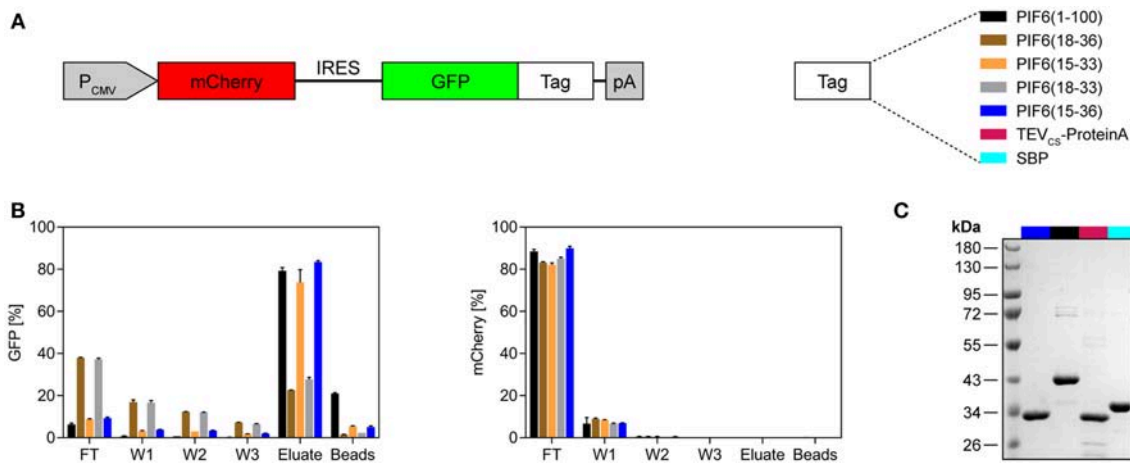


FIGURE 2 | Evaluation of different truncated variants of PIF6 for the application as an affinity tag. **(A)** Design of the expression construct for testing of different affinity tags. GFP was fused to the depicted PIF6 variants or to the affinity tags TEV_{CS}-ProteinA or SBP. The fusion proteins were expressed together with the untagged red fluorescent protein mCherry by a constitutive CMV promoter in human embryonic kidney cells (HEK-293T). IRES, internal ribosomal entry site; pA, poly(A) tail. **(B)** Monitoring of the light-controlled affinity purification process. Fluorescence of GFP and of the background control mCherry was measured using a plate reader and is shown as percentage normalized to the fluorescence of the initial cell lysate. For each replicate, 4×10^6 cells were lysed in 500 μ l lysis buffer and proteins were purified with 50 μ l of PhyB* beads. The proteins were eluted at 740 nm light for 30 min. FT, flow-through; W1-W3, wash 1-3. Data are means $\pm$ s.d. ($n = 2$). **(C)** Comparison of the purity of light-controlled affinity purification [PIF6(15-36) and PIF6(1-100)] with established purification methods (TEV_{CS}-ProteinA and SBP). For each purification, 125×10^6 cells were lysed with 500 μ l of lysis buffer and purified with 100 μ l of beads and four washing steps. Coomassie-stained SDS-PAGE gel of the eluates is shown.

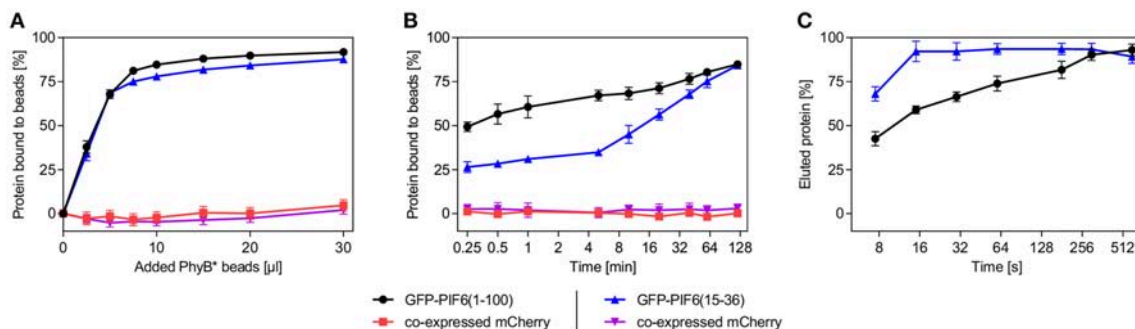


FIGURE 3 | Characterization of the light-controlled affinity purification system. Lysates of HEK-293T cells containing mCherry and GFP-PIF6(1-100) or GFP-PIF6(15-36) as described in **Figure 2A** were used. **(A)** Binding efficiency and capacity of PhyB* beads. Different amounts of PhyB* beads were each incubated with 250 μ l of cell lysate containing 1.5 nmol GFP-PIF6(1-100) or GFP-PIF6(15-36) for 2 h at 660 nm illumination. Afterwards, the ratio of bound protein was determined by measuring fluorescence of unbound GFP and mCherry. **(B)** Binding kinetics. Equal amounts of PhyB* beads (20 μ l) were incubated with 125 μ l of cell lysate containing 0.75 nmol of GFP-PIF6(1-100) or GFP-PIF6(15-36) for the indicated time periods under 660 nm light. Subsequently, the ratio of bound protein was determined by measuring fluorescence of unbound GFP and mCherry. **(C)** Elution kinetics. Equal amounts of PhyB* beads (40 μ l) were incubated with 500 μ l of cell lysate containing 3.0 nmol of GFP-PIF6(1-100) or GFP-PIF6(15-36) for 1 h at 660 nm illumination. After washing, the beads were incubated in 500 μ l of wash buffer for the indicated times at 740 nm light for elution. The percentage of eluted protein was calculated by determining GFP fluorescence in the supernatant in comparison to the GFP amount on the beads before elution. All data are means $\pm$ s.d. ($n = 3$).

the same purification with Jurkat cells expressing non-tagged ZAP70. As expected, the eluates of the stable cell lines contained PIF-tagged ZAP70 of the correct molecular weight whereas the eluates of the Jurkat cells did not contain detectable amounts of ZAP70. The eluates of all purified cells showed a massive reduction in the GAPDH level compared to the cell lysates. This experiment demonstrates that we were able to specifically purify our POI, in this case ZAP70, using the new light-controlled affinity purification approach.

To validate whether we are able to identify interaction partners of ZAP70 by our light-controlled purification approach and whether we can eliminate false positives, we used the unstimulated P116 cells expressing PIF6(1-100)- or PIF6(15-36)-tagged human ZAP70. The usage of unstimulated cells has the following advantage. ZAP70 was shown to interact with the TCR in resting cells (7) thus this interaction serves as our positive control. Importantly, this ZAP70 is not phosphorylated on tyrosines (7). Since these tyrosines have to be phosphorylated

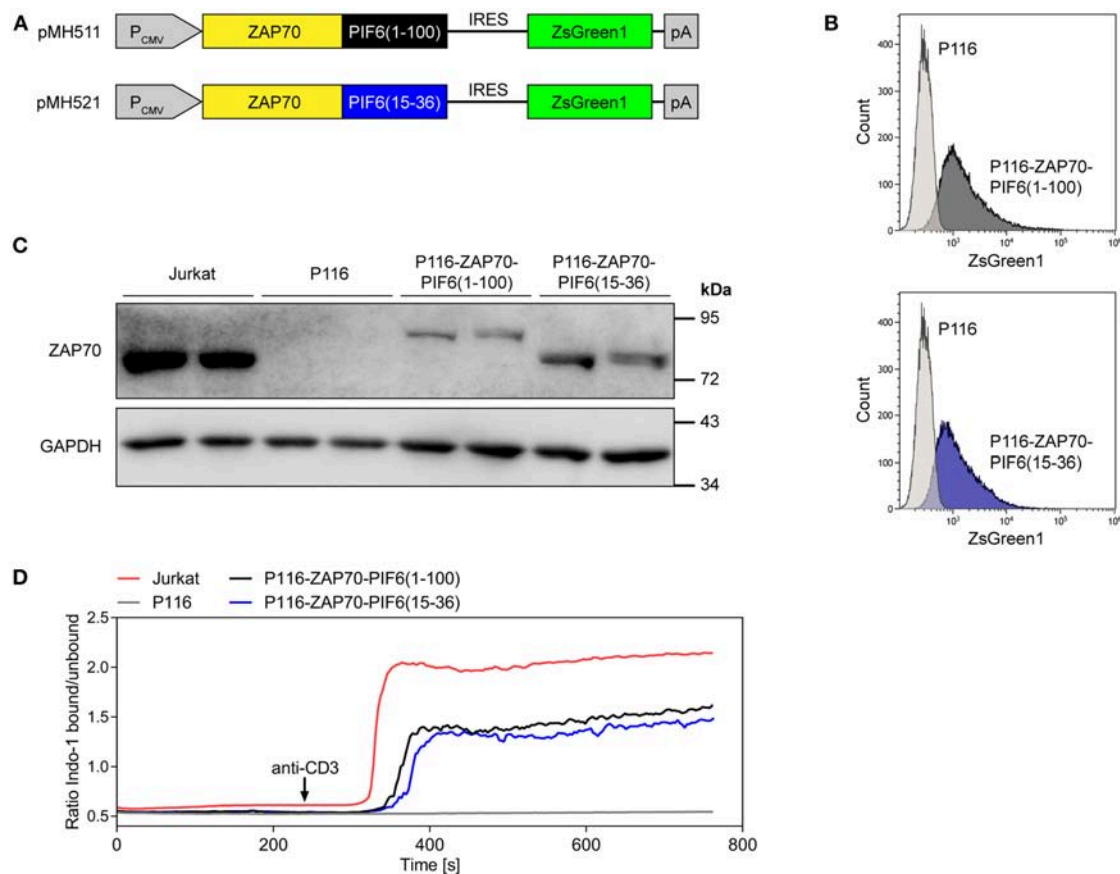


FIGURE 4 | Generation and characterization of P116-based cell lines stably expressing ZAP70-PIF6(1-100) or ZAP70-PIF6(15-36). **(A)** Lentiviral vectors encoding the depicted constructs for expression of ZAP70-PIF6(1-100) or ZAP70-PIF6(15-36) and the fluorescent protein ZsGreen1 under control of the constitutive CMV promoter (P_{CMV}). Transduced P116 cells were sorted based on ZsGreen1 fluorescence. **(B)** After culturing the two sorted cell lines generated in **(A)**, ZsGreen1 fluorescence of the cell lines in comparison to parental P116 cells was analyzed by flow cytometry. **(C)** Expression of ZAP70 in different cell lines. The cell lines indicated were analyzed for ZAP70 and GAPDH expression by SDS-PAGE and Western blotting. **(D)** Restoring anti-CD3 induced Ca²⁺-flux in P116 cells stably expressing ZAP70-PIF6(1-100) or ZAP70-PIF6(15-36). The Indo-1 fluorescence of Indo-1-stained cells was measured by flow cytometry, as a readout for the intracellular Ca²⁺ concentration. After 240 s of the recording the cells were stimulated with 1 $\mu\text{g ml}^{-1}$ of an anti-CD3 antibody (indicated by the arrow).

to serve as interactions sites for further signaling proteins (8), we do not expect any other interaction partners besides the TCR. Thus, we can assume that other proteins detected by mass spectrometry are false positives.

Next, we analyzed the eluates after 740 nm illumination and the flow-throughs of the last washing step of the above described unstimulated samples by mass spectrometry (3 biological replicates each, **Supplementary Table 1**). We first looked for proteins that were significantly enriched in the eluates derived from each ZAP70-PIF6 cell line compared to the eluates derived from the Jurkat cell line. We observed that for both ZAP70-PIF6 cell lines the 20 top hits ($p < 0.05$, sorted on protein enrichment) contained besides ZAP70 as the POI, the proteins CD247-i3 (i3, isoform 3), CD3D, CD3E, CD3G, and TRBC1/2 (**Figure 5B**). The different CD3 proteins, CD247 (also called CD3 ζ) as well as TRBC1/2 (also called TCR β chains) are all subunits of the TCR (28) which is known to associate with ZAP70 (5, 6, 29). As stated above, ZAP70 associates with the TCR in unstimulated primary T

cells (7) and most likely also in unstimulated Jurkat cells although to lower levels (30, 31). Since ZAP70 that is associated with the resting TCR is not phosphorylated (7) and thus might not have other binding partners, we conclude that the other proteins might be false positives. The two PIF6 tags showed a similar performance as shown by the high overlap of the 20 best hits (14 out of 20) and as visualized by the clustering of the proteins along the diagonal when plotting the enriched proteins against each other (**Figure 5C**, **Supplementary Table 2**). Together, this indicates that both PIF6(1-100) and PIF6(15-36) are suitable affinity tags that allow purification, and identification of a POI together with its interaction partners.

As our light-controlled purification approach is characterized by the very mild elution condition (740 nm light), we next asked whether we could use the flow-throughs of the last washing step performed under 660 nm illumination as background control instead of the eluates from the Jurkat cell line (expressing untagged ZAP70). Indeed, using this procedure we could identify

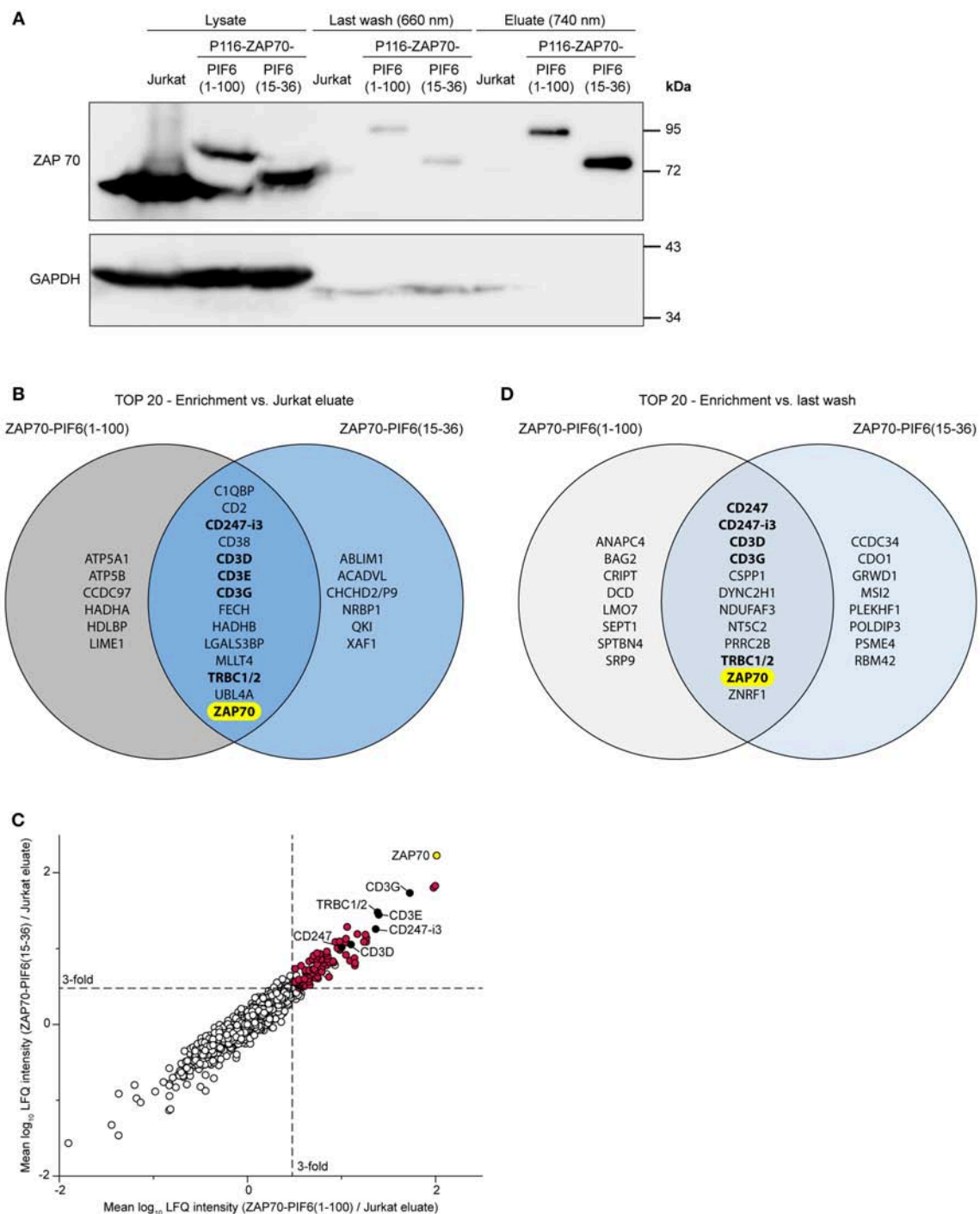


FIGURE 5 | Analysis of light-controlled ZAP70-PIF purification by mass spectrometry. Jurkat cells and P116-derived cell lines stably expressing ZAP70-PIF6(1-100) or ZAP70-PIF6(15-36) were lysed and PIF-tagged proteins were light-controlled purified using PhyB⁺ beads ($n = 3$ biological replicates). All eluates and the flow-throughs of the last washing were analyzed by mass spectrometry. **(A)** Analysis of the cell lysates, the flow-throughs of the last washing step and the eluates by SDS-PAGE and Western blot against ZAP70 and GAPDH. **(B)** Comparison of the 20 best hits ($p < 0.05$, sorted on protein enrichment) enriched in the eluates of each ZAP70-PIF6 cell line vs. the eluates from the Jurkat cell line. Known interaction partners of ZAP70 are written in bold. **(C)** Enrichment of proteins identified in the ZAP70-PIF6(1-100) eluate vs. the Jurkat eluate (x-axis) plotted against the enrichment of proteins in the ZAP70-PIF6(15-36) eluate vs. the Jurkat eluate (y-axis). The enrichment for each protein is shown as the mean log₁₀ ratio of the label-free quantification (LFQ) intensities (3 biological replicates) of the compared samples. Known interaction partners of ZAP70 identified in **(B)** are highlighted in black and are labeled. All significant hits ($p < 0.05$) in the upper right quadrant are highlighted in red. The POI ZAP70 is highlighted in yellow. Dashed lines represent a ratio cut-off corresponding to a 3-fold enrichment compared to the Jurkat eluate. **(D)** Comparison of the 20 best hits ($p < 0.05$, sorted on protein enrichment) enriched in the eluates of each ZAP70-PIF6 cell line vs. the flow-throughs of the last washing step. Known interaction partners of ZAP70 are written in bold. CHCHD2/P9, CHCHD2, CHCHD2P9; CD247-i3, CD247 isoform 3; TRBC1/2, TRBC1, TRBC2.

a similar enrichment of ZAP70 for both PIF6 tags and the known interaction partners CD247, CD247-i3, CD3D, CD3G, and TRBC1/2 within the 20 best hits ($p < 0.05$, sorted on protein enrichment, **Figure 5D**). Therefore, the flow-throughs of the last washing step offer an alternative as control to filter out unspecific and false positive proteins.

Finally, we asked whether we could combine the Jurkat eluates and the flow-throughs of the last washing step to confine the interaction partner analysis to the most relevant hits. To this end, we compared for each PIF6 tag separately the 20 best hits ($p < 0.05$, sorted on protein enrichment) enriched in the eluates compared to the Jurkat eluates and to the flow-throughs of the last washing step (**Figure 6**, **Supplementary Tables 3, 4**). Identical for both PIF6 tags only ZAP70 and the known interaction partners CD247-i3, CD3D, CD3G, TRBC1/2 were overlapping. This suggests that the other proteins were indeed false positives that we were able to filter out. Of note, CD3E was classified as a false positive although it is a known component of the TCR and thus an interaction partner of ZAP70. Together our data suggest that the combined usage of the Jurkat eluates and the flow-throughs of the last washing step as controls can be used to confine the identified proteins to true interaction partners eliminating false positives.

CONCLUSION

In this study we established an optogenetics-based light-controlled affinity purification method making use of the plant PhyB/PIF system. The POI-PIF6 fusion protein is recombinantly produced and bound to a PhyB* resin. It is eluted after affinity purification by changing the wavelength of light from 660 to 740 nm. Compared to other affinity purification methods (see introduction), in which the biochemical or biophysical properties of the elution buffer are changed compared to the washing buffers, light in the red spectrum at the used low intensities is a mild elution condition. Thus, we can reduce the identification of false positives, especially if one uses two controls, namely the flow-through of the last washing step and control cells expressing the untagged POI.

The fast kinetics of the binding and dissociation of the PhyB/PIF system enable to conduct the affinity purification within a short time window, which is beneficial for the investigation of transient and weak interactions. Finally, we reduced the size of the PhyB-interacting fragment of PIF6 from 100 amino acids [PIF6(1-100)] to 22 amino acids [PIF6(15-36)]. The size reduction of the PIF6 tag increases its suitability as affinity tag by decreasing the probability to interfere with the function of the POI and to interact with other proteins. Furthermore, the 22 amino acids PIF6 fragment might also allow novel optogenetic applications using the PhyB/PIF system (19, 32).

MATERIALS AND METHODS

Construction of Plasmids

The design and construction of the plasmids generated in this study are described in **Supplementary Table 5**

and the oligonucleotides used for this purpose in **Supplementary Table 6**. DNA-fragments were amplified by polymerase chain reaction and assembled by an isothermal, enzymatic DNA assembly reaction as described previously (Gibson cloning) (33).

Production of PhyB* and Protein Quantification

PhyB* encoded by plasmid pMH17 was expressed in *E. coli* together with the biosynthesis genes for phycocyanobilin (PCB) encoded by plasmid p171 and purified by immobilized metal ion affinity chromatography (IMAC) as described previously (24). The fluorescent protein GFP-PIF6(1-100)-His₆ encoded by plasmid pHB111 was used as fluorescence standard and was produced in *E. coli* and purified by IMAC as described previously (34). The protein concentrations of the purified proteins were determined by Bradford assay (Bio-Rad, Hercules, CA, cat. no. 500-0006) using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, cat. no. 05479) as standard.

The fluorescent proteins GFP and mCherry were quantified by measuring their fluorescence (GFP: excitation: 488 nm, emission: 522 nm; mCherry: excitation: 588 nm, emission: 620 nm) with an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). The concentration of all GFP-containing proteins expressed in HEK-293T cells was calculated based on the fluorescence using purified GFP-PIF6(1-100)-His₆ in wash buffer [20 mM Tris/HCl, 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, pH 7.4] as standard. In **Supplementary Figure 1**, the concentration of unbound PhyB* was determined by measuring PhyB* fluorescence (excitation: 620 nm, emission: 680 nm) after 660 nm illumination (10 min, 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with an Infinite M200 Pro microplate reader.

Maintenance of Mammalian Cells

HEK-293T cells were maintained in DMEM complete medium [DMEM (PAN Biotech, Aidenbach, Germany, cat. no. P04-03550), 10% (v/v) fetal calf serum (FCS, PAN Biotech, cat. no. P30-3602), 100 U ml⁻¹ penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin]. Jurkat, P116 (27) and transduced P116 cells were cultivated in RPMI complete medium [RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, cat. no. 31870-025), 10% (v/v) FCS (PeproTech, Hamburg, Germany, cat. no. 200-02), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, 10 mM HEPES (Thermo Fisher Scientific, cat. no. 15630-056)]. All cells were incubated in a humidified atmosphere at 37°C, 5% CO₂.

Expression and Harvesting of GFP Fused to Different Affinity Tags

All constructs encoding untagged mCherry and GFP fused to different affinity tags (**Figure 2A**, **Supplementary Table 5**) were expressed in HEK-293T cells. To this aim, 6×10^6 HEK-293T cells were seeded per 15 cm culture dish and transfected 24 h later with the corresponding plasmid. Per 15 cm dish, 60 μg plasmid DNA, and 200 μg PEI (linear, MW: 25 kDa, Polysciences, Warrington, PA, cat. no. 23966-2) were mixed in 3 ml OptiMEM (Thermo Fisher Scientific, cat. no. 22600-134), incubated for 15 min at RT and added dropwise to the

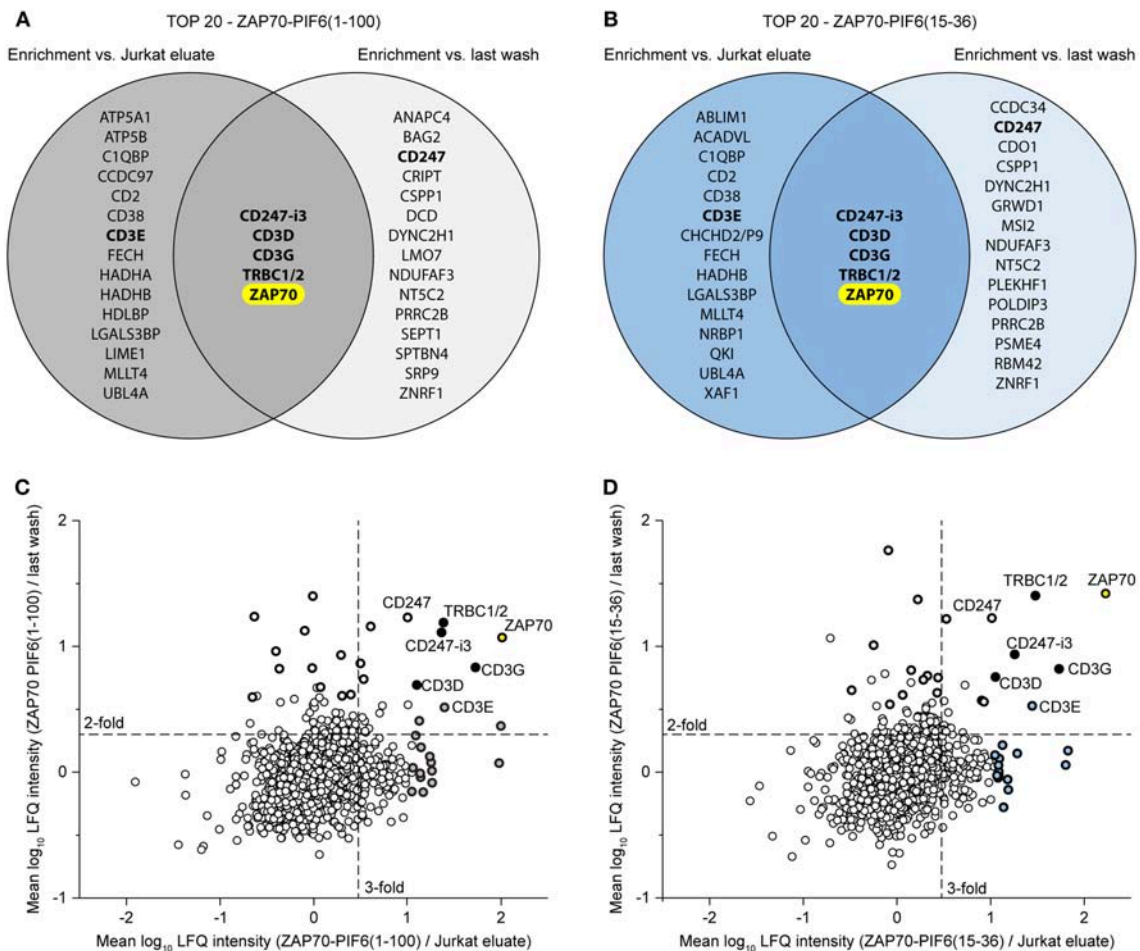


FIGURE 6 | Usage of two controls to confine the data to the most relevant interaction partners of the POI ZAP70. **(A,B)** Comparison of the 20 best hits ($p < 0.05$, sorted on protein enrichment) enriched in the eluates of the ZAP70-PIF6(1-100) **(A)**/ZAP70-PIF6(15-36) **(B)** cell line vs. the eluates from the Jurkat cell line or the flow-throughs of the last washing step. Known interaction partners of ZAP70 are written in bold. **(C,D)** Enrichment of proteins identified in the ZAP70-PIF6(1-100) **(C)**/ZAP70-PIF6(15-36) **(D)** eluates vs. the Jurkat eluates (x-axis) plotted against the enrichment of proteins in the ZAP70-PIF6(1-100) **(C)**/ZAP70-PIF6(15-36) **(D)** eluates vs. the corresponding flow-throughs of the last washing step (y-axis). The enrichment for each protein is shown as the mean $\log_{10}$ ratio of the label-free quantification (LFQ) intensities (3 biological replicates) of the compared samples. Known double-positive interaction partners of ZAP70 identified within the TOP 20 hits in **(A)**/**(B)** are highlighted in black and the names are indicated; the remaining hits of the TOP 20 are highlighted as bold black circles filled with the color corresponding to the group color used in the VENN diagram in **(A)**/**(B)**. The POI ZAP70 is highlighted in yellow. Dashed lines represent a ratio cut-off corresponding to a 3-fold or 2-fold enrichment. CHCHD2/P9, CHCHD2, CHCHD2P9; CD247-i3, CD247 isoform 3; TRBC1/2, TRBC1, TRBC2.

cells. After incubation for 5 h, the medium was exchanged and proteins were expressed for 48 h. Before harvesting, the cells were washed once with DBPS containing Ca^{2+} and Mg^{2+} (PAN Biotech, cat. no. P04-35500) and cells were lysed—if not stated otherwise—with 3 ml of lysis buffer [20 mM Tris/HCl, 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, pH 7.4, protease inhibitor cocktail (Sigma-Aldrich, cat. no. SRE0055), 0.5% (v/v) Brij97 (Sigma-Aldrich, cat. no. 431281-100ML)] per 15 cm culture dish for 20 min on ice. After clarifying the lysate by centrifugation at $16,500 \times g$ for 15 min, the supernatant was either immediately further processed or shock-frozen in liquid nitrogen and stored at -80°C . The average yield of GFP-PIF6(1-100) was $\sim 720 \mu\text{g}$ of purified protein per 15 cm dish.

Light-Controlled Affinity Purification

NeutrAvidin agarose beads (Thermo Fisher Scientific, cat. no. 29202) were washed once with Ni-Elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 250 mM imidazole, pH 8.0) and incubated afterwards with purified PhyB* in Ni-Elution buffer supplemented with 1 mM 2-mercaptoethanol (final PhyB* concentration: $\sim 2 \text{ mg ml}^{-1}$) for 2 h at 4°C under slight agitation. If not otherwise stated, $1 \mu\text{l}$ NeutrAvidin beads were incubated with 0.27 nmol PhyB*. After washing the beads twice with wash buffer, the beads were resuspended in lysis buffer and the indicated amounts of beads were transferred into a spin column (Thermo Fisher Scientific, cat. no. 69725). Subsequently, the cell lysate was added to the beads and the closed spin column was incubated on a rotator for 60 min under 660 nm illumination

(10 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 4°C. All following steps were performed under green safe light at 4°C. After incubation, the beads were washed by centrifugation of the spin column at $150 \times g$ for 30 s, addition of 500 μl of wash buffer and incubation of the closed spin column for 10 min on a rotator under 660 nm illumination. This washing step was repeated for the indicated number of times (3 x if not indicated otherwise). Afterwards, the column was centrifuged before 500 μl of wash buffer were added and the column was incubated under 740 nm light (70 $\mu\text{mol m}^{-2} \text{s}^{-1}$) on a rotator for 10 min. Finally, the eluted proteins were collected by centrifugation of the spin column.

Affinity Purification of GFP With SBP Tag

The indicated number of HEK-293T cells transfected with plasmid pMH501 (as described above) were lysed 48 h after transfection with 500 μl of lysis buffer. All following steps were performed at 4°C. The soluble fraction of the cell lysate was incubated with the indicated amount of prewashed streptavidin Sepharose beads (GE Healthcare, Freiburg, Germany, cat. no. 17-5113-01) within a closed spin column on a rotator at 4°C for 2 h. Afterwards, the beads were washed by centrifugation ($150 \times g$, 30 s) of the column, addition of 500 μl of wash buffer and incubation of the closed spin column for 10 min on a rotator. After repeating this washing step for three times, the column was centrifuged and 500 μl of elution buffer (wash buffer supplemented with 2 mM biotin) was added and the closed spin column was incubated on a rotator at 4°C for 20 min. Finally, the eluted proteins were collected by centrifugation.

Affinity Purification of GFP With TEV_{CS}-ProteinA Tag

The indicated number of HEK-293T cells transfected with plasmid pMH502 (as described above) were lysed 48 h after transfection with 500 μl of lysis buffer. All following steps were performed at 4°C. The soluble fraction of the lysate was incubated with the indicated amount of prewashed IgG Sepharose beads (35) within a closed spin column (Mobicol Classic with 35 μm pore size filter, MoBiTec, Göttingen, Germany, cat. no. M1003 and M513515) on a rotator at 4°C overnight. Afterwards, the beads were washed by centrifugation ($150 \times g$, 30 s) of the column, addition of 500 μl of wash buffer and incubation of the closed spin column for 10 min on a rotator. After repeating this washing step three times, the columns were centrifuged and the beads were resuspended in 500 μl of wash buffer containing TEV protease (1 U TEV per 1 μl of beads, Thermo Fisher Scientific, cat. no. 12575015). After incubation on a shaker at 16°C for 3 h, prewashed Ni-NTA agarose beads (1 μl Ni-NTA beads per 2 μl of IgG beads) were added to remove the His₆-tagged TEV protease and the sample was incubated for further 2 h. Finally, the eluted proteins were collected by centrifugation.

Generation of Stable Cell Lines

The P116-based cell lines stably expressing ZAP70-PIF6(1-100) or ZAP70-PIF6(15-36) were generated by transduction with lentiviral particles. For lentiviral particle production, 8×10^6 HEK293-T cells were seeded in DMEM lenti [Advanced

DMEM (Thermo Fisher Scientific, cat. no. 12491015), 2% (v/v) FCS, 100 U ml^{-1} penicillin, and 100 $\mu\text{g ml}^{-1}$ streptomycin, 10 μM cholesterol, 10 μM egg lecithin (Serva Electrophoresis, Heidelberg, Germany, cat. no. 27608), 1 x chemical defined lipid concentrate (Thermo Fisher Scientific, cat. no. 11905031)] per 15 cm dish. On the next day, the cells were transfected as described above with the plasmids pMH511 or pMH521, pLTR-G (36), and pCD/NL-BH* $\Delta\Delta\Delta$ (37) in a mass ratio of 2:1:1 and the medium was replaced after 5 h with fresh DMEM lenti. After 48 h, the supernatant containing the lentiviral particle was harvested and filtered through a 0.45 μm filter. For transduction, P116 cells at a density of 3×10^5 cells ml^{-1} were diluted 1:2 with the filtered lentiviral particle-containing supernatant. After 96 h, cells with low expression of the fluorescent protein ZsGreen1 were sorted using an S3e Cell Sorter (Bio-Rad).

Lysis of T Cells

For Western blot analysis (Figure 4C) and for each mass spectrometry measurement, 1×10^6 or 250×10^6 cells were resuspended in 50 or 500 μl of lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 0.5 mM sodium orthovanadate, and 1 mM NaF, respectively. After incubation for 15 min on ice the lysate was clarified by centrifugation for 15 min at $16,500 \times g$. The supernatant was either mixed with SDS loading buffer for Western Blot analysis or used for light-controlled affinity purification (200 μl of PhyB* beads, four washing steps) and subsequent mass spectrometry analysis.

Western Blots

For Western blots, proteins were transferred after SDS-PAGE onto PVDF membranes and the membranes were blocked with TBS-T [TBS (50 mM Tris/HCl, 150 mM NaCl, pH 7.4) with 0.05% (v/v) Tween-20] containing 5% (w/v) milk powder for 1 h at RT. Afterwards, the membranes were incubated with the primary antibody (anti-ZAP70, dilution: 1:500, Santa Cruz Biotechnology, Dallas, TX, cat. no. sc-30674 or anti-GAPDH, dilution: 1:100,000, Cell Signaling, Danvers, MA, cat. no. 5174) diluted in TBS-T supplemented with 3% (w/v) BSA and 0.02% (w/v) sodium azide at 4°C overnight. After washing the membrane three times with TBS-T, they were incubated with the secondary antibody (ZAP70: rabbit anti-goat IgG-HRP, dilution: 1:10,000, Thermo Fisher Scientific, cat. no. 31402 or GAPDH: goat anti-rabbit IgG-HRP, dilution: 1:10,000, Thermo Fisher Scientific, cat. no. 31460) diluted in TBS-T supplemented with 3% (w/v) BSA and 3% (w/v) milk powder for 1 h at RT. Following three washing steps with TBS-T, chemiluminescence was detected using ECL substrate and an ImageQuant LAS-4000 mini system (GE Healthcare).

Ca²⁺-Flux Measurements

For measuring of Ca²⁺-flux, 5×10^6 cells were washed with PBS and incubated in 1 ml starvation medium (RPMI complete containing 1% (v/v) FCS) containing 4 μM Indo-1 (Thermo Fisher Scientific, cat. no. I1223) and 0.1% (v/v) Pluronic F-127 (Thermo Fisher Scientific, cat. no. P3000MP) for 1 h at 37°C, 5% CO₂. Afterwards, the cells were pelleted by centrifugation,

resuspended in 500 μ l of starvation medium and kept on ice in the dark until the measurement. Analyses of Ca^{2+} -bound and unbound Indo-1 was performed with a customized MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) using a 355 nm laser and 405/20 nm and 530/30 nm emission filters, respectively. Cells were stimulated with 1 μ g ml^{-1} anti-CD3 antibody (UCHT1, Dr. Beverly, UK).

Mass Spectrometry

The proteins present within 400 μ l of the sample (either eluate or flow-through of the last washing step from the light-controlled affinity purification) were precipitated by addition of 1.6 ml of ice-cold acetone and incubation at -20°C overnight. Afterwards, the precipitated proteins were pelleted by centrifugation at $10,000 \times g$ for 10 min at 4°C and the air-dried pellet was resolubilized in 10 μ l of buffer (60% (v/v) methanol in 50 mM NH_4HCO_3). The disulfide bonds were then reduced with 5 mM tris(2-carboxyethyl)phosphine (TCEP) for 30 min at 65°C , and afterwards alkylated with 50 mM 2-chloroacetamide for 30 min at RT. The reaction was quenched by addition of 25 mM dithiothreitol (DTT), diluted with 25 μ l buffer and the proteins were digested with 1 μ g trypsin (Promega, Mannheim Germany, cat. no. V5111) at 42°C for 4 h. The samples were dried in a vacuum concentrator and resuspended in 15 μ l of 0.1% (v/v) trifluoroacetic acid (TFA) prior to mass spectrometry (MS) analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed with an UltiMate 3000 RLSCnano HPLC System (Thermo Fisher Scientific) coupled online to a Q Exactive Plus instrument (Thermo Fisher Scientific) essentially as described previously (38). Samples were washed on a C18 pre-column ($\varnothing$ 0.3 $\times$ 5 mm; PepMap, Thermo Fisher Scientific) using 0.1% (v/v) TFA for 15 min, which was thereafter switched in line with the analytical column (Acclaim PepMap (ID: 75 $\mu\text{m} \times$ 250 mm, 2 μm , 100 $\text{\AA}$, Dionex LC Packings/Thermo Fisher Scientific), equilibrated in 96% solvent A [0.1% (v/v) formic acid (FA)] and 4% solvent B (0.1% (v/v) FA, 86% (v/v) CH_3CN). A gradient of 100 min (4–40% solvent B in 95 min and 40–95% solvent B in 5 min) at a flow rate of 0.250 $\mu\text{l min}^{-1}$ was applied to separate the peptide mixture. The column was washed for 5 min with 95% solvent B, before re-equilibration for 15 min. The Q Exactive Plus acquired mass spectra from m/z 375 to 1,700, with a resolution of 70,000 at m/z 200 [parameters: automatic gain control (AGC), 3×10^6 ions; maximum fill time, 60 ms]. The instrument operated in the data-dependent mode, using a TOP 15 method for the isolation of precursor ions (parameters: AGC target, 1×10^5 ions; fill time, 120 ms; isolation window, 3 m/z ; normalized collision energy, 28; underfill ratio, 1.2%; dynamic exclusion, 45 s).

Mass Spectrometry Data Analysis

For mass spectrometry data analysis, MaxQuant version 1.5.5.1 with its integrated search engine Andromeda was used to search peak lists against the Uniprot proteome set *H. sapiens* (database 19/09/2018, 95109 entries) (39, 40). The search was conducted with default parameters (e.g., FDR 1% on protein and peptide level; precursor mass tolerance 20 ppm for the first search, 4.5 ppm for the main search), except the

following adjustments: trypsin was selected as proteolytic enzyme and up to three missed cleavages were allowed. As variable modifications oxidation on methionine and acetylation on protein N-termini were selected. Cysteine carbamidomethylation was set as fixed modification. Protein identification required at least one unique peptide. Label-free quantification (LFQ) (41) was enabled, the LFQ minimal ratio count was set to 2 and fast LFQ was disabled. “Match between runs” was enabled with default parameters.

The LFQ intensities from the proteingroups.txt output file of MaxQuant were loaded into Perseus version 1.5.5.3 (42). Entries from contaminants, reverse hits and hits only identified by modified peptides were discarded. LFQ intensities were $\log_{10}$ -transformed and proteingroups with 3 out of 3 valid values (3 independent biological replicates) in the ZAP70-PIF pulldown samples were used for further data analysis. Subsequently, for missing values of the control and wash samples random numbers were imputed from normal distribution (width of the distribution 0.5, down shift 1.8) to simulate values below the detection limit. To identify significantly enriched proteins, a right-sided, two-sample *t*-test was performed. Results were imported into Origin Pro 2017 (OriginLab, Northampton, MA) and visualized as scatter plots.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (43) partner repository with the dataset identifier PXD012156. All other raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MH and JE planned and performed all experiments to set up the new purification protocol, and MH, OY, and JE the ones involving the P116T cells. JS performed interactome studies by LC-MS followed by quantitative data analysis. MH, OY, BW, WW, and WS were involved in the design, supervision, and analysis of all experiments. MH, JE, and WS wrote the manuscript with input from all authors.

FUNDING

This work was funded by the German Research Foundation (DFG) under the Excellence Initiative (BIOSS - EXC-294 (to WW, WS, and BW) and SGBM - GSC-4 (to MH, OY, and JS)) and the Excellence Strategy (CIBSS - EXC-2189 - Project ID 390939984) to WW, WS, and BW as well as through the collaborative research center DFG-TRR 130 to BW.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00226/full#supplementary-material>

REFERENCES

- Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A, et al. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* (2006) 440:637–43. doi: 10.1038/nature04670
- Huttlin EL, Bruckner RJ, Paulo JA, Cannon JR, Ting L, Baltier K, et al. Architecture of the human interactome defines protein communities and disease networks. *Nature* (2017) 545:505–9. doi: 10.1038/nature22366
- Balogopalan L, Kortum RL, Coussens NP, Barr VA, Samelson LE. The linker for activation of T cells (LAT) signaling hub: from signaling complexes to microclusters. *J Biol Chem.* (2015) 290:26422–9. doi: 10.1074/jbc.R115.665869
- Lork M, Staal J, Beyaert R. Ubiquitination and phosphorylation of the CARD11-BCL10-MALT1 signalosome in T cells. *Cell Immunol.* (2018) doi: 10.1016/j.cellimm.2018.11.001. [Epub ahead of print].
- Chan AC, Iwashima M, Turck CW, Weiss A. Zap-70 - a 70 Kd protein-tyrosine kinase that associates with the Tcr Zeta-Chain. *Cell* (1992) 71:649–62. doi: 10.1016/0092-8674(92)90598-7
- Iwashima M, Irving BA, van Oers NS, Chan AC, Weiss A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* (1994) 263:1136–9. doi: 10.1126/science.7509083
- van Oers NSC, Killeen N, Weiss A. Zap-70 is constitutively associated with tyrosine-phosphorylated tcr-Zeta in murine thymocytes and lymph-node T-cells. *Immunity* (1994) 1:675–85. doi: 10.1016/1074-7613(94)90038-8
- Au-Yeung BB, Deindl S, Hsu LY, Palacios EH, Levin SE, Kuriyan J, et al. The structure, regulation, and function of ZAP-70. *Immunol Rev.* (2009) 228:41–57. doi: 10.1111/j.1600-065X.2008.00753.x
- Katz ZB, Novotna L, Blount A, Lillemeier BF. A cycle of Zap70 kinase activation and release from the TCR amplifies and disperses antigenic stimuli. *Nat Immunol.* (2017) 18:86–95. doi: 10.1038/ni.3631
- Roncagalli R, Hauri S, Fiore F, Liang Y, Chen Z, Sansoni A, et al. Quantitative proteomics analysis of signalosome dynamics in primary T cells identifies the surface receptor CD6 as a Lat adaptor-independent TCR signaling hub. *Nat Immunol.* (2014) 15:384–92. doi: 10.1038/ni.2843
- Gingras AC, Gstaiger M, Raught B, Aebersold R. Analysis of protein complexes using mass spectrometry. *Nat Rev Mol Cell Biol.* (2007) 8:645–54. doi: 10.1038/nrm2208
- Pardo M, Choudhary JS. Assignment of protein interactions from affinity purification/mass spectrometry data. *J Proteome Res.* (2012) 11:1462–74. doi: 10.1021/pr2011632
- Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol.* (2003) 60:523–33. doi: 10.1007/s00253-002-1158-6
- Pina AS, Lowe CR, Roque AC. Challenges and opportunities in the purification of recombinant tagged proteins. *Biotechnol Adv.* (2014) 32:366–81. doi: 10.1016/j.biotechadv.2013.12.001
- Parks TD, Leuther KK, Howard ED, Johnston SA, Dougherty WG. Release of proteins and peptides from fusion proteins using a recombinant plant-virus proteinase. *Anal Biochem.* (1994) 216:413–7. doi: 10.1006/abio.1994.1060
- Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol.* (1999) 17:1030–2. doi: 10.1038/13732
- Khanna R, Huq E, Kikis EA, Al-Sady B, Lanzatella C, Quail PH. A novel molecular recognition motif necessary for targeting photoactivated phytochrome signaling to specific basic helix-loop-helix transcription factors. *Plant Cell* (2004) 16:3033–44. doi: 10.1105/tpc.104.025643
- Levskaya A, Weiner OD, Lim WA, Voigt CA. Spatiotemporal control of cell signalling using a light-switchable protein interaction. *Nature* (2009) 461:997–1001. doi: 10.1038/nature08446
- Kolar K, Knobloch C, Stork H, Znidaric M, Weber W. OptoBase: a web platform for molecular optogenetics. *ACS Synth Biol.* (2018) 7:1825–8. doi: 10.1021/acssynbio.8b00120
- Adrian M, Nijenhuis W, Hoogstraaten RI, Willems J, Kapitein LC. A phytochrome-derived photoswitch for intracellular transport. *ACS Synth Biol.* (2017) 6:1248–56. doi: 10.1021/acssynbio.6b00333
- Toettcher JE, Weiner OD, Lim WA. Using optogenetics to interrogate the dynamic control of signal transmission by the ras/erk module. *Cell* (2013) 155:1422–34. doi: 10.1016/j.cell.2013.11.004
- Beyer HM, Juillot S, Herbst K, Samodelov SL, Müller K, Schamel WW, et al. Red light-regulated reversible nuclear localization of proteins in mammalian cells and zebrafish. *ACS Synth Biol.* (2015) 4:951–8. doi: 10.1021/acssynbio.5b00004
- Müller K, Engesser R, Metzger S, Schulz S, Kämpf MM, Busacker M, et al. A red/far-red light-responsive bi-stable toggle switch to control gene expression in mammalian cells. *Nucleic Acids Res.* (2013) 41:e77. doi: 10.1093/nar/gkt002
- Smith RW, Helwig B, Westphal AH, Pel E, Hörner M, Beyer HM, et al. Unearthing the transition rates between photoreceptor conformers. *BMC Syst Biol.* (2016) 10:110. doi: 10.1186/s12918-016-0368-y
- Keefe AD, Wilson DS, Seelig B, Szostak JW. One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-tag. *Protein Expr Purif.* (2001) 23:440–6. doi: 10.1006/prep.2001.1515
- Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K, et al. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* (1999) 15:963–72. doi: 10.1002/(Sici)1097-0061(199907)15:10b<963::Aid-Yea399>3.0.Co;2-W
- Williams BL, Schreiber KL, Zhang W, Wange RL, Samelson LE, Leibson PJ, et al. Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol Cell Biol.* (1998) 18:1388–99. doi: 10.1128/MCB.18.3.1388
- Schamel WW, Alarcon B. Organization of the resting TCR in nanoscale oligomers. *Immunol Rev.* (2013) 251:13–20. doi: 10.1111/imr.12019
- Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. *Cell* (1994) 76:263–74. doi: 10.1016/0092-8674(94)90334-4
- Qian DP, Mollenauer MN, Weiss A. Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. *J Exp Med.* (1996) 183:611–20. doi: 10.1084/jem.183.2.611
- Roose JP, Diehn M, Tomlinson MG, Lin J, Alizadeh AA, Botstein D, et al. T cell receptor-independent basal signaling via Erk and Abl kinases suppresses RAG gene expression. *PLoS Biol.* (2003) 1:271–87. doi: 10.1371/journal.pbio.0000053
- Tischer D, Weiner OD. Illuminating cell signalling with optogenetic tools. *Nat Rev Mol Cell Biol.* (2014) 15:551–8. doi: 10.1038/nrm3837
- Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA III, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* (2009) 6:343–5. doi: 10.1038/nmeth.1318
- Beyer HM, Thomas OS, Riegel N, Zurbriggen MD, Weber W, Hörner M. Generic and reversible opto-trapping of biomolecules. *Acta Biomater.* (2018) 79:276–82. doi: 10.1016/j.actbio.2018.08.032
- Piechura H, Oeljeklaus S, Warscheid B. SILAC for the study of mammalian cell lines and yeast protein complexes. *Methods Mol Biol.* (2012) 893:201–21. doi: 10.1007/978-1-61779-885-6_14
- Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci USA.* (1996) 93:15266–71.
- Zhang XY, La Russa VF, Reiser J. Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins. *J Virol.* (2004) 78:1219–29. doi: 10.1128/JVI.78.3.1219-1229.2004
- Reimann L, Wiese H, Leber Y, Schwable AN, Fricke AL, Rohland A, et al. Myofibrillar Z-discs are a protein phosphorylation hot spot with protein kinase C (PKC α) modulating protein dynamics. *Mol Cell Proteomics* (2017) 16:346–67. doi: 10.1074/mcp.M116.065425

39. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol.* (2008) 26:1367–72. doi: 10.1038/nbt.1511
40. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the maxquant environment. *J Proteome Res.* (2011) 10:1794–805. doi: 10.1021/pr101065j
41. Cox J, Hein MY, Lubner CA, Paron I, Nagaraj N, Mann M. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* (2014) 13:2513–26. doi: 10.1074/mcp.M113.031591
42. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* (2016) 13:731–40. doi: 10.1038/Nmeth.3901
43. Vizcaino JA, Csordas A, Del-Toro N, Dienes JA, Griss J, Lavidas I, et al. 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* (2016) 44:D447–56. doi: 10.1093/nar/gkw880

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fate of a Naive T Cell: A Stochastic Journey

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
United Kingdom Research and
Innovation, United Kingdom

Reviewed by:

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equally to this work

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 28 June 2018

Accepted: 23 January 2019

Published: 06 March 2019

Citation:

de la Higuera L, López-García M,
Castro M, Abourashchi N, Lythe G
and Molina-París C (2019) Fate of a
Naive T Cell: A Stochastic Journey.
Front. Immunol. 10:194.
doi: 10.3389/fimmu.2019.00194

The homeostasis of T cell populations depends on migration, division and death of individual cells (1). T cells migrate between spatial compartments (spleen, lymph nodes, lung, liver, etc.), where they may divide or differentiate, and eventually die (2). The kinetics of recirculation influences the speed at which local infections are detected and controlled (3). New experimental techniques have been developed to measure the lifespan of cells, and their migration dynamics; for example, fluorescence-activated cell sorting (4), *in vitro* time-lapse microscopy (5), or *in vivo* stable isotope labeling (e.g., deuterium) (6). When combined with mathematical and computational models, they allow estimation of rates of migration, division, differentiation and death (6, 7). In this work, we develop a stochastic model of a single cell migrating between spatial compartments, dividing and eventually dying. We calculate the number of division events during a T cell's journey, its lifespan, the probability of dying in each compartment and the number of progeny cells. A fast-migration approximation allows us to compute these quantities when migration rates are larger than division and death rates. Making use of published rates: (i) we analyse how perturbations in a given spatial compartment impact the dynamics of a T cell, (ii) we study the accuracy of the fast-migration approximation, and (iii) we quantify the role played by direct migration (not via the blood) between some compartments.

Keywords: T cell, stochastic model, continuous-time Markov chain, single cell, cellular fate, migration, division, apoptosis

1. INTRODUCTION

T cells are descendants of bone marrow progenitors that migrated to the thymus and underwent processes of maturation, gene rearrangement and selection (8). The surface of a T cell is populated with tens of thousands of copies of a T-cell receptor. A repertoire of T cells is maintained in a mammal's body that enables recognition of and response to the many benign and pathogenic microorganisms that are encountered over its lifetime, although the T-cell receptor of any individual cell only recognizes a tiny fraction of them (9, 10). An individual T cell may circulate between different tissues of the body for months or years, never encountering cognate antigen. Their interactions with self antigens, generally weak, are occasionally strong enough to cause one round of cell division. Strong interaction between the T-cell receptor and non-self antigens, mounted on MHC on the surface of antigen-presenting cells in lymph nodes (11), initiates a programme of multiple rounds of cell division and phenotypic changes that generate effector and memory T cells with different lifetimes and migration patterns (2, 12–15).

Blood is a dynamic conduit through which T cells pass, in homeostasis and during immune responses (16). Blood is also the only tissue from which it is easy to obtain samples of T cells from healthy humans, although only about two percent of the body's T cells are in the blood at any one time (17, 18). The fraction of T cells found in a particular tissue depends on how likely a T cell is to enter the tissue and on how long it stays there. At any one time, for example, the fraction of T cells in lymph nodes and spleen is large, not because a T cell in the blood is most likely to go there, but because, when they do enter, they remain there a long time (3). Direct counts of T cell numbers in organs of mice are sometimes possible (19, 20); direct measurement of the kinetics of recirculation is more difficult. Mathematical models of the full kinetics of recirculation are the basis of a systematic extrapolation from measurements to residence times and migration probabilities.

Ganusov and Auerbach (3) constructed a model, based on experimental data, in which the migration history of a T cell consists of short intervals in the blood (less than a minute each) between longer sojourns in lung, liver, spleen and lymph nodes. We adopt their star-shaped migration topology pattern here. We also adopt a Markov description, in which the next event in the lifetime of a T cell (migration, division or death) is stochastic, but governed by parameters that depend only on the cell's current position. We treat a division event as the birth of one new cell, that follows the same rules as its mother, and a continuation of the life of another. In our modeling, we have in mind the homeostasis of naive CD4⁺ T cells, without explicitly taking the effect of aging (15, 19, 21) into account.

Novel labeling techniques are providing an increasing amount of information about recirculation and other properties at the single cell level (4–6), which lead to new hypotheses and new experiments aimed at elucidating the kinetic properties of a cell's journey. Techniques such as staining or barcoding are ideal for quantifying dynamics at the single cell level, since they are able to track individual cells, their interactions with the extra-cellular environment and other cells and to help understand single cell lifetime dynamics (22, 23).

Although they are able to provide a substantial amount of quantitative data, experimental techniques are still far from being able to provide a full picture of lymphocyte dynamics *in vivo*, even in mice (24, 25). Thus, a partnership between experimental and *in silico* approaches is required. Deterministic continuous time models (based on ordinary differential equations) are the usual approach to study the kinetics of cell recirculation (7, 26, 27) when describing large cell populations. On the other hand, these deterministic approaches can miss some crucial behavior due to the stochastic nature of cellular heterogeneity and cellular interactions (28, 29). Stochastic processes are more appropriate when studying observables at the single cell level, instead of at the population level (30, 31).

This work is inspired by these new experimental techniques, and by the work of Ganusov and Auerbach (3), where the authors analyse the kinetics of lymphocyte recirculation. Our aim is to show how new analytical approaches can be applied to these systems to study the stochastic journey of a single cell during its lifetime. Based on the assumption that there are many more

migration events than division and death events, we propose a *fast-migration approximation*. Finally, we carry out a range of numerical experiments to test the approximation, and to show the impact that cellular events occurring in a given spatial compartment can have on the whole system.

2. THEORY

2.1. Description of the General Model

We consider a model of a T cell that migrates between different spatial compartments, where it may divide one or more times, before ultimately dying. Inspired by the representation of Ganusov and Auerbach (3, Figure 2), these compartments can represent blood, lymph nodes, lung, liver, spleen and Peyer's patches. We denote the blood compartment by B and denote M additional compartments by $\{C_1, \dots, C_M\}$ (see Figure 1).

The journey of a T cell during its lifetime is summarized by the diagram in Figure 1. A cell can migrate between compartments, divide or die (reaching the state $\emptyset$). Our model is an absorbing continuous-time Markov chain (CTMC) $\mathcal{Y} = \{Y(t) : t \geq 0\}$ defined on the space of states $\mathcal{S} = \{B, C_1, \dots, C_M, \emptyset\}$, where $Y(t)$ identifies the position of the cell at time $t \geq 0$. We note that division does not affect the position of the cell, $Y(t)$, and therefore, we keep division events in our description as events that leave the process in the same state, as described in Figure 1. When tracking a given T cell, if a division event occurs, one of the two resulting cells is the daughter, while the other is taken to be the original cell.

Our aims are: (i) to show how the dynamics of a T cell (see Figure 1) can be studied by means of a number of summary statistics (or *stochastic descriptors*) in section 2.2, inspired by current single cell experimental techniques; (ii) to present in section 2.3 a fast-migration approximation which allows us to simplify the analysis when migration rates are much larger than division and death rates; and (iii) to quantify the impact of changes occurring in a single spatial compartment (section 3).

2.2. Single Cell Descriptors

Recent studies have highlighted the importance of improving the existing experimental and analytic toolset for continuous single cell dynamics. While some tools such as TimeLapseAnalyzer (32) or TLM-Tracker (33) are fully automated, successful *in vitro* single cell tracking by long-term time-lapse microscopy usually requires combined automated methods and manual curation. It is worth mentioning here the recently developed single cell tracking and quantification software toolset consisting of The Tracking Tool and qTFy (34), which allows for robust and efficient analysis of large amounts of time-lapse imaging data, is not limited to specific cell types, and allows for some degree of manual curation after automated processing.

These and similar tools have led to the quantification of cellular dynamics corresponding to a single cell or the whole lineage descended from a *founder* cell. When this cellular dynamics is represented in terms of a stochastic process consisting of division, migration and death events, such as the one in Figure 1, our aim is to define and analyse a number of summary statistics that can be compared to the dynamics

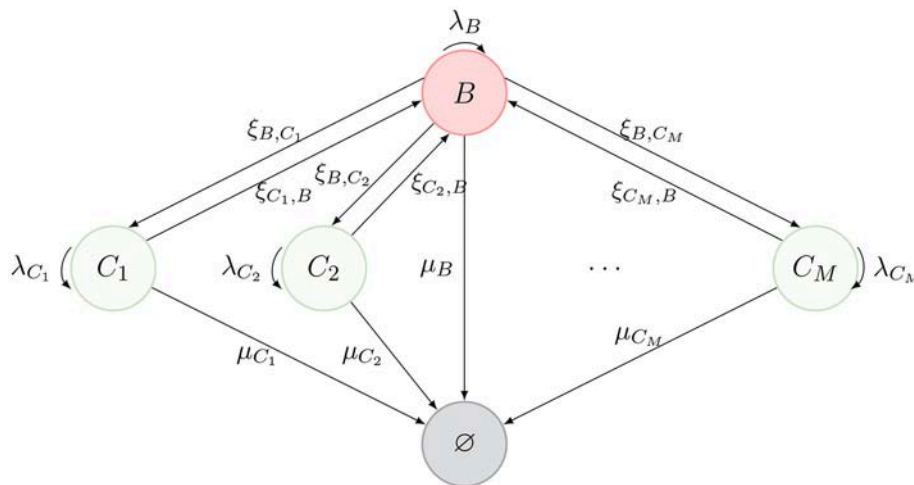


FIGURE 1 | Schematic description of the model. The nodes represent spatial compartments where the CD4⁺ T cell can be located at a given time. The arrows connecting them represent the migration of the cell between compartments, with migration rates $\{\xi_{B,C_j}, \xi_{C_j,B}\}$, $j \in \{1, \dots, M\}$. Division events are represented by curved arrows with rates λ_B and $\{\lambda_{C_j}, j \in \{1, \dots, M\}\}$. Finally, the state $\emptyset$ represents the death of the cell (with different death rates μ_B and $\{\mu_{C_j}, j \in \{1, \dots, M\}\}$, depending on the location where cell death takes place).

observed experimentally, at least in *in vitro* experiments. In particular, the Markovian representation of the process in **Figure 1** allows us to make use of first-step arguments to analyse a number of summary statistics for the cellular dynamics. In this section, we present the summary statistics of interest together with exact formulae for their computation, while the mathematical details to obtain these expressions can be found in the **Appendix**.

These summary statistics are directly inspired by data obtained from the experimental analysis of single cell dynamics and cell *pedigrees*. For example, when analysing a single founder B cell in *in vitro* experiments, Hawkins et al. (35) were able to obtain data regarding its lineage tree and quantified the times for cell division and death of the founder and descendent cells [see Figure 2A in Hawkins et al. (35, **Supplementary Material**)]. Similar dynamics and analysis can be found in Piltti et al. (36, Figure 2) for *in vitro* experiments with neural stem cells. On the other hand, if one was to consider a simulation of the stochastic process described in **Figure 1**, a realization would resemble **Figure 2**. In the same manner, in Reinhardt et al. (37), the authors show how the time-course of OT-II counts can be tracked in different locations *in vivo* (blood, spleen, lymph nodes, ...). This experimental setup contains valuable information about total counts or even cumulative numbers in each spatial compartment. For long enough times, these counts could be directly linked to the total number of divisions in each compartment. This kind of long-time experiments can be found, for instance, in Masopust et al. (38) where CD8⁺ T cells were tracked for almost three months, or in Sathaliyawala et al. (39) where the count is made in humans at the time of death of the donors.

Motivated by these experimental achievements, we introduce different stochastic descriptors (also known as summary

statistics). Not all of them can be straightforwardly quantified but, interestingly, combined they give information about specific aspects of the cellular dynamics that are unattainable using standard population dynamics approaches. In particular, the process in **Figure 2**, similarly to that in Hawkins et al. (35, **Supplementary Material**, Figure 2A) or Piltti et al. (36, Figure 2), can be quantified in terms of the following statistics:

- Lifetime of a CD4⁺ T cell and number of division events of this cell during its lifetime,

$$T_i = \text{"lifetime of a given cell starting in compartment } i"$$

$$= \inf\{t \geq 0 : Y(t) = \emptyset \mid Y(0) = i\},$$

$$N_i = \text{"for a given cell starting in compartment } i,$$

$$\text{number of division events until it dies"},$$

for $i \in \{B, C_1, \dots, C_M\}$. If we define $m_i = \mathbb{E}(T_i)$ and $\hat{m}_i = \mathbb{E}(N_i)$, one can show that

$$m_B = \left(\mu_B + \sum_{i=1}^M \xi_{B,C_i} - \sum_{i=1}^M \xi_{B,C_i} \xi_{C_i,B} (\mu_{C_i} + \xi_{C_i,B})^{-1} \right)^{-1}$$

$$\times \left(\sum_{i=1}^M \xi_{B,C_i} (\mu_{C_i} + \xi_{C_i,B})^{-1} + 1 \right),$$

$$m_{C_i} = (\mu_{C_i} + \xi_{C_i,B})^{-1} (\xi_{C_i,B} m_B + 1), \quad i \in \{1, \dots, M\},$$

$$\hat{m}_B = \left(\mu_B + \sum_{i=1}^M \xi_{B,C_i} - \sum_{i=1}^M \xi_{B,C_i} \xi_{C_i,B} (\mu_{C_i} + \xi_{C_i,B})^{-1} \right)^{-1}$$

$$\times \left(\sum_{i=1}^M \xi_{B,C_i} \lambda_{C_i} (\mu_{C_i} + \xi_{C_i,B})^{-1} + \lambda_B \right),$$

$$\hat{m}_{C_i} = (\mu_{C_i} + \xi_{C_i,B})^{-1} (\xi_{C_i,B} \hat{m}_B + \lambda_{C_i}), \quad i \in \{1, \dots, M\}.$$

of the random variable

G_i = “total number of cells within the genealogy of a cell which starts in compartment i ”,

and the mean values of these random variables, $\tilde{m}_i = \mathbb{E}(G_i)$, can be computed as

$$\begin{aligned}\tilde{m}_B &= \left(\mu_B + \sum_{i=1}^M \xi_{B,C_i} - \lambda_B - \sum_{i=1}^M \xi_{B,C_i} (\mu_{C_i} + \xi_{C_i,B} - \lambda_{C_i})^{-1} \right. \\ &\quad \left. \times \xi_{C_i,B} \right)^{-1} \left(\sum_{i=1}^M \xi_{B,C_i} (\mu_{C_i} + \xi_{C_i,B} - \lambda_{C_i})^{-1} 2\lambda_{C_i} + 2\lambda_B \right), \\ \tilde{m}_{C_i} &= (\mu_{C_i} + \xi_{C_i,B} - \lambda_{C_i})^{-1} (\xi_{C_i,B} \tilde{m}_B + 2\lambda_{C_i}), \\ i &\in \{1, \dots, M\}.\end{aligned}$$

For a particular realization of the stochastic process described by **Figure 1**, we show in **Figure 2**, the definition of this summary statistics.

We note that if on average, a larger number of division events take place than death ones, the corresponding branching process depicted in **Figure 2** might explode. This means that, depending on the parameter values, one might have $\mathbb{P}(G_i = +\infty) > 0$ and thus, $\mathbb{E}(G_i) = +\infty$. We find that sufficient conditions on the parameters to ensure $\mathbb{P}(G_i = +\infty) = 0$, are given by

$$\xi_{C_i,B} + \mu_{C_i} > \lambda_{C_i}, \quad \forall i \in \{1, \dots, M\}, \quad (1)$$

$$\sum_{i=1}^M \xi_{B,C_i} + \mu_B > \lambda_B + \sum_{i=1}^M \frac{\xi_{B,C_i} \xi_{C_i,B}}{\xi_{C_i,B} + \mu_{C_i} - \lambda_{C_i}}. \quad (2)$$

We also note that there is an intuitive interpretation of these conditions. In particular, for each spatial compartment C_i , the total rate of removing cells from this compartment (migration of cells, $\xi_{C_i,B}$, or death, μ_{C_i}) needs to be larger than the corresponding division rate λ_{C_i} , so that cells do not indefinitely accumulate in this compartment. This is represented by Equation (1). On the other hand, it is not enough to export these cells to a different compartment if these cells cannot die sufficiently fast in a different compartment after they migrate, which is summarized by Equation (2), where blood acts as a special migration hub.

2.3. Fast-Migration Approximation

As we show in section 3 for CD4⁺ T cells in mice, the migration rates, $\{\xi_{B,C_i}, \xi_{C_i,B}, i \in \{1, \dots, M\}\}$, are of the order of min^{-1} , and division, $(\lambda_B, \lambda_{C_1}, \dots, \lambda_{C_M})$, and death rates, $(\mu_B, \mu_{C_1}, \dots, \mu_{C_M})$, are of the order of days^{-1} . Thus, migration is several orders of magnitude faster. One can use this fact to propose a fast-migration approximation for the summary statistics above, and thus, to study a much simpler birth-and-death (or branching) process without spatial compartments.

We propose to approximate the journey of the cell under analysis, and its progeny, by considering a birth-and-death

stochastic process within a single spatial compartment, with birth and death rates given by

$$\bar{\lambda} = f_B \lambda_B + \sum_{i=1}^M f_{C_i} \lambda_{C_i}, \quad \bar{\mu} = f_B \mu_B + \sum_{i=1}^M f_{C_i} \mu_{C_i},$$

where f_j represents the fraction of time that the cell under study spends in each spatial compartment $j \in \{B, C_1, \dots, C_M\}$, in the absence of division and death (i.e., if only migration is considered in **Figure 1**). One could imagine that this birth-and-death process would approximate well the division and death dynamics of the original one when migration occurs at a much faster rate than division and death, so that steady state conditions (i.e., f_j values) for the spatial location of the cell can be assumed before any division or death event occurs.

In order to compute the fraction f_j , one needs to calculate the steady state probabilities for the process in **Figure 1**, in the absence of division and death, which satisfy the following system of equations

$$\begin{aligned}(f_B \ f_{C_1} \ \dots \ f_{C_M}) &\begin{pmatrix} -\sum_i \xi_{B,C_i} & \xi_{B,C_1} & \xi_{B,C_2} & \dots & \xi_{B,C_M} \\ \xi_{C_1,B} & -\xi_{C_1,B} & 0 & \dots & 0 \\ \xi_{C_2,B} & 0 & -\xi_{C_2,B} & \dots & 0 \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ \xi_{C_M,B} & 0 & 0 & \dots & -\xi_{C_M,B} \end{pmatrix} = 0, \\ (f_B \ f_{C_1} \ \dots \ f_{C_M}) &\begin{pmatrix} 1 \\ 1 \\ \vdots \\ 1 \end{pmatrix} = 1,\end{aligned}$$

which leads to the solution

$$f_B = \frac{1}{1 + \sum_{i=1}^M K_i}, \quad f_{C_j} = K_j \frac{1}{1 + \sum_{i=1}^M K_i}, \quad j \in \{1, \dots, M\} \quad (3)$$

where we have introduced

$$K_i = \frac{\xi_{B,C_i}}{\xi_{C_i,B}}, \quad i \in \{1, \dots, M\}.$$

Once this birth-and-death approximation has been introduced, one can propose the following simplifications:

- The average lifetime of the cell in the original model can be approximated by $\mathbb{E}(T_i) \approx \bar{\mu}^{-1}$, which is its average lifetime in the fast-migration approximation, and does not depend on the initial compartment i . From now on, and when implementing the fast-migration approximation, we remove the initial compartment (labeled by i) in the notation.
- The average number of division events during the lifetime of a given cell in the original model can be approximated by $\mathbb{E}(N) \approx \frac{\bar{\lambda}}{\bar{\mu}}$, which is the average number of division events in the fast-migration approximation. Since $N = N(B) + \sum_{k=1}^M N(C_k)$, as explained in section 2.2, where $N(j)$ is the number of division events in compartment $j \in \{B, C_1, \dots, C_M\}$, one can propose $\mathbb{E}(N(j)) \approx \frac{\bar{\lambda}}{\bar{\mu}} f_j$.

- The time for the progeny of a single cell to become extinct in the original process is not easy to analyse, and was not considered in section 2.2. Yet, it can be approximated by the time to extinction of a birth-and-death process with birth rate $\bar{\lambda}$ and death rate $\bar{\mu}$, for one single cell starting the process. This time follows a phase-type distribution (40), and its mean is given by $-\bar{\lambda}^{-1} \log \left(1 - \frac{\bar{\lambda}}{\bar{\mu}}\right)$ (41).
- The probability of the original cell dying in compartment $j \in \{B, C_1, \dots, C_M\}$ can be approximated by $\beta(j) \approx \frac{\mu_j f_j}{\bar{\mu}}$.
- The number of cells in the genealogy of a single cell in the original process can be approximated by the number of cells in the genealogy of a branching process with division rate $\bar{\lambda}$ and death rate $\bar{\mu}$. In particular, we can write (see section 1 in the **Appendix** for further details)

$$\mathbb{E}(G) \approx \frac{\bar{\mu} + \bar{\lambda}}{\bar{\mu} - \bar{\lambda}}. \quad (4)$$

2.4. The Effect of Non-blood Mediated Migration

Thus far, we have considered that, as described in **Figure 1**, cells can only migrate from one compartment to another through blood. However, in Ganusov and Auerbach (3), the authors made a compelling case for direct migration between compartments, namely, non-mediated by blood. In this section, we make use of the same model as before, but allow for cells in compartment C_{1a} to migrate directly to compartment C_{1b} . The new scenario is described in **Figure 3**, where the dashed arrow is the new migration rate. Note that, following Ganusov and Auerbach (3), we do not allow for migration from C_{1a} to blood.

In order to keep the notation consistent, we split the former compartment C_1 into two compartments, labeled C_{1a} and C_{1b} , respectively. Thus, the process of **Figure 1** represents the dynamics of **Figure 3**, when one is not interested in deciphering where exactly a given cell is located in C_1 (i.e., if the cell is in C_{1a} or C_{1b}).

The summary statistics defined in section 2.2 could be analyzed for the process of **Figure 3** in a similar way, but we do not present the details here. On the other hand, the fast-migration approximation can be implemented by considering the steady state migration dynamics of **Figure 3**, which leads to the new set of equations

$$\begin{pmatrix} f_B & f_{C_{1a}} & f_{C_{1b}} & \dots & f_{C_M} \end{pmatrix} \times \begin{pmatrix} -\sum_i \xi_{B,C_i} & \xi_{B,C_{1a}} & \xi_{B,C_{1b}} & \xi_{B,C_2} & \dots & \xi_{B,C_M} \\ 0 & -\xi_{C_{1a},C_{1b}} & \xi_{C_{1a},C_{1b}} & 0 & \dots & 0 \\ \xi_{C_{1b},B} & 0 & -\xi_{C_{1b},B} & 0 & \dots & 0 \\ \vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\ \xi_{C_M,B} & 0 & 0 & 0 & \dots & -\xi_{C_M,B} \end{pmatrix} = 0,$$

$$\begin{pmatrix} f_B & f_{C_{1a}} & f_{C_{1b}} & \dots & f_{C_M} \end{pmatrix} \begin{pmatrix} 1 \\ 1 \\ \vdots \\ 1 \end{pmatrix} = 1.$$

One can solve this system of equations to find

$$\begin{aligned} f_{C_{1a}} &= \frac{f_B \xi_{B,C_{1a}}}{\xi_{C_{1a},C_{1b}}}, & f_{C_{1b}} &= \frac{f_B (\xi_{B,C_{1a}} + \xi_{B,C_{1b}})}{\xi_{C_{1b},B}}, \\ f_{C_i} &= \frac{f_B \xi_{B,C_i}}{\xi_{C_i,B}}, & i &\in \{2, \dots, M\}. \end{aligned} \quad (5)$$

Let us introduce

$$K_{1a} = \frac{\xi_{B,C_{1a}}}{\xi_{C_{1a},C_{1b}}}, \quad K_{1b} = \frac{\xi_{B,C_{1b}}}{\xi_{C_{1b},B}}, \quad K_{1a,b} = \frac{\xi_{C_{1a},C_{1b}}}{\xi_{C_{1b},B}},$$

and $K = K_{1a}K_{1a,b} + K_{1a} + K_{1b} + \sum_{i=2}^M K_i$, to be able to write

$$\begin{aligned} f_B &= \frac{1}{K+1}, & f_{C_{1a}} &= \frac{K_{1a}}{K+1}, & f_{C_{1b}} &= \frac{K_{1a}K_{1a,b} + K_{1b}}{K+1}, \\ f_{C_i} &= \frac{K_i}{K+1}, & i &\in \{2, \dots, M\}. \end{aligned} \quad (6)$$

Interestingly, by adding the fractions in compartments C_{1a} and C_{1b} , we can map this model to the previous one if we define

$$\xi_{B,C_1} = \xi_{B,C_{1a}} + \xi_{B,C_{1b}}, \quad (7)$$

and

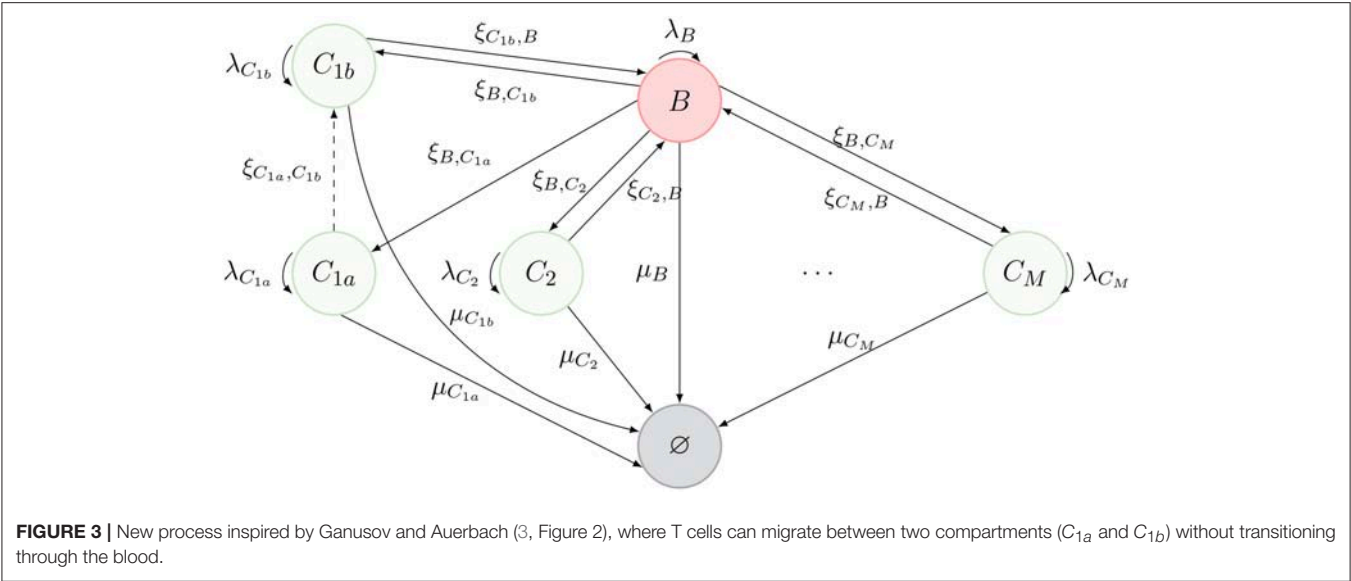
$$\begin{aligned} \frac{1}{\xi_{C_1,B}} &= \frac{\xi_{B,C_{1a}}}{\xi_{C_{1a},C_{1b}}(\xi_{B,C_{1a}} + \xi_{B,C_{1b}})} + \frac{\xi_{B,C_{1a}}}{\xi_{C_{1b},B}(\xi_{B,C_{1a}} + \xi_{B,C_{1b}})} \\ &\quad + \frac{\xi_{B,C_{1b}}}{\xi_{C_{1b},B}(\xi_{B,C_{1a}} + \xi_{B,C_{1b}})}. \end{aligned} \quad (8)$$

We note that Equations (7)–(8) imply that the parameters $(\xi_{C_1,B}, \xi_{B,C_1})$ can be considered as *effective* migration rates between the blood and compartment C_1 , when C_1 is merged from compartments C_{1a} and C_{1b} . The rate of a cell migrating from the blood to C_{1a} or C_{1b} , if one is not interested in where exactly it migrates to (i.e., migration to C_1), would then be given by $\xi_{B,C_1} = \xi_{B,C_{1a}} + \xi_{B,C_{1b}}$ [see Equation (7)]. On the other hand, for a cell in C_1 , the mean time to reach the blood ($\mathbb{E}(T_{C_1 \rightarrow B}) = \xi_{C_1,B}^{-1}$) can be computed from the following analysis

$$\begin{aligned} \xi_{C_1,B}^{-1} &= \mathbb{E}(T_{C_1 \rightarrow B} \mid \text{cell is at } C_{1a}) \mathbb{P}(\text{cell is at } C_{1a}) \\ &\quad + \mathbb{E}(T_{C_1 \rightarrow B} \mid \text{cell is at } C_{1b}) \mathbb{P}(\text{cell is at } C_{1b}). \end{aligned}$$

Finally, Equation (8) can be derived by noting that

$$\begin{aligned} \mathbb{P}(\text{cell is at } C_{1a}) &= \frac{\xi_{B,C_{1a}}}{\xi_{B,C_{1a}} + \xi_{B,C_{1b}}}, \\ \mathbb{P}(\text{cell is at } C_{1b}) &= \frac{\xi_{B,C_{1b}}}{\xi_{B,C_{1a}} + \xi_{B,C_{1b}}}, \\ \mathbb{E}(T_{C_1 \rightarrow B} \mid \text{cell is at } C_{1a}) &= \xi_{C_{1a},C_{1b}}^{-1} + \xi_{C_{1b},B}^{-1}, \\ \mathbb{E}(T_{C_1 \rightarrow B} \mid \text{cell is at } C_{1b}) &= \xi_{C_{1b},B}^{-1}. \end{aligned}$$



3. NUMERICAL RESULTS

In this section we carry out a numerical study to illustrate our analytical results and the fast-migration approximation, to compare our analytical results with those obtained from stochastic numerical simulations, and to show how dynamics occurring in a particular compartment can have a significant impact on the whole system. We propose in section 3.1 parameter values for the process described by **Figure 1**, based on those considered in den Braber et al. (1) and Ganusov and Auerbach (3). In section 3.2, we compare analytical and numerical results with those obtained from our fast-migration approximation. We analyse in section 3.3 the role played by the asymmetry in the death rates of the different spatial compartments. We focus in section 3.4 on the potential impact of (not blood-mediated) migration between compartments, inspired by the model considered in Ganusov and Auerbach (3).

3.1. Parameters

In **Table 1**, we provide baseline parameter values obtained from den Braber et al. (1) and Ganusov and Auerbach (3), for the model described in **Figure 1** with $M = 5$. These spatial compartments represent, according to Ganusov and Auerbach (3), the blood (B), mesenteric lymph nodes and Peyer's patches (C_1), lung (C_2), liver (C_3), spleen (C_4) and subcutaneous lymph nodes (C_5). In order to show the goodness of the fast-migration approximation, and to show the impact of spatial asymmetry in this system, we vary in section 3.2, section 3.3 and section 3.4 the division and death rates in the different spatial compartments, so that the values μ and λ in **Table 1** should be considered baseline parameters for $CD4^+$ T cells according to den Braber et al. (1).

We also note that the model in Ganusov and Auerbach (3) considers Peyer's patches and mesenteric lymph nodes as two different compartments (i.e., C_{1a} and C_{1b} , respectively, described

TABLE 1 | Parameter values considered in the numerical study. Blood and $M = 5$ additional spatial compartments as in Ganusov and Auerbach (3, Figure 2).

Parameter	References	Value (min^{-1})
$\xi_{B,C_{1a}}$	(3)	0.53×10^{-2}
$\xi_{B,C_{1b}}$	(3)	1.06×10^{-2}
$\xi_{C_{1b},B}$	(3)	0.34×10^{-2}
$\xi_{C_{1a},C_{1b}}$	(3)	0.34×10^{-2}
ξ_{B,C_1}	Equation (7)*	1.59×10^{-2}
$\xi_{C_1,B}$	Equation (8)*	2.60×10^{-3}
ξ_{B,C_2}	(3)	1.83
$\xi_{C_2,B}$	(3)	2.17
ξ_{B,C_3}	(3)	0.41
$\xi_{C_3,B}$	(3)	1.14
ξ_{B,C_4}	(3)	5.6×10^{-2}
$\xi_{C_4,B}$	(3)	7.0×10^{-3}
ξ_{B,C_5}	(3)	2.6×10^{-2}
$\xi_{C_5,B}$	(3)	3.4×10^{-3}
μ	(1)	$1.478 \times 10^{-5} (\sim 1/47 \text{ days}^{-1})$
λ	(1)	$1.458 \times 10^{-5} (\sim 1/48 \text{ days}^{-1})$

C_1 : Peyer's patches (C_{1a}) and mesenteric lymph nodes (C_{1b}); C_2 : lungs; C_3 : liver; C_4 : spleen; and C_5 : subcutaneous lymph nodes. Time units: min^{-1} . (*) We have estimated these parameters combining the parameters in Ganusov and Auerbach (3) according to Equations (7)–(8).

in section 2.4). We propose in section 3.2 and section 3.3 to merge these compartments and analyse the cellular dynamics without deciphering where exactly a given cell in C_1 is (i.e., if the cell is in the Peyer's patches or in the mesenteric lymph nodes), by using Equations (7)–(8) to obtain *effective* migration rates ($\xi_{B,C_1}, \xi_{C_1,B}$) in **Table 1**. In section 3.4 we carry out a numerical study of our second model, described in **Figure 3**, where the migration rates ($\xi_{B,C_{1a}}, \xi_{B,C_{1b}}, \xi_{C_{1b},B}, \xi_{C_{1a},C_{1b}}$) are those in Ganusov and Auerbach (3). Finally, we note that for all the parameter values considered in this section, Equations (1)–(2) are satisfied.

3.2. Gillespie Simulations, Analytic Results, and Fast-Migration Approximation

In this section we set the migration rates $\{(\xi_{B,C_i}, \xi_{C_i,B}), i \in \{1, \dots, 5\}\}$ as given in **Table 1**. In order to show the goodness of the fast-migration approximation we set

$$\begin{aligned}\mu_B &= R\mu, & \mu_{C_i} &= R\mu, & i \in \{1, \dots, 5\}, \\ \lambda_B &= \lambda, & \lambda_{C_i} &= R\lambda, & i \in \{1, \dots, 5\},\end{aligned}$$

for varying values of $R > 0$. We note that in the rest of the paper, but this section, we set $R = 1$. $R = 1$ corresponds to a completely symmetric scenario, where all compartments have death rate μ and division rate λ . For increasing values of R , the scenario becomes asymmetric, where division in the blood is less likely to occur compared to division in other compartments, while death rates are still the same in all spatial compartments. However, division and death rates for large values of R become comparable (similar order of magnitude) to migration rates.

In **Figure 4**, we plot (starting with a single cell in the blood) *a*) the mean number of division events in the blood and *b*) the mean number of division events in compartments $\{B, C_1, C_2\}$, as a function of $\log(R)$. The fast-migration (FM) approximation mostly provides reliable results when $\log(R) < 1.0$. In this case, results obtained by simulations agree with the analytic results (obtained as detailed in section 2.2), and with the fast-migration approximation (computed as explained in section 2.3). We note that values $\log(R) < 1.0$ correspond to division and apoptotic rates of the order of $10^{-5} - 10^{-4} \text{ min}^{-1}$, and migration rates are of the order of $10^{-3} - 10^0 \text{ min}^{-1}$. For $\log(R) > 1.0$, division and death rates become comparable to some of the migration rates, and the fast-migration approximation provides results in **Figure 4** which significantly differ from those obtained by numerical simulations and analytic methods. We also note that even for small values of $\log(R)$, other variables of interest cannot be well captured by the fast-migration approximation. This is the case for $\hat{m}_B(B) = \mathbb{E}(N_B(B))$, the mean number of division events occurring in the blood for a cell starting in the blood. While the fast-migration approximation provides reliable results for $\log(R) = 0$, once $\log(R) > 0$ the true (analytic) value of $\hat{m}_B(B)$ fastly decays to zero. This behavior is captured by the stochastic simulations in **Figure 4B**, but not by the fast-migration approximation.

In **Figure 5**, we plot *a*) the probability of the original cell dying in compartments $\{B, C_1, C_2\}$, and *b*) the mean total number of cells in the genealogy of the original cell, as a function of $\log(R)$ and for a cell starting in the blood. Similar comments to the ones above apply to the results in **Figure 5A**, where the fast-migration approximation behaves well for $\log(R) < 1.0$. We also note that for small values of R we get $\beta_B(C_1) > \beta_B(B) > \beta_B(C_2)$ for the cell starting in the blood, which shows the importance of the migration dynamics in the fate of a given cell. However, the probability of the cell dying in the blood increases with increasing values of R as one would expect, since if division and death rates increase, the starting position of the cell has a larger impact on its proliferation and death dynamics and the impact of migration rates accordingly decreases. It is also interesting to note that the fast-migration approximation provides reliable results for all the

values of R explored in **Figure 5B**. In this case, we study the mean number of cells in the genealogy, which is a population-based descriptor rather than a descriptor only related to a given cell. This result is striking, since division and death rates for $\log(R) > 2.0$ are of the order of $10^{-3} - 10^{-2} \text{ min}^{-1}$, which are comparable to the migration rates in **Table 1**. This might indicate that the fast-migration approximation could behave better when dealing with population-based summary statistics, while it is reliable when analysing single cell descriptors only for small enough values of the division and death rates (compared to migration rates).

It is also worth noting that simulating stochastic processes with different timescales is a challenging problem from a computational point of view, which in our case means simulating the migration of cells (timescales of the order of minutes), until some division or death events occur (in days). This is even more challenging when dealing with a population of cells (see **Figure 5B**) rather than a single cell (**Figures 4, 5A**). For these computational reasons, 10^3 stochastic simulations were used to compute the values in **Figure 5B**, compared to 10^4 simulations for **Figures 4, 5A**. Thus, our results in **Figure 5B** illustrate the need for developing the analytical results of section 2.2, or related approximations, such as the one in section 2.3, instead of using standard stochastic simulations to analyse cellular dynamics in these systems.

3.3. The Role of Different Death Rates in Different Compartments

We have assumed that death rates are the same in all compartments. However, taking into account that migration rates determine the relative weight of each compartment in the overall behavior of the system, in this section we analyse the role of different death rates in compartments C_1 (where migration from blood is around six times faster than the reverse) and C_3 (where migration from blood is around one third slower than the reverse) (3).

Figure 6A shows the impact of varying the death rate μ_{C_1} in compartment C_1 (with respect to the one in **Table 1** $\mu_{C_1} = \mu$, shown as a vertical solid line). It is interesting to note how compartment C_4 is one of the most sensitive to this parameter in spite of the fact that we are only changing the death rate of compartment C_1 . The rationale behind this result is related to the relative *immigration* to *emigration* rates in each compartment. In particular, using the migration rates of **Table 1** we find $K_1 = 6.115, K_2 = 0.843, K_3 = 0.360, K_4 = 8.000, K_5 = 7.647$ (namely, immigration in C_1 is 6.115 times larger than emigration). The three compartments with higher immigration/emigration ratios are C_1, C_4 and C_5 . Thus, when μ_{C_1} increases, the weight of the dynamics is shifted to the compartment with the highest such ratio. On the other hand, in **Figure 6B**, as compartment C_3 has low $K_3 = 0.360$, the most sensitive probability of death with respect to parameter μ_{C_3} is $\beta_B(C_3)$.

Similarly, **Figure 7A** shows the effect of changing μ_{C_1} on the mean lifetime of a cell. Again, the role of K_i is very relevant. While in both cases (changing μ_{C_1} or μ_{C_3}) the mean lifetime decreases (as expected), the effect is milder in the case of μ_{C_3} . This is related to the fact that, due to the reduced immigration rate into

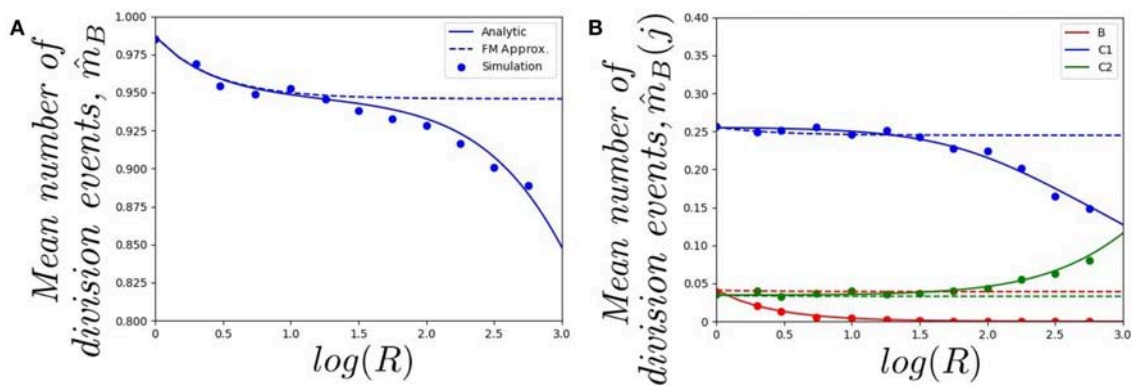


FIGURE 4 | Effect of changing the value of R on (A) the mean total number of division events $\hat{m}_B$, and (B) the mean number of division events occurring in compartment j , $\hat{m}_B(j)$, for $j \in \{B, C_1, C_2\}$. We consider a single cell starting in the blood. Analytic solution (solid), fast-migration approximation (dashed) and 10^4 stochastic simulations (dots).

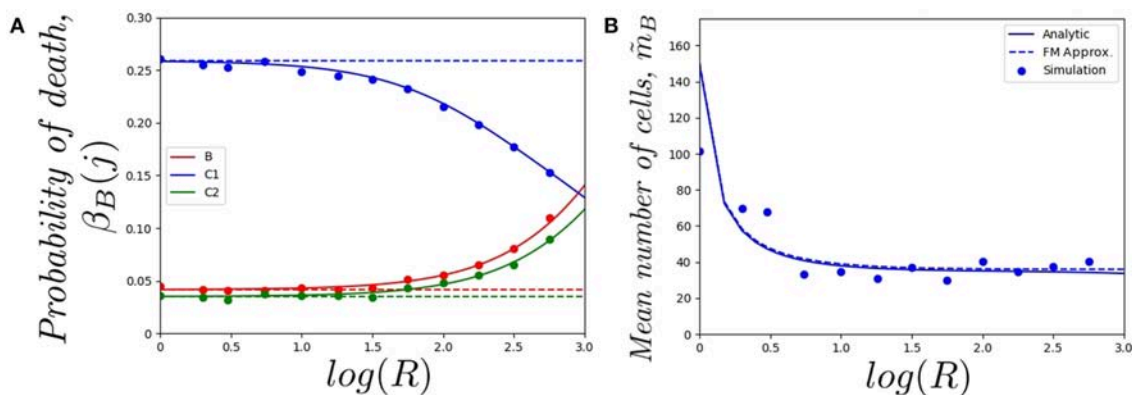


FIGURE 5 | Effect of changing the value of R on (A) the probability of the cell dying in compartment j , for $j \in \{B, C_1, C_2\}$, and (B) the mean total number of cells in the genealogy of the original cell, $\hat{m}_B = E(G_B)$. We consider a single cell starting in the blood. Analytic result (solid), fast-migration approximation (dashed) and 10^4 , for (A), and 10^3 , for (B), stochastic simulations (dots).

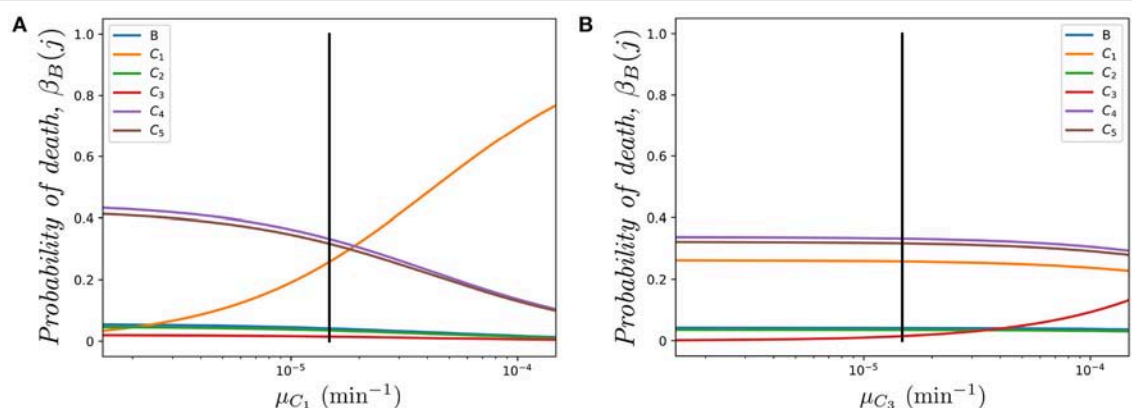


FIGURE 6 | Effect of varying μ_{C_i} on the probability $\beta_B(j)$ of dying in different compartments $j \in \{B, C_1, \dots, C_5\}$, for a cell starting at blood. (A) Varying μ_{C_1} ; (B) Varying μ_{C_3} . The vertical black line represents the value μ .

compartment C_3 , the odds of finding the cell in that compartment are relatively low. On the contrary, as the cell spends more time in compartment C_1 , varying the death rate in that compartment increases/decreases quickly the mean lifetime when the death rate decreases/increases, respectively.

Finally, in **Figure 7B** we show the mean number of cells produced by a single cell during its lifetime (the size of the offspring tree). Again, in the case of compartment C_1 , reducing slightly the death rate produces a transition from finite to infinite number of descendants, which is directly related to the conditions given by Equations (1)–(2), and relates to the asymptotic behavior observed in **Figure 7B**. In this case, small

where the rows stand for $f_B, f_{C_1}, \dots, f_{C_5}$ and the columns for $\xi_{B,C_1}, \xi_{C_1,B}, \dots, \xi_{C_5,B}$. That is, matrix $\mathbf{S}$ is defined so that

$$\mathbf{S}_{12} = \frac{\partial f_B}{\partial \xi_{C_1,B}} \quad \mathbf{S}_{21} = \frac{\partial f_{C_1}}{\partial \xi_{B,C_1}}, \dots$$

While this matrix is informative, it does not reflect a property of Equation (3); namely, that the relevant quantities are not migration rates themselves but, rather, the *immigration/emigration* ratio for each spatial compartment. Thus, one can obtain a simpler version of the sensitivity matrix with respect to the ratios K_i , $i \in \{1, \dots, 5\}$:

$$\tilde{\mathbf{S}} = \begin{pmatrix} -1.7 \times 10^{-3} & -1.7 \times 10^{-3} & -1.7 \times 10^{-3} & -1.7 \times 10^{-3} & -1.7 \times 10^{-3} \\ +3.1 \times 10^{-2} & -1.1 \times 10^{-2} & -1.1 \times 10^{-2} & -1.1 \times 10^{-2} & -1.1 \times 10^{-2} \\ -1.5 \times 10^{-3} & +4.0 \times 10^{-2} & -1.5 \times 10^{-3} & -1.5 \times 10^{-3} & -1.5 \times 10^{-3} \\ -6.3 \times 10^{-4} & -6.3 \times 10^{-4} & +4.1 \times 10^{-2} & -6.3 \times 10^{-4} & -6.3 \times 10^{-4} \\ -1.4 \times 10^{-2} & -1.4 \times 10^{-2} & -1.4 \times 10^{-2} & +2.8 \times 10^{-2} & -1.4 \times 10^{-2} \\ -1.3 \times 10^{-2} & -1.3 \times 10^{-2} & -1.3 \times 10^{-2} & -1.3 \times 10^{-2} & +2.8 \times 10^{-2} \end{pmatrix},$$

changes in the parameters might have a huge impact on the cell population dynamics.

Overall, these analyses show that, not only migration rates, but the ratio between immigration and emigration rates affect the overall dynamics of the system. Thus, analysing these ratios, and their interplay with the apoptotic and proliferation rates, can help to identify the most relevant locations of the immune system related to the fate of a single cell.

3.4. The Role of Direct Migration Between Compartments

To test the relevance of migration between compartments (as emphasized in Ganusov and Auerbach (3)) we compute the fractions, $f_B, f_{C_1}, \dots, f_{C_5}$ using the parameters from **Table 1**. We have

$$f_B = 0.042, \quad f_{C_1} = 0.255, \quad f_{C_2} = 0.035, \quad f_{C_3} = 0.015, \\ f_{C_4} = 0.334, \quad f_{C_5} = 0.319,$$

where, as we defined above, f_j represents the fraction of time that the cell under study spends in each spatial compartment $j \in \{B, C_1, \dots, C_M\}$, in the absence of division and death. Furthermore, in **Figure 8** we show the dependence of these fractions on the rate connecting compartments C_{1a} and C_{1b} , $\xi_{C_{1a},C_{1b}}$. Clearly, as $\xi_{C_{1a},C_{1b}} \rightarrow 0$, compartment C_{1a} becomes a *sink* of cells so that $f_{C_{1a}} \rightarrow 1$ and the rest tend to 0.

3.5. Sensitivity Analysis

For the original model in **Figure 1**, we can use Equation (3) to compute the sensitivity matrix $\mathbf{S}$. Using the parameters in **Table 1**, we find that the matrix $\mathbf{S}$ is given by

$$\begin{pmatrix} -6.7 \cdot 10^{-1} & 4.1 & -8 \cdot 10^{-4} & 6.8 \cdot 10^{-4} & -1.5 \cdot 10^{-3} & 5.5 \cdot 10^{-4} & -2.5 \cdot 10^{-1} & 2.0 & -5.1 \cdot 10^{-1} & 3.9 \\ 1.2 \cdot 10^1 & -7.3 \cdot 10^1 & -4.9 \cdot 10^{-3} & 4.1 \cdot 10^{-3} & -9.3 \cdot 10^{-3} & 3.4 \cdot 10^{-3} & -1.5 & 1.2 \cdot 10^1 & -3.1 & 2.4 \cdot 10^1 \\ -5.6 \cdot 10^{-1} & 3.5 & 1.9 \cdot 10^{-2} & -1.6 \cdot 10^{-2} & -1.3 \cdot 10^{-3} & 4.6 \cdot 10^{-4} & -2.1 \cdot 10^{-1} & 1.7 & -4.3 \cdot 10^{-1} & 3.3 \\ -2.4 \cdot 10^{-1} & 1.5 & -2.9 \cdot 10^{-4} & 2.4 \cdot 10^{-4} & 3.6 \cdot 10^{-2} & -1.3 \cdot 10^{-2} & -8.9 \cdot 10^{-2} & 7.2 \cdot 10^{-1} & -1.8 \cdot 10^{-1} & 1.4 \\ -5.4 & 3.3 \cdot 10^1 & -6.4 \cdot 10^{-3} & 5.4 \cdot 10^{-3} & -1.2 \cdot 10^{-2} & 4.4 \cdot 10^{-3} & 4.0 & -3.2 \cdot 10^1 & -4.1 & 3.1 \cdot 10^1 \\ -5.1 & 3.1 \cdot 10^1 & -6.1 \cdot 10^{-3} & 5.2 \cdot 10^{-3} & -1.2 \cdot 10^{-2} & 4.2 \cdot 10^{-3} & -1.9 & 1.5 \cdot 10^1 & 8.4 & -6.4 \cdot 10^1 \end{pmatrix},$$

where each row corresponds to a given fraction, f_j , and with the columns representing $(K_1, K_2, K_3, K_4, K_5)$, where $K_1 = 6.115$, $K_2 = 0.843$, $K_3 = 0.360$, $K_4 = 8.000$, $K_5 = 7.647$, so that

$$\tilde{\mathbf{S}}_{ij} = \begin{cases} \frac{\partial f_j}{\partial K_i} & \text{if } i = j, \\ \frac{\partial f_{C_{i-1}}}{\partial K_j} & \text{if } i \neq j. \end{cases}$$

Due to some symmetries in Equation (3) with respect to K_i , the reduced sensitivity matrix has many repeated entries. Biologically, this is a remarkable result as it shows that events occurring in different compartments can affect equally a given one. Another conclusion can be derived from the sign of the elements of $\tilde{\mathbf{S}}$. These signs can be easily understood by noting that, since K_i represents the fraction of *immigration* to *emigration* events in a given compartment, the higher K_i the higher the probability of finding a cell in compartment C_i .

4. DISCUSSION AND CONCLUSIONS

We propose a mathematical model for the migration, proliferation and death of a CD4⁺ T cell, and focus on a number of observables which refer to the single cell journey during its lifetime, as well as to the dynamics of its progeny. We have presented analytical methods to study these observables and have provided conditions for this cellular system not to explode. A fast-migration approximation can be proposed when migration events occur significantly faster than division and death, so that steady state conditions can be assumed for the spatial location of the cell.

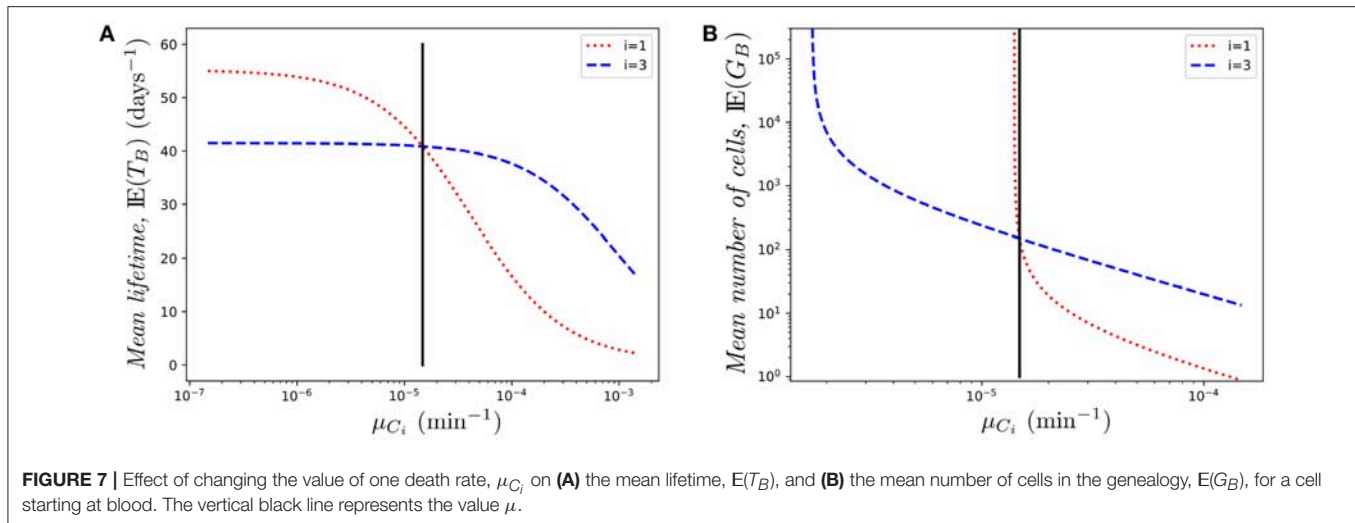


FIGURE 7 | Effect of changing the value of one death rate, μ_{C_i} on (A) the mean lifetime, $E(T_B)$, and (B) the mean number of cells in the genealogy, $E(G_B)$, for a cell starting at blood. The vertical black line represents the value μ .

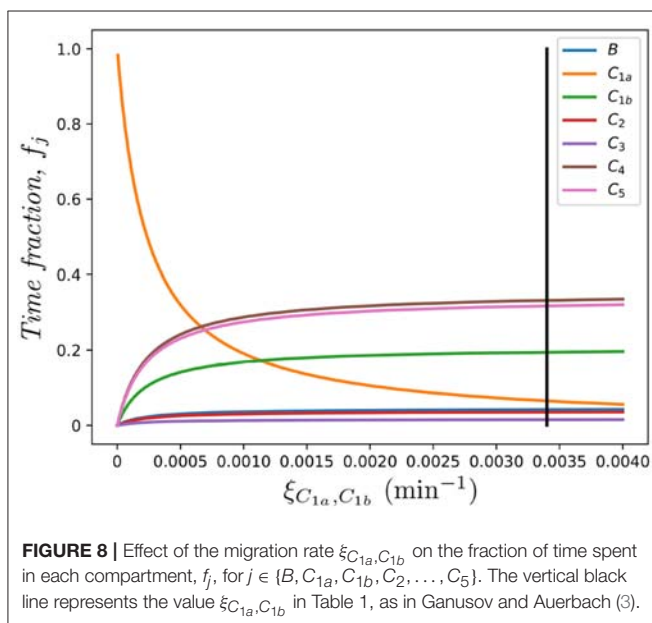


FIGURE 8 | Effect of the migration rate $\xi_{C_{1a}, C_{1b}}$ on the fraction of time spent in each compartment, f_j , for $j \in \{B, C_{1a}, C_{1b}, C_2, \dots, C_5\}$. The vertical black line represents the value $\xi_{C_{1a}, C_{1b}}$ in Table 1, as in Ganusov and Auerbach (3).

Our numerical results show that most of the stochastic observables under study can be properly captured by means of the fast-migration approximation, when migration rates are (at least) one order of magnitude larger than division and death rates. The fast-migration approximation is able to appropriately capture the mean number of cells in the genealogy of a given cell, even when migration events occur at a similar rate to those of division and death events. It is also worth mentioning that our numerical results illustrate how perturbing a single rate in a given spatial compartment can have a significant impact on the cellular dynamics and observables corresponding to other compartments, which indicates the clear interplay between cellular dynamics in the different spatial compartments, and which depends on the specific migration structure (and rates) of the system. Finally, a particular feature of our analytical approach is that it allows for an exact sensitivity analysis of each of the observables with respect to each kinetic rate. In this way the

contribution that each particular rate (i.e., event) has on a given stochastic observable can be assessed.

Recent experimental advances allow us to observe biological processes at the single cell level, but at the same time these new experimental techniques are still far from being able to provide a full picture of migration, division and death events *in vivo*. Thus, experimental observations need to be combined with mathematical and computational models which allow one to test hypotheses, shed some light on cellular dynamics or to design new or different experiments. As a consequence, two main challenges that directly arise are: (i) to develop new analytical techniques to study different observables in these cellular systems, which can be compared to experimental measurements, and (ii) to propose new and more advanced methodologies for calibrating these mathematical models by using experimental data. Although our focus in this work is on (i), our results can have a direct impact on (ii), since the distributions and mean values computed in section 2 could be used to calculate the likelihood function when applying Bayesian statistical methods for parameter estimation and model calibration.

Finally, it is worth noting that, unlike population dynamics models (based on collective counts of cells), our stochastic descriptors have two clear advantages. First of all, they represent variables directly related to the dynamics of a single cell, and thus, allow us to bridge between the novel experimental techniques described in section 2.2 and specific proliferation, death or migration rates at the cell level. Secondly, our descriptors allow us to quantify these individual rates. For instance, in practice only the net division rate can be measured but, combining descriptors, we can separate birth from death or migration rates. Naturally, these estimates require the use of both more sophisticated experiments and parameter estimation techniques (such as the Bayesian methods mentioned above).

AUTHOR CONTRIBUTIONS

ML-G, MC, and GL designed and analyzed the summary statistics and the fast-migration approximation. ML-G, MC, NA, and LdlH wrote the numerical codes and performed the numerical

simulations. ML-G, MC, LdlH, GL, and CM-P contributed to writing the manuscript. All authors contributed to the development of the mathematical model and reviewing the literature and to revising the manuscript.

FUNDING

This work has been supported by Arthritis Research UK A25929 and Cancer Research UK C62155 (CM-P). This work has been also partially supported by grant FIS2016-78883-C2-2-P (Ministerio de Economía, Industria y Competitividad - Agencia Estatal de Investigación) and PRX 16/00287

(MC), PIRSES-GA-2012-317893 (MC, ML-G, GL and CM-P), PITN-GA-2012-317040 (LdlH, GL, and CM-P), the European Union H2020 Programme under agreement 764698 MSCA-ITN-2017, QuantII ETN (GL and CM-P), and the Spanish Ministry of Economy and Competitiveness MTM2014-58091-P (ML-G).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00194/full#supplementary-material>

REFERENCES

- den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mögling R, de Boer AB, et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity*. (2012) 36:288–97. doi: 10.1016/j.immuni.2012.02.006
- Thome JJ, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawa T, et al. Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell*. (2014) 159:814–28. doi: 10.1016/j.cell.2014.10.026
- Ganusov VV, Auerbach J. Mathematical modeling reveals kinetics of lymphocyte recirculation in the whole organism. *PLoS Comput Biol*. (2014) 10:e1003586. doi: 10.1371/journal.pcbi.1003586
- Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem*. (2002) 48:1819–27. Available online at: <http://clinchem.aaccjnl.org/content/clinchem/48/10/1819.full.pdf>
- Johnson J, Nowicki MO, Lee CH, Chiocia EA, Viapiano MS, Lawler SE, et al. Quantitative analysis of complex glioma cell migration on electrospun polycaprolactone using time-lapse microscopy. *Tiss Eng C Methods*. (2009) 15:531–40. doi: 10.1089/ten.tec.2008.0486
- Westera L, Drylewicz J, Den Braber I, Mugwagwa T, Van Der Maas I, Kwast L, et al. Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans. *Blood*. (2013) 122:2205–12. doi: 10.1182/blood-2013-03-488411
- Gosling JP, Krishnan SM, Lythe G, Chain B, MacKay C, Molina-París C. A mathematical study of CD8⁺ T cell responses calibrated with human data. *arXiv [preprint]*. arXiv:180205094. (2018).
- Seddon B, Yates AJ. The natural history of naive T cells from birth to maturity. *Immunol Rev*. (2018) 285:218–32. doi: 10.1111/imr.12694
- Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol Tod*. (1998) 19:395–404. doi: 10.1016/S0167-5699(98)01299-7
- Jenkins MK, Chu HH, McLachlan JB, Moon JJ. On the composition of the preimmune repertoire of T cells specific for peptide-major histocompatibility complex ligands. *Ann Rev Immunol*. (2010) 28:275–94. doi: 10.1146/annurev-immunol-030409-101253
- Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. *Nature Immunol*. (2003) 4:733–9. doi: 10.1038/ni957
- Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. (1999) 402:34–8. doi: 10.1038/35005534
- Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. *Nat Rev Immunol*. (2013) 13:309–20. doi: 10.1038/nri3442
- Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol*. (2014) 14:24. doi: 10.1038/nri3567
- Kumar BV, Connors TJ, Farber DL. Human T cell development, localization, and function throughout life. *Immunity*. (2018) 48:202–13. doi: 10.1016/j.immuni.2018.01.007
- Davis MM, Brodin P. Rebooting human immunology. *Ann Rev Immunol*. (2018) 36:843–64. doi: 10.1146/annurev-immunol-042617-053206
- Blum KS, Pabst R. Lymphocyte numbers and subsets in the human blood: do they mirror the situation in all organs? *Immunol Lett*. (2007) 108:45–51. doi: 10.1016/j.imlet.2006.10.009
- Ganusov VV, De Boer RJ. Do most lymphocytes in humans really reside in the gut? *Trends Immunol*. (2007) 28:514–8. doi: 10.1016/j.it.2007.08.009
- Hogan T, Gossel G, Yates AJ, Seddon B. Temporal fate mapping reveals age-linked heterogeneity in naive T lymphocytes in mice. *Proc Natl Acad Sci USA*. (2015) 112:E6917–26. doi: 10.1073/pnas.1517246112
- Gonçalves P, Ferrarini M, Molina-París C, Lythe G, Vasseur F, Lim A, et al. A new mechanism shapes the naive CD8⁺ T cell repertoire: the selection for full diversity. *Mol Immunol*. (2017) 85:66–80. doi: 10.1016/j.molimm.2017.01.026
- Baliu-Piqué M, Kurniawan H, Ravesloot L, Verheij M, Drylewicz J, Lievaert-Peterson K, et al. Age-related distribution and dynamics of T cells in blood and lymphoid tissues of goats. *Dev Comp Immunol*. (2018) 93:1–10. doi: 10.1016/j.dci.2018.12.004
- Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B, et al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med*. (2002) 8:631. doi: 10.1038/nm0602-631
- Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods*. (2006) 3:361. doi: 10.1038/nmeth872
- Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. *Nat Immunol*. (2007) 8:1295. doi: 10.1038/ni1545
- Westermann J, Söllner S, Ehlers EM, Nohroudi K, Bleszenohl M, Kalies K. Analyzing the migration of labeled T cells *in vivo*: an essential approach with challenging features. *Lab Invest*. (2003) 83:459. doi: 10.1097/01.LAB.0000062852.80567.90
- Pham K, Shimoni R, Charnley M, Ludford-Menting MJ, Hawkins ED, Ramsbottom K, et al. Asymmetric cell division during T cell development controls downstream fate. *J Cell Biol*. (2015) 210:933–50. doi: 10.1083/jcb.201502053
- Sawicka M, Stritesky G, Reynolds J, Abourashchi N, Lythe G, Molina-París C, et al. From pre-DP, post-DP, SP4, and SP8 thymocyte cell counts to a dynamical model of cortical and medullary selection. *Front Immunol*. (2014) 5:19. doi: 10.3389/fimmu.2014.00019
- Artalejo J, Gómez-Corral A, López-García M, Molina-París C. Stochastic descriptors to study the fate and potential of naive T cell clonotypes in the periphery. *J Math Biol*. (2017) 74:673–708. doi: 10.1007/s00285-016-1020-6
- Reynolds J, Coles M, Lythe G, Molina-París C. Deterministic and stochastic naive T cell population dynamics: symmetric and asymmetric cell division. *Dyn Syst*. (2012) 27:75–103. doi: 10.1080/14689367.2011.645447
- Donovan GM, Lythe G. T-cell movement on the reticular network. *J Theor Biol*. (2012) 295:59–67. doi: 10.1016/j.jtbi.2011.11.001
- Currie J, Castro M, Lythe G, Palmer E, Molina-París C. A stochastic T cell response criterion. *J R Soc Interface*. (2012) 9:2856–70. doi: 10.1098/rsif.2012.0205

32. Huth J, Buchholz M, Kraus JM, Mølhave K, Gradinaru C, Wichert Gv, et al. TimeLapseAnalyzer: multi-target analysis for live-cell imaging and time-lapse microscopy. *Comput Methods Progr Biomed.* (2011) 104:227–34. doi: 10.1016/j.cmpb.2011.06.002
33. Klein J, Leupold S, Biegler I, Biedendieck R, Münch R, Jahn D. TLM-Tracker: software for cell segmentation, tracking and lineage analysis in time-lapse microscopy movies. *Bioinformatics.* (2012) 28:2276–7. doi: 10.1093/bioinformatics/bts424
34. Hilsenbeck O, Schwarzfischer M, Skylaki S, Schauburger B, Hoppe PS, Loeffler D, et al. Software tools for single-cell tracking and quantification of cellular and molecular properties. *Nat Biotechnol.* (2016) 34:703. doi: 10.1038/nbt.3626
35. Hawkins E, Markham J, McGuinness L, Hodgkin P. A single-cell pedigree analysis of alternative stochastic lymphocyte fates. *Proc Natl Acad Sci USA.* (2009) 106:13457–62. doi: 10.1073/pnas.0905629106
36. Piltti KM, Cummings BJ, Carta K, Manughian-Peter A, Worne CL, Singh K, et al. Live-cell time-lapse imaging and single-cell tracking of *in vitro* cultured neural stem cells—Tools for analyzing dynamics of cell cycle, migration, and lineage selection. *Methods.* (2018) 133:81–90. doi: 10.1016/j.ymeth.2017.10.003
37. Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature.* (2001) 410:101. doi: 10.1038/35065111
38. Masopust D, Vezys V, Marzo AL, Lefrançois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science.* (2001) 291:2413–7. doi: 10.1126/science.1058867
39. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJ, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity.* (2013) 38:187–97. doi: 10.1016/j.immuni.2012.09.020
40. He QM. *Fundamentals of Matrix-Analytic Methods.* Springer (2014).
41. Matis JH, Kiffe TR. *Stochastic Population Models: A Compartmental Perspective.* Vol. 145. New York, NY: Springer Science & Business Media (2012).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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T-Cell Mechanobiology: Force Sensation, Potentiation, and Translation

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A T cell is a sensitive self-referential mechanical sensor. Mechanical forces influence the recognition, activation, differentiation, and function throughout the lifetime of a T cell. T cells constantly perceive and respond to physical stimuli through their surface receptors, cytoskeleton, and subcellular structures. Surface receptors receive physical cues in the form of forces generated through receptor-ligand binding events, which are dynamically regulated by contact tension, shear stress, and substrate rigidity. The resulting mechanotransduction not only influences T-cell recognition and signaling but also possibly modulates cell metabolism and gene expression. Moreover, forces also dynamically regulate the deformation, organization, and translocation of cytoskeleton and subcellular structures, leading to changes in T-cell mobility, migration, and infiltration. However, the roles and mechanisms of how mechanical forces modulate T-cell recognition, signaling, metabolism, and gene expression, are largely unknown and underappreciated. Here, we review recent technological and scientific advances in T-cell mechanobiology, discuss possible roles and mechanisms of T-cell mechanotransduction, and propose new research directions of this emerging field in health and disease.

Keywords: T cell, force, mechanotransduction, receptor-ligand interaction, T-cell recognition, T-cell activation, T-cell differentiation, metabolism

OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
Imperial College London,
United Kingdom

Reviewed by:

Brian Evavold,
The University of Utah, United States
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Specialty section:

This article was submitted to
Biomedical Physics,
a section of the journal
Frontiers in Physics

Received: 28 October 2018

Accepted: 08 March 2019

Published: 02 April 2019

Citation:

Harrison DL, Fang Y and Huang J
(2019) T-Cell Mechanobiology: Force
Sensation, Potentiation, and
Translation. *Front. Phys.* 7:45.
doi: 10.3389/fphy.2019.00045

INTRODUCTION

T lymphocytes or T cells are white blood cells that play a central role in the adaptive immune system, where the body adapts specificity to foreign antigens. T cells are derived from hematopoietic stem cells and mature in the thymus, thus named T cells. Most T cells in the thymus express $\alpha\beta$ T-cell receptors (TCRs) and approximately 5% bear the $\gamma\delta$ TCRs. $\alpha\beta$ T cells have been extensively studied while $\gamma\delta$ T cells are much less known. This review will focus on $\alpha\beta$ T cells which can be generally divided into two major populations based on their co-receptors and immune functions: CD4⁺ helper T cells and CD8⁺ cytotoxic T cells. CD4⁺ helper T cells orchestrate the full panoply of immune responses by releasing cytokines and chemokines to help other immune cells while CD8⁺ cytotoxic T cells can directly kill pathogen-infected cells [1–3]. Due to the essential role of T cells in the adaptive immune system, they have been extensively studied in immunology. In recent years, the development of T-cell based cancer immunotherapy has shifted the paradigm of cancer treatment. Both checkpoint inhibitors and chimeric antigen receptors (CAR) T cells have been approved by FDA for cancer therapy and showed unprecedented success in clinics [4–9]. Throughout the lifetime and phenotypical trajectory of T cells, they constantly evolve themselves

and execute their immune functions in different biochemical and biomechanical coupled environments. The role of biochemical cues in regulating T cells have been extensively studied in the context of T-cell development, migration, activation, differentiation, and function. However, the role of mechanical force, despite its critical importance, is much less investigated, understood, and appreciated. In this paper, we will review some recent advances in T-cell mechanobiology with focuses on T-cell recognition, signaling, metabolism, and genetics, discuss possible key questions and challenges in T-cell selection, differentiation, metabolism, and fate decision and propose new biomechanical approaches to tackle these important problems.

FORCE IN T-CELL RECOGNITION

T cells patrol the body, using their TCRs to search for foreign antigens presented on antigen-presenting cells (APCs) and trigger antigen-specific immune responses, a process called antigen recognition. T-cell antigen recognition is essential to cell-mediated adaptive immune responses. It has been found that T cells can specifically [10, 11] and sensitively [12–14] detect a small number of antigenic peptide-bound major histocompatibility complexes (pMHCs) in the ocean of endogenous ligands displayed on the surface of professional APCs, including B cells [12–17] and dendritic cells [18]. How T cells achieve this sensitivity to antigenic but not endogenous ligands is still enigmatic, but mechanical forces can critically regulate the entire process of T-cell recognition (**Figure 1**). It has been observed that T cells migrate to the infection sites, deform their shapes, interact with their target APCs, survey their antigens, reorient their cell organelles, and release cytokines or cytotoxins to mediate the antigen-specific adaptive immune responses. All these steps require and involve mechanical forces, which are largely understudied and/or under appreciated. T-cell recognition is the first and essential step for initiating T-cell signaling and activation. Here we review the challenges in fully understanding T-cell recognition, examine current knowledge in T-cell recognition, and discuss how mechanical forces regulate T-cell recognition at the molecular and cellular levels.

Challenges in T-Cell Recognition

There is considerable controversy about the molecular mechanism of antigen recognition by T cells, which is a complex process by which TCRs bind to pMHCs displayed on APCs' surface, propagate surface binding across the plasma membrane, trigger T-cell signaling, and ultimately influence immune function. Mechanical forces are involved throughout each step of the recognition process, including cell trafficking, antigen survey, cell adhesion, cytoskeleton reorientation, transmembrane signaling, and cytokine release or cell killing. The spatial scale ranges from a single molecule (~ 10 nm) to a single cell (~ 10 μ m), and the temporal scale spans from molecular interactions ($\sim$ ms) to cellular immune responses ($\sim$ hr). T-cell recognition has the following important characteristics: (1) it is very sensitive—TCRs can recognize even a single foreign pMHC in the presence of abundant self-pMHCs, (2) it is very specific—TCRs can discriminate

between closely related amino acids, and (3) it is subjected to substantial mechanical forces. The complexity reflects the uniquely demanding nature of T-cell recognition, which requires the detection of a weak “signal” (very rare foreign pMHCs) in the presence of considerable “noise” (abundant self-pMHCs) at the surface of the cell being surveyed, and precise propagation of such a weak recognition signal across the cell membrane via mechanical force. Many models have been proposed based on the structure, thermodynamics, kinetics, and signaling, but the molecular mechanism of T-cell recognition remains elusive [37–41]. One of the major caveats is that most of these existing models neglect the importance of mechanical forces. In addition, most state-of-the-art mechanical methods utilize artificial or surrogate APCs to present antigens to T cells [11, 19, 42, 43]. It is important to note that future studies should consider using real and physiological relevant APCs such as dendritic cells, B cells and macrophages to study T-cell recognition, as the cellular environment and type of APC could significantly affect the force, signaling, and function of T cells. To fully explain the entire process of T-cell recognition including signal reception, signal transduction, and cellular response, one needs to measure the binding-signaling coupled mechanotransduction of TCRs with enough spatiotemporal resolution under physiological conditions.

The Mechanosensor TCR TCR-CD3 Complex and TCR Diversity

The cell surface TCR-CD3 complex serves as the unique mechanosensor for T-cell recognition and signaling. Structurally, an extracellular TCR $\alpha\beta$ domain is noncovalently associated with a multisubunit CD3 signaling apparatus, consisting of one CD3 $\epsilon\gamma$ heterodimer, one CD3 $\epsilon\delta$ heterodimer, and one CD3 $\zeta\zeta$ homodimer, which collectively form the TCR-CD3 complex. The CD3 $\epsilon\gamma$ and CD3 $\epsilon\delta$ subunits each consist of a single extracellular Ig domain and a single immunoreceptor tyrosine-based activation motif (ITAM), whereas CD3 ζ has a short extracellular domain and three ITAMs [44–47]. It is generally thought that the cell surface TCR binds to the pMHC and the CD3 initiates T-cell signaling through phosphorylation. However, far less is known about how TCRs relay the binding of antigens to initiate the CD3 intracellular signaling. Although mechanical force is generally believed to be one of the critical factors in mediating this signaling propagation process, the molecular details remain unclear. Possible solution requires simultaneous examination of TCR-pMHC binding, TCR-CD3 conformational dynamics, and CD3 phosphorylation with high spatiotemporal resolution.

To combat the enormously diverse types of antigens in infections and cancer, the human body needs to generate enough TCR clonotypes with high antigen specificity. Genetically, the diversity of the TCR repertoire is generated by V(D)J recombination in the thymus in a nearly random fashion that rearranges of variable (V), joining (J), and in some cases, diversity (D) gene segments [37]. After recombination, random insertion, deletion, and substitution, a small set of TCR genes can theoretically generate 10^{15} to 10^{20} different TCR clonotypes [48]. Although the actual diversity of a person's TCR repertoire

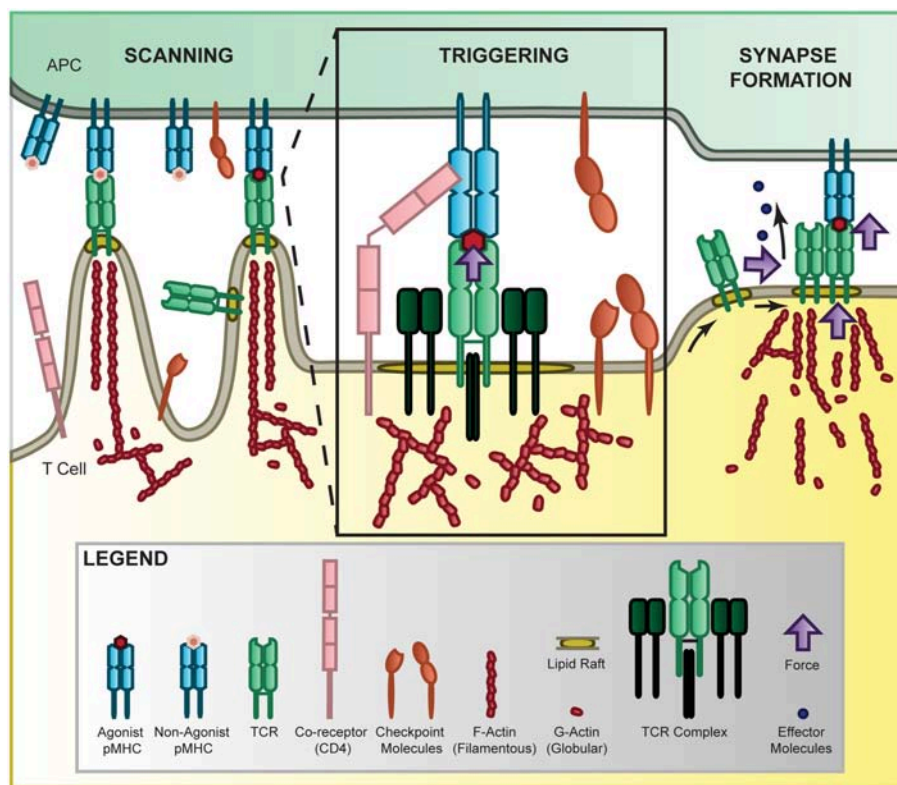


FIGURE 1 | Force in T-cell recognition. T cells scan APCs where they are able to sensitively discriminate activation cues from a large pool of non-activation cues by the TCR binding to MHC presenting self or non-self ligands [10–14]. The 2D agonist pMHC/TCR interaction is further mediated by co-receptor binding [19–22], checkpoint inhibition [23–25], and force through a catch-bond mechanism [11, 26–28]. Once sufficiently triggered, the TCR complexes accumulate together, sliding along the membrane in lipid rafts where they form microclusters and eventually the immunological synapse to localize internal signaling and release of effector molecules [14, 29–33]. This process is mediated by force through the cytoskeleton [34] where polymerizing actin provides tension in the microvilli that scan APC surfaces [35], at the membrane to stabilize the receptor and facilitate receptor sliding [36], and at the synapse to promote adhesion and release of effector molecules.

is much less [49] and difficult to accurately estimate [48], this process results in amino acid sequences in the antigen-binding regions of TCRs that allow for the recognition of antigens from nearly all foreign pathogens as well as mutated self-antigens as seen in cancer [50]. Because T cells stimulate both humoral and cellular immune responses, it is crucially important that T cells only react with foreign antigens while ignoring the large excess of self-peptides. It remains enigmatic how T cells maintain such a diverse TCR repertoire with high specificity to foreign antigens. TCR-pMHC binding kinetics and affinity have been used to explain the molecular mechanism of TCR antigen recognition. So far, most studies measured the kinetic parameters using purified TCRs and pMHCs in solution in which both proteins can diffuse three dimensionally, and therefore termed as 3D binding kinetics and affinity [38]. However, in the physiological conditions, both TCRs and pMHCs are anchored on the live cell membrane, where their orientation, diffusion, association, and dissociation are occurring at the two-dimensional cellular environment subjected to mechanical forces [51], governing the signaling, activation, function, and survival of T cells.

Two-Dimensional TCR Binding Kinetics

Different from molecular interactions between antibody and antigen or cytokine and its receptor, where at least one molecule is in solution and can freely diffuse in a three-dimensional (3D) environment, the antigen recognition by T cells is mediated by molecular interactions occurring at the two-dimensional (2D) immunological synapse formed between a T cell and an APC [17, 52], depicted in **Figure 1**. Most studies have used surface plasmon resonance, a sensitive technique to measure molecular interactions using purified receptors and ligands in a 3D fluid phase [53–56]. Some other studies used pMHC tetramers to study the binding kinetics [57–59]. Although these *in vitro* 3D measurements nevertheless provide the basic kinetic information of TCR-pMHC interactions, they cannot faithfully reveal the physiological binding properties of TCRs *in situ*, as these measurements removed the molecules from their cellular environment, where they initiate signaling and function. Most 2D TCR-pMHC measurements at cell membrane are significantly different from 3D TCR-pMHC binding in solution. These differences have prompted us to re-examine our current views of TCR recognition.

The kinetic differences between 2D and 3D TCR-pMHC interactions come from both the reaction dimension and the complexity of the cellular microenvironment. Firstly, these two interactions are geometrically different. For 3D interactions, at least one molecule is in solution that can diffuse three dimensionally without the constraints of the cell to encounter its counterpart molecule for binding. While for 2D interactions, both the receptors and ligands are surface-bound and only can diffuse at the cell membrane. In order to have 2D interactions, two cells must first make contact so that the surface receptors and ligands can encounter and interact with each other. The dimensional differences result in different units for binding affinity K_a (molecular concentration, M^{-1} for 3D and molecular density, m^2 for 2D) and on-rate k_{on} for 2D or 3D interactions, although they have the same unit of off-rate k_{off} (time, s^{-1}). The second difference is from the complex cellular environment in which the molecules reside. Surface molecules are often linked to cytoskeleton, sitting on the lipid bilayer of the cell membrane, or associated with intracellular signaling molecules. It has been found that the molecular length, dimension and orientation, cell surface roughness, membrane nanostructure, cytoskeleton polymerization, lipid composition, and molecular/cellular signaling can profoundly impact 2D receptor-ligand binding kinetics [11, 19, 29–31, 60–66]. Several 2D techniques have been used to measure the molecular interactions at live T-cell membranes with pMHCs presented by surrogate APCs or supported planar lipid bilayers. These techniques can be generally classified as mechanical-based and fluorescence-based methods. The pros and cons of some of the 2D techniques have been summarized in a previous review paper [67]. Here we will review some representative papers and summarize their results.

There are several major fluorescence-based 2D kinetic measurements, including a single-molecule fluorescence resonance energy transfer (smFRET) [20], a single-molecule diffusion assay [68], a single-molecule TCR-pMHC-ZAP70 tracking assay [69], and a DNA-based TCR imaging method [70]. Here we will mainly focus on the smFRET assay to highlight the uniqueness and importance of measuring 2D receptor-ligand interactions in the physiological cellular microenvironment [20]. To use smFRET to measure the kinetics of TCR-pMHC binding *in situ*, individual TCRs on the live primary T-cell surface and single pMHCs embedded in the planar lipid bilayer are, respectively, labeled with a pair of FRET donor and acceptor fluorophores. The TCR-pMHC interaction brings the donor and acceptor into close proximity and trigger smFRET. The off-rate is derived by monitoring the duration of smFRET signals during synapse formation after photobleaching correction. The 2D binding affinity is estimated by measuring the concentrations of free TCRs, free pMHCs and TCR-pMHC complexes in the 2D immunological synapse as well as individual TCR microclusters. Compared to 3D measurements, the 2D on-rate and off-rate increased ~ 100 -fold and ~ 10 -fold, respectively. Molecular orientation and clustering of TCRs have been suggested by the authors to be the causes of 2D and 3D kinetic differences. In a following study, Klein used a same FRET system to measure the 2D off-rate of 5C.C7 TCR in bulk [71]. However, his bulk

FRET results ($k_{off} = 0.17 s^{-1}$) are significantly different from those obtained by smFRET ($k_{off} = 0.41\text{--}6.36 s^{-1}$) [20]. Such differences were further revealed by a study from the Groves group, who used a single-molecule tracking 2D assay to measure the dwelling time of 5C.C7 TCR and found a value of 5.2 s ($k_{off} = 0.19 s^{-1}$) [69]. Although it is not clear which factors caused such a discrepancy among different studies, more work need to be done to fully address this issue, which is of critical importance in understanding TCR recognition.

Simultaneously, we used two mechanical methods, the micropipette adhesion frequency assay and the thermal fluctuation assay, to measure the TCR-pMHC interactions at the live T-cell membrane [11]. Both mechanical methods, described in detail later in section Methods to Study Force-Mediated Receptor-Ligand Interactions, used a human red blood cell (RBC) as sensitive force sensor to detect single TCR-pMHC bond with piconewton sensitivity. Unexpectedly, the 2D off-rates are up to 8,300-fold faster than the 3D off-rate. Although we cannot directly compare on-rate and affinity between 2D and 3D measurements because they have different units in this case, we have found that 2D affinities and on-rates of the TCR for a panel of pMHC ligands possess far broader dynamic ranges and they are excellent predictors of T-cell responses [11]. Please note that the kinetics and affinities measured here were in the absence of external force (or zero force). In the following sections, we will discuss how force regulates TCR-pMHC binding kinetics and bond lifetime. Our two biomechanical assays consistently show fast kinetics in the 2D cellular environment. Also, disruption of cytoskeleton and lipid rafts by pharmacological agents dramatically changed the 2D binding kinetics and affinity in our biomechanical assays, highlighting the importance of cellular environment to the TCR-pMHC interaction *in situ*.

There are major differences between fluorescent assays and biomechanical methods [51]. The mechanical methods probe the formation of a small number of bonds within a few seconds of transient cell contacts, while fluorescent assays measure interactions over minutes of continuous cell contacts at the ordered structure of immunological synapse (although the measurements are at the single-molecule level). Another difference is that fluorescent assays measure the TCR-pMHC interactions in the presence of adhesion molecules such as B7 and ICAM-1, while mechanical methods purely measure the TCR-pMHC interaction without other interactions. The presence of other receptor-ligand interactions in an ordered structure could greatly affect TCR ligand binding. Also, directly comparing 2D affinities and on-rates between fluorescent assays and mechanical methods are not established, one of the difficulties being precise measurements of the effective contact areas in both assays.

Force Measurements of TCR/pMHC Interaction

During T-cell recognition, T cells actively migrate to the infection sites, contact with target cells, scan target cell surface antigens, form immunological synapses, and initiate immune responses (Figure 1). T cells are subject to external forces as well as generate their own internal forces. The external forces include shear stress, compression, and tension in the circulation system while internal forces are generated by cell adhesion, membrane tension,

and actomyosin cytoskeleton contraction, depicted in **Figure 1**. Several studies have suggested that the TCR is a mechanosensor [72–74]. As TCR/pMHC interaction is the key molecular event that initiates and governs the T-cell immune response, we need to fully understand how mechanical forces regulate TCR recognition in a physiological setting. It has been shown that mechanical forces trigger T-cell signaling via TCR/pMHC interactions but not other receptor-ligand interactions on the T-cell surface [75]. Also, the forces mediating T cell/dendritic cell interactions are peptide-dependent and match the potencies of the peptide in activating T-cells [18], shown as force controlled calcium signaling and IL-2 secretion. In addition, mechanical forces generated from receptor sliding or movement during clustering as well as from actin flow, drive TCR-dependent recognition and signaling [76, 77]. It has been shown that mechanical force regulates the TCR/pMHC binding kinetics [78] as well as increases the T-cell recognition sensitivity by a magnitude of ~100-fold [79]. Recently, a type of bond that changes its kinetics in response to force, named catch bond, has been identified as an important mechanism for antigen discrimination by the mechanosensor TCR [26, 27, 78], and we will discuss this in details below.

TCR-pMHC Catch Bond

A catch bond is a noncovalent bond whose lifetime increases with tensile force applied to the bond. Catch bond is counterintuitive because bond lifetimes are expected to decrease with force, termed as slip bond [80]. Catch bond was initially proposed by Dembo et al. [81] and was first decisively observed in selectin-ligand interactions by Zhu and colleagues in 2003 [82]. Since then, catch bond have been found in many other receptor-ligand interactions [83–88]. Recently, Zhu and colleagues further found that TCR-pMHC interaction forms catch bond [26, 78]. Using the biomembrane force probe (BFP, described in section Methods to Study Force-Mediated Receptor-Ligand Interactions) and CD8⁺ OT-I transgenic TCR system, Zhu and colleagues found that force regulates bond lifetimes and activation levels in a peptide-specific manner. Importantly, catch bond was found for agonists while slip bond was found for antagonists in both CD4⁺ and CD8⁺ T-cell systems. The peak lifetimes of catch bond and slip bond are at ~10 pN and 0 pN, respectively. This provides an explanation for the puzzling negative correlation between peptide potencies and off-rates at zero force, because it is hard to directly explain the observation that the off-rate of an agonist is faster than those of weak ligands [11, 78]. When the force reaches ~10 pN, the negative correlation is reversed to positive correlation by the catch bond mechanism, shown as that the agonist has the longest lifetime (slowest off-rate) compared to other weak ligands [26]. The catch bond mechanism reconciles the appearing contradiction of TCR dwelling time [11] and the kinetic proofreading model [28, 89–91]. In addition, it has been found that both the magnitude and duration, i.e., the accumulation of force are important for the activation of the T-cells. In summary, force reinforces antigen discrimination between closely related peptides through this catch-slip mechanism. Mechanistically, the fast off-rate at zero force allows fast scanning of antigens by TCRs. Following binding, forces generated from the cellular

environment drive the formation of catch bonds to stabilize the TCR-pMHC interaction of agonist but not the weak ligands, thereby selectively prolonging the cumulative bond lifetime of agonist ligands to trigger antigen-specific activation of T-cells.

The TCR-pMHC catch bond was also found using optical tweezers, a method described later in section Methods to Study Force-Mediated Receptor-Ligand Interactions [27]. This work shows that TCR is a mechanosensor that can be activated by force upon pMHC ligation. The authors discovered a catch-and-release TCR structural conversion correlating with ligand potency wherein a strongly binding/compact state transitions to a weakly binding/extended state. They proposed an allosteric mechanism that the C β FG loop region allosterically controls the V domain module's catch bond lifetime and peptide discrimination via force-driven conformational transitions.

Catch bond also can be used to examine whether a TCR-pMHC interaction is productive. We previously found the 2D TCR-pMHC binding affinity correlates with TCR activation using a panel of pMHC ligands [11]. However, Garcia et al. found that high frequency of human TCRs are refractory to activation by pMHC ligands with high binding affinity [28]. Analysis of 3D affinity, 2D dwell time, and crystal structures of stimulatory vs. non-stimulatory TCR-pMHC interactions failed to explain their differences in signaling outcome. Recently, they found that TCRs use the catch bond mechanism to differentiate stimulatory from non-stimulatory interactions with same robust binding. Therefore, force-dependent catch bonds may serve as a checkpoint in governing TCR immune responses.

Co-receptor CD4/8 and PD-1 Molecule

In addition to TCRs, T-cell recognition also involves many other surface receptors. Here we will focus on co-receptor CD4/CD8 and programmed cell death protein 1 (PD-1). The co-receptor CD4/CD8 plays a critical role in T-cell signaling. It is generally thought that co-receptor CD4/CD8 binding to MHC brings Lck to mediate the phosphorylation of CD3 and ZAP-70. It has been found that blocking co-receptor CD4/CD8 significantly reduces T-cell signaling by more than 100-fold [15, 20, 92–95]. However, co-receptors CD4 and CD8 bind to MHC ligands with very low 3D affinities (K_D , ~100 μ M) [21, 96–98]. Consistently, 2D CD8-MHC interaction has a very low 2D affinity [99] and 2D CD4-MHC binding is too weak to precisely quantify by current techniques [78]. Clearly, there is a significant discrepancy between the strong signaling and weak binding of co-receptor CD4/CD8. In addition, there might be differences between CD4 and CD8 as well. It has been previously found that CD8 greatly promotes TCR recognition in 2D binding [19, 22]. In sharp contrast, CD4 does not appear to affect the both the 2D and 3D kinetics of TCR-pMHC interactions [20, 21]. We speculate that forces might play an important role in mediating co-receptor CD4/CD8 binding and signaling. To our limited knowledge, we did not find any conclusive force experiments that quantitatively measure how forces regulate the interactions between co-receptor CD4/CD8 and MHC ligand. However, recent studies show CD8 participates in a trimolecular catch bond with TCR-pMHC in a peptide specific manner, beginning to address the link between 2D affinity, force, and CD8 co-receptors,

postulating a possible mechanism for thymocyte selection [42, 100]. Such force measurements will be critical to elucidate the major discrepancy between signaling and binding of co-receptors as well as the differences between CD4 and CD8.

PD-1, one of the checkpoint and key molecules for immunotherapy [101–103], has been extensively studied biochemically, although its detailed mechanisms regarding modulation of T-cell immune responses are still not fully understood. It has been found that PD-1 mediated inhibition is through both CD28 [104, 105] and the TCR complex [23–25], with CD28 as the primary target. Interestingly, PD-1 mediates not only trans PD-1/PD-L1 interactions between the T cell and the target cell, but also cis PD-1/PD-L1 and PD-L1/B7-1 interactions at the same cell surface [106, 107]. The co-existing trans- and cis-interactions suggest a much more complex regulation network of T-cell activation and inhibition. Zhu and colleagues have measured 2D binding kinetics for PD-1 and ligand interactions [43]. However, no force measurements have been performed for PD-1 yet. It is highly possible that, similar to the TCR or integrin, forces could regulate the binding and signaling of PD-1-mediated inhibition. Therefore, further mechanotransduction studies should include PD-1 as an important research focus. Such investigations will not only advance our understanding of the inhibitory signaling pathway of T cells, but also can guide the rational design of immunotherapy for effective cancer treatment.

Cytoskeleton, Lipid and Receptor Nanostructure

The cytoskeleton is a highly dynamic network of filamentous proteins that exists in the three-dimensional space to connect and integrate different regions and components of a T cell. Upon TCR-pMHC ligation, T cells undergo a series of complex cytoskeleton-dependent, force-mediated activities, including cell adhesion [108–110], receptor clustering [29–31], cellular polarization [111], actin depletion [112], CD45 exclusion [113], synapse formation [17, 114], signaling [34, 79, 115, 116], and immune functions [112, 117, 118] (**Figure 1**). The cytoskeleton provides the dynamic cellular framework to orchestrate these processes to ultimately control T-cell signaling [111]. We have previously found that disruption of the cytoskeleton dramatically reduced 2D TCR-pMHC binding [11] and 3D pMHC dodecamer staining [119]. Evidently, T cells generate and apply mechanical forces to regulate molecular and cellular events through their cytoskeleton. Accumulating evidence further supports the importance of force in T-cell signaling and function that directly involves cytoskeleton regulations [79, 116, 117], including a recent study showing that forces generated by T cells are regulated by dynamic microtubules at the interface [120]. Actin polymerization and turnover are energy dependent and essential for force generation by T cells. Actin polymerization requires ATP and some metabolic enzymes have been shown to be dynamically regulated by the cytoskeleton [121–124]. To fully elucidate the T-cell recognition mechanism, the role of forces generated and transduced by the cytoskeleton during antigen recognition needs to be fully addressed and understood at the

molecular and cellular levels: one needs to understand how the multifunctional cytoskeleton provides active transportation of TCRs and other surface molecules, how the cytoskeleton uses energy to generate and transduce mechanical force, how the cytoskeleton works as an efficient machine to transduce signals in a fast manner, and how these processes are integrated together to facilitate T-cell recognition.

Simultaneously, TCRs and other surface receptors are residing at the T-cell plasma membrane, which consists of a lipid bilayer with embedded proteins. It has been suggested that most plasma membrane-associated proteins are clustered in cholesterol-enriched “islands” (lipid rafts) that are separated by “protein-free” and cholesterol-low membrane [29, 36], shown in **Figure 1**. The composition of the membrane is regulated by lipid metabolism, which has been implicated in generating lipid rafts to facilitate T-cell function but the details remain unclear [125]. Epifluorescence, total internal reflection fluorescence (TIRF), and super-resolution microscopy experiments have together shown that TCRs form microclusters for effective antigen recognition and signaling [14, 29–33]. TCR microclusters are considered to be the signaling hotspots [32, 33], where the large CD45 protein tyrosine phosphatase molecules [126] are excluded to facilitate the binding and signaling of TCRs [32, 33, 61, 62, 113, 127]. The formation of TCR clusters is sensitive to the disruption of lipid rafts and the cytoskeleton [34, 36] and a recent study shows that cholesterol sulfate inhibits T-cell signaling [16]. We have also previously found that disruption of lipid rafts greatly reduced the 2D binding affinity of TCRs [11]. These studies together highlight the importance of the membrane lipid microenvironment in modulating cell surface receptor activation. However, the force regulation of lipid membrane signaling is not well studied. Recently, Xu and colleagues used single-molecule atomic force microscopy (AFM) to study conformational dynamics of the CD3 ϵ cytoplasmic domain binding to the lipid plasma membrane and reveal multiple conformational states with different openness of three functional motifs [128]. This study suggests the fundamental importance of force in regulating lipid and T-cell signaling. To fully understand how mechanical forces regulate lipid rafts and TCR binding and signaling, more mechanistic studies combining force, metabolism, and signaling measurements are required to fully elucidate this interplay between forces and lipid structures at the T-cell membrane.

T-CELL MECHANOTRANSDUCTION STRATEGIES

T cells live in a three-dimensional microenvironment in which they not only contribute to but also exert and respond to mechanical forces of varying magnitude, direction, and frequency [129]. Throughout their life cycles, T cells are exposed to a wide range of tissues with distinct mechanical microenvironments and subjected to varying hemodynamic forces in the lymphatic and blood circulation [67]. In addition to the external forces, internally generated forces such as actin cytoskeleton contraction and ligand-receptor catch bond have also been implicated in

regulation of major T-cell functions as described in previous sections, and further depicted here [73, 111]. Here we review biomechanical regulation of T-cell functions, describe a cohort of bioengineering tools applicable to investigate these functions, and discuss several force sensors for quantification of physical forces exerted by T cells over multiple time and lengths scales.

Bioengineering Strategies to Study External Mechanical Force Regulation on T-Cells Function

Application of fundamental engineering principles and recent advances in manufacturing biomechanics, materials sciences, and tissue engineering enable specific mechanical perturbations at the cellular and subcellular levels for mechanistic mechanobiology studies. Here we discuss a few bioengineering tools that model pathophysiological mechanical forces exerted by T cells at the cellular or subcellular levels for mechanistic investigation of T-cell biology.

Extracellular Matrix (ECM) Manipulation for Microenvironmental Mechanical Cues

T cells encounter and function in a wide range of tissues comprising distinct cell populations and extracellular matrix (ECM) components through their development and during surveillance (**Figure 2**). Mechanical forces applied to and exerted by T cells are therefore dictated by the composition, architecture, and crosslinking of cell–extracellular matrix in these tissues with very distinct ECM elasticities. For instance, the Young's modulus is estimated ~ 50 Pa in mucus and circulation, 0.5–1.5 kPa in bone marrow, 3–15 kPa in spleen, and 1,000–1,500 in cartilage [135]. It is important to note that the Young's modulus is $\sim 100,000$ kPa in tissue culture plastic and $\sim 70,000,000$ kPa in rigid glass slides which are commonly used to culture T cells but do a poor job to recapitulate native microenvironmental mechanical cues. ECM mechanics are instrumental to cellular migration, growth, differentiation, morphogenesis, and tissue homeostasis [136], important biological processes intimately linked to T-cell functions and biology. Therefore, *in vitro* T-cell studies incorporating controlled matrix stiffness mimicking mechanical environments of tissues of interest may significantly strengthen the *in vivo* relevance of the findings. An array of biomaterials has been employed to engineer *in vitro* culture systems mimicking the *in vivo* mechanical properties of endogenous ECM mainly composed of elastic fibers, fibrillar collagens, glycosaminoglycans (GAGs), and proteoglycans (PGs). For instance, polyacrylamide hydrogels (in both 2D and 3D formats) have been widely used to engineer the microenvironments of variable stiffness for cellular studies in adhesion, differentiation, migration, proliferation, force generation, and cell–matrix interaction [130, 137, 138]. The elasticity of polyacrylamide hydrogels can be tuned precisely by altering the ratio of acrylamide monomer to the cross-linker of bis-acrylamide. Cellular responses to varying matrix stiffness from a few to hundreds of kPa have been investigated utilizing this tunable polyacrylamide hydrogel system. In addition to polyacrylamide, other materials such as Poly(dimethylsiloxane), Poly(ethylene glycol), alginate, and

hyaluronic acid have also been utilized to engineer hydrogels with tunable elasticity for cell culture [139]. Using a 2D culture composed of poly(dimethylsiloxane)-based silicone elastomer, O'Connor et al. reported that *ex vivo* proliferation of human CD4⁺ and CD8⁺ T cells is significantly increased when cells are seeded in a substrates with Young's modulus <100 kPa when compared to those on stiffer substrates with Young's modulus >2 MPa [131]. In addition, the numbers of IFN γ -producing Th1 T cells are considerably increased when naïve CD4⁺ T cells are expanded on softer substrates ($E < 100$ kPa) when compared with stiffer substrates (>2 MPa) [131]. Besides controlling mechanical properties of the tissues, ECM molecules connect to the cells through integrins, syndecans, and other receptors. Synthetic polymers with functional groups therefore are ideal to engineer hydrogels conjugating ECM proteins to study the biological consequences of different matrix protein–integrin pairs. Indeed, integrins on T cells not only bind to receptors on APCs and endothelium but also ECM proteins such as collagen, laminin, and fibronectin. For instance, fibronectin has been shown to co-stimulate T-cell proliferation via integrins $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ [132]. Nevertheless, the interplay between ECM elasticity and ECM protein composition in regulating T-cell action remains largely unexplored at the molecular level.

Flow Devices for Defined Hemodynamics

In the lymphatic and blood circulation as well as in the interstitial space, T cells are constantly exposed to hemodynamic forces generated by the flowing fluid, as shown in **Figure 2**. For instance, during immune surveillance naïve T cells dynamically circulate between the vasculature and lymph nodes where the interactions of fluid flow with local vessel geometry create complex hemodynamic characteristics including heterogeneous spatiotemporal shear stresses on the vessel wall. Hemodynamic shear stresses therefore not only govern major vascular functions but also play an important role in regulating critical T-cell functions such as crawling and extravasation (diapedesis) at the endothelial interface. Although underused in studying T-cell biology, an array of systems has been developed to apply well-defined hemodynamics investigating cellular responses to complex hemodynamic forces observed in the lymphatic and blood circulation as well as in interstitial space. For instance, parallel-plate flow chambers have been widely utilized to simulate fluid shear stresses on various cell types such as endothelial cells, smooth muscle cells, osteoblasts, osteocytes, cancer cells, and immune cells including leukocytes and T cells [133, 140–142]. In addition, cone-and-plate rheometers have been adapted to apply complex flow waveforms to cells that successfully elucidate novel mechanobiology insights related to cell morphology, gene expression, metabolic switch, and epigenome regulation [143–146]. A few studies using above-mentioned systems have provided lines of evidence supporting the importance of hemodynamic forces in regulating key T-cell functions. Steiner et al. employed a parallel flow chamber system showing T cells preferentially crawl against the direction of flow on the blood–brain barrier endothelial surface [133]. Using a cone-and-plate device, Schreiber et al. reported that hemodynamic shear stress promotes T-cell pseudopodial protrusions and consequent

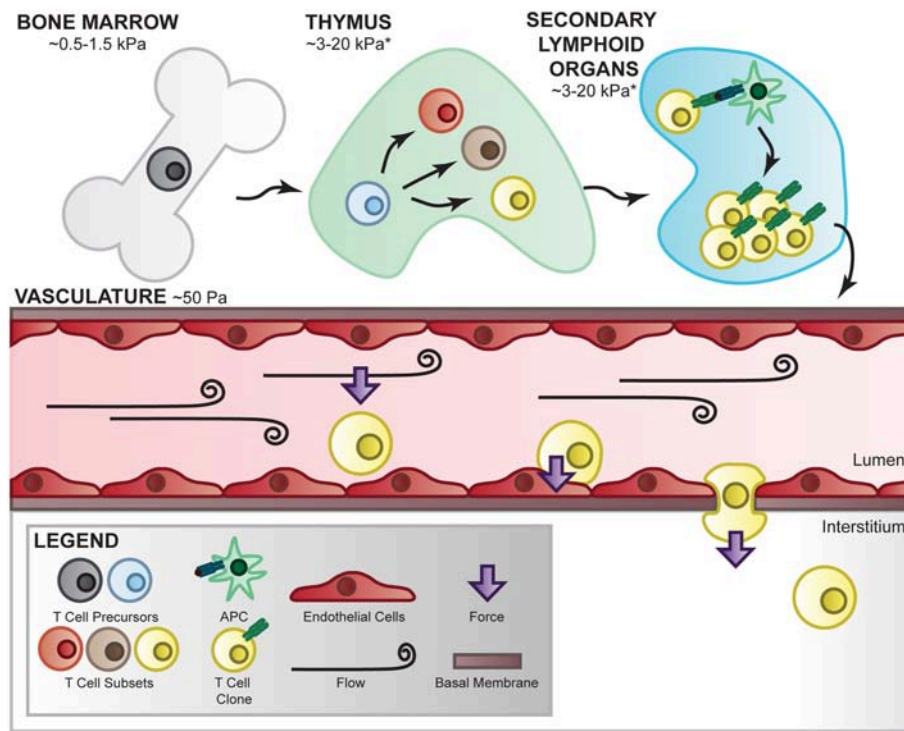


FIGURE 2 | T-cell mechanical environment. T cells are subjected to various mechanical environments throughout their lifetime. During development and differentiation, T cells migrate between tissues of varying elasticity and extracellular matrix components which has been shown to affect their signaling and differentiation [130–132]. In the periphery, they are subjected to fluid flow-mediated forces which apply shear stress to the cells and their receptor/ligand interactions. In this environment, T cells are able to crawl along the vascular bed, adhere at the correct location, deform their shape, and propel themselves into the interstitial space to perform their immune function, all of which requires internally generated force as well as external mechanical regulation [133, 134]. *Estimation based on measured Young's modulus on similar organs [135].

chemotaxis during lymphocyte extravasation from vascular endothelial cell monolayer [134]. Moreover, Woolf et al. reported using a parallel-plate flow chamber that shear flow interacts with CCL21 and integrin ligands to promote robust integrin-mediated adhesion of T cells to the endothelial monolayer [147]. However, molecular insights related to hemodynamic regulation of T-cell biology remain poorly understood. Recent advances in microfluidic devices that simultaneously model multiple mechanical cues (ECM stiffness and fluid flow) [148] could provide a powerful platform for future investigations of single T-cell responses to biophysical stimuli in a high-throughput fashion.

Methods to Study Force-Mediated Receptor-Ligand Interactions

T cells are able to sense and respond to external mechanical stimuli through a milieu of surface receptors, whose binding kinetics are mediated by force. For example, the TCR catch-bond forms selectively between an agonistic peptide but not an antagonistic peptide, described in section TCR-pMHC Catch Bond and depicted in **Figure 1**. Therefore, applying and measuring force to single surface molecules is critical to understanding how cells interpret and propagate mechanical signals to influence cell functions.

A common method for applying and measuring forces exerted by cells is Atomic Force Microscopy (AFM) [149]. In AFM, deflection of a spring-like cantilever scanned across a substrate can give topography of a sample at resolution on the order of fractions of a nanometer. As the tip of the cantilever comes into close proximity of the sample, the cantilever bends due to the force between the tip and sample. This deflection is monitored and is proportional to the force. Because the spring properties of the cantilever are known, and the tip can be tightly controlled spatially and temporally to make contact with the sample, AFM can be used not only as a means of imaging but also to both measure and apply forces at the few piconewton level. The tip can be functionalized to study receptor-ligand dynamics by adding a biotinylated ligand of interest to the streptavidin coated tip. This has been done to quantify adhesion forces and frequencies of the TCR/pMHC in both CD8⁺ [22] and CD4⁺ [150] T cells. AFM can be coupled with fluorescence microscopy to allow for multiplexing fluorescent readouts such as calcium sensitive dyes, labeled receptors, or cytoskeletal rearrangement, enabling powerful real-time imaging.

Another method capable of studying single-molecule interactions is optical tweezers, which utilize the angular and linear momentum of light to manipulate microscopic objects [151, 152]. Optical traps are formed by tightly focusing an

infrared laser beam to create a gradient of light intensity. Objects will be attracted to the point of highest intensity and “trapped” in three dimensions. The trapped object, normally a dielectric object such as a bead, will act as a Hookean spring where displacements can be correlated to force. Optical tweezers can be used to both measure or apply nanometer displacements corresponding to piconewton forces on single molecules attached to the bead, sometimes in the presence of an aspirated cell. For example, applying or measuring force at the TCR/pMHC interaction [27, 74, 79]. However, the use of isolated molecules functionalized to beads limits the physiological relevance of each measurement. Moreover, the beads can achieve angstrom spatial scale and second timescale but users have reported high experimental noise at longer timescales. Though the optical system allows for multiplexing other imaging modalities such as fluorescence or multiple optical traps, there are limitations to the amount of molecules that can be monitored at once which is a significant challenge for capturing the molecules’ native environment, especially in the context of T cells where multiple activation, co-stimulatory, checkpoint, and adhesion receptors cluster together and dynamically regulate each other’s kinetics.

The majority of force measuring methods utilize cells interacting with molecules functionalized to a bead, tip, or substrate. In order to encompass the complexity of a single molecules environment, methods that utilize cell-cell interactions can be used to monitor force such as the micropipette aspiration system [153]. In this system, single cells are held in place by aspiration into a micropipette. The pipettes can be driven by a piezoelectric translator to ensure contact of the two cells. Though one cell can be replaced by a bead, the strength of this system is being able to keep molecules within their semi-native environment to take real-time 2D kinetic measurements which has been shown to play an integral role in T-cell responsiveness [11, 154]. For TCR/pMHC interactions, an RBC is used as a surrogate APC to present pMHC, which is brought in and out of contact with a primary T cell aspirated by another micropipette with precisely controlled contact area and duration to yield an adhesion frequency. After generating adhesion frequencies over a range of contact durations, the binding affinity and off-rate are extracted using a monovalent binding kinetic model [153].

In another micropipette set-up, a functionalized probe bead can be attached to an aspirated cell and brought into close contact with another target bead. Named the biomembrane force probe (BFP), the RBC acts as an ultrasensitive force transducer, whose spring properties and deflection can be translated to sub-piconewton forces. For the thermal fluctuation assay [155], the RBC is attached by a pMHC-coated glass bead to form a biomembrane force probe, which is real-time tracked with nanometer spatial and sub-millisecond temporal resolution. The thermal fluctuation of the BFP is damped by the formation of a TCR-pMHC bond, allowing direct visualization of bond formation and dissociation and precise determination of bond lifetime in real time. The off-rate of single TCR-pMHC bond is extracted from the distribution of lifetime using a first-order irreversible dissociation model [11, 155]. A dual BFP has been developed to allow for multiple for temporally distinct ligand presentation to study crosstalk between surface receptors

[156]. Finally, deformations of the aspirated cell morphology can be used to monitor pushing and pulling forces, which has been applied to studying T-cell responses to antibodies [157]. Similarly, to the aforementioned methods, any of these micropipette systems can incorporate fluorescent probes into the existing imaging system to monitor receptor clustering, and downstream signaling of binding events.

Biophysical Force Sensors to Measure Internal Force Translation and Generation in T Cells

Recent biomechanical studies in cell biology have led to a cohort of force measurement systems that enable dynamic quantification of physical forces in cells on multiple scales from the cellular, subcellular, to molecular levels [158]. Here we review a few recently developed force sensors that serve as physical devices of known mechanical properties to receive biomechanical stimuli and transform them into measurable physical quantities. Particularly, force sensors that dynamically detect intracellular force changes due to cytoskeleton remodeling will certainly inform new molecular insights of T-cell biology given that T-cell trafficking and immunological synapse formation are actively regulated by the structure and function of cytoskeleton [73].

Traction Force Measured by Microscopy

Traction force microscopy (TFM) was developed to map and quantify the forces generated by cells against their substrates at the cellular and subcellular levels [159, 160]. Briefly, cells of interest are embedded in well-defined elastic hydrogel matrices (2D or 3D) that contain fluorescent microspheres. Traction forces exerted by the adherent cells are transmitted to the ECM via focal adhesions composed of structural and signaling molecules that form physical links between actin cytoskeleton and ECM. The deformation of the substrate, the result of cell contractile forces generated by the actomyosin cytoskeleton, can be detected by the displacement of fluorescent beads and quantified by elastic mechanics of matrices to yield vector maps of traction forces at the subcellular level. Notably, the substrate can be coated with varying ECM proteins to mimic the extracellular matrix of interest or with a ligand to study forces generated at the receptor-ligand interface [161]. TFM using ICAM-1-coated silicone-based gel was utilized to demonstrate that integrin-mediated force transmission in T cells requires actin-binding protein filamin A [162]. In addition to the continuous elastic substrates such as hydrogels, the elastic substrate can be microfabricated to form pillar arrays instead of a flat substrate so that each pillar acts as an independent cantilever [163]. TFM with micrometer-scale elastomer pillar arrays was used to show that T cells generate significant traction forces through TCRs and CD28 [76]. TFM can be easily integrated into existing fluorescent microscopy set-ups with modular cost and modification. However, TFM only reports cell-generated forces on an artificial substrate and is therefore not suitable for applying specific force to the cell or for studying cell-cell interactions.

Intramolecular Forces Measured by FRET

As described above, common methods for quantifying cellular forces can achieve physiological range but all either measure force generation onto extracellular substrates or apply known forces extracellularly to observe how cells respond. Since extracellular mechanical force is transmitted through surface receptors to the cytoskeleton and organelles, a method to measure internal forces between specific molecules is critical to understand the physiological relevance and mechanism of mechanosensitivity. In order to interrogate these mechanical interactions between single molecules at or beneath the cell surface, researchers have developed tension probes capable of entering cells that rely on the principles of FRET to reliably quantify force. FRET is commonly used to observe and quantify molecular interactions due to the distance dependent transfer of photons between two fluorophores. Because the efficiency of this transfer is dependent on proximity, changes in FRET efficiency can correspond to force-induced displacement of a small fluorophore labeled tensor [164]. One such tool utilizes DNA as the tension sensitive molecule to quantify TCR force transmission [165]. DNA is a popular tension sensor due to its simple synthesis, ease of fluorophore conjugation, and ability to tune the force required for unfolding at the piconewton level based on GC content and stem-loop structure [166, 167]. The FRET pair can also be connected via a flexible polypeptide linker to generate a completely genetically encoded tension sensor [168]. The sensor can be encoded between different molecules to measure intra- and inter-molecular tension such as between α and β integrin domains and between integrins and cytoskeleton within T-cells [65]. These DNA based molecular sensors allow for interrogation of single-molecule force transmission intracellularly which will be critical to understanding T-cell internal mechanobiology.

TCR Signaling as a Result of Force

Following force-mediated activation via the TCR, there are multiple signaling pathways that propagate throughout the cell to coordinate the effector function of the lymphocyte. Here we will focus on the major signaling pathways that follow T cell activation via the TCR, though they are coupled with co-stimulatory receptor and cytokine receptor signals, and describe the limited but promising evidence linking force to signaling. Receptor clustering following antigen recognition and activation at the immunological synapse localizes the internal signaling domains of the TCR co-receptors, CD4 and CD8, which bind the Src family protein tyrosine kinase Lck. Lck phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 cytoplasmic domains [169], which are able to recruit SH2-domain-containing proteins including ZAP70 to the TCR complex. Lck will also activate ZAP70, which will phosphorylate scaffolding transmembrane adaptor linker for activation of T cells (LAT) [170], creating docking sites for adaptor molecules which will recruit and activate multiple signaling molecules [171], serving as a activation signaling hub. For example, VAV1 activates RHO-family GTPases RAC and cell division control protein 42 (CDC42) to initiate actin polymerization and gene transcription. Cytoskeletal rearrangement is critical for synapse formation and cell motility but also can propagate

force to other cellular components including the endoplasmic reticulum (ER), nucleus and mitochondria, possibly linking force at the synapse to T cell function, as depicted in **Figure 3**. Another LAT-docked signaling molecule is PLC γ 1 which will catalyze the production of second messengers to increase intracellular calcium by ER release and membrane channel opening, thereby activating calcineurin which will induce the nuclear localization of key transcription factor nuclear factor of activated T cells (NFAT). Both cytoskeleton rearrangement and intracellular calcium levels are critical to carrying out T cell function and a feedback mechanism between the two helps sustain this activation [172]. Interestingly, the translocation of the mitochondria close to the plasma membrane by the cytoskeleton can sustain calcium influx, thereby maintaining activation of T cells. Additional transcription factors including activator protein 1 (AP-1) and nuclear factor- κ B (NF- κ B) will become activated or localized to the nucleus by other signaling effectors, mitogen-activated protein (MAP) kinases and protein kinase C (PKC), collectively resulting in cell proliferation, cytokine production, and proliferation. Though TCR signaling has been extensively studied, how force regulates TCR signaling remains largely unknown.

Recent studies have started to assess canonical downstream T-cell signaling in the context of force. However, more studies need to be done to fully address how the mechanical environment and properties of T cells influence signaling and consequent function. The force-dependent TCR catch bond, described earlier in section TCR-pMHC Catch Bond, is required for triggering and sustaining calcium mobilization by prolonging the TCR-pMHC lifetime [26]. High calcium levels required early and rapid accumulation of TCR-pMHC bond lifetimes. High interaction forces between T cells and peptide ligands displayed on dendritic cells promoted calcium mobilization and IL-2 secretion, a key cytokine produced by activated T cells to induce proliferation [18]. Extracellular matrix stiffness has been shown to significantly influence key signaling pathways in T cells. Seeding on softer substrates (<100 kPa) has been shown to increase proliferation of both CD4⁺ and CD8⁺ T cells and activated effector Th1 T cells, or IFN γ -producing, increased after naïve CD4⁺ T cells were grown on softer substrates [131]. Similarly, naïve CD4⁺ T cells activation, measured by attachment and IL-2 secretion correlates with increasing the Young's modulus of the substrate up to 200 kPa. Phosphorylation of signaling molecules including ZAP70 was also increased, indicating the mechanical environment is playing a role in the activation and downstream signaling of these cells [161]. In addition to the biomechanical cues provided by the extracellular matrix, T cells are constantly exposed and respond to the hemodynamic forces generated by the blood flow and interstitial fluid. For instance, fluid shear stress regulates transendothelial migration of T cells via cytoskeleton and Gi protein-mediated chemokine signaling [173] and modulates lymph node chemokine-induced T cell migration by integrin-dependent signaling [147]. Though the readouts of the key T cell mechanobiology studies reviewed here include calcium flux, signaling protein phosphorylation, and cytokine production, the links between purely mechanical stimuli and

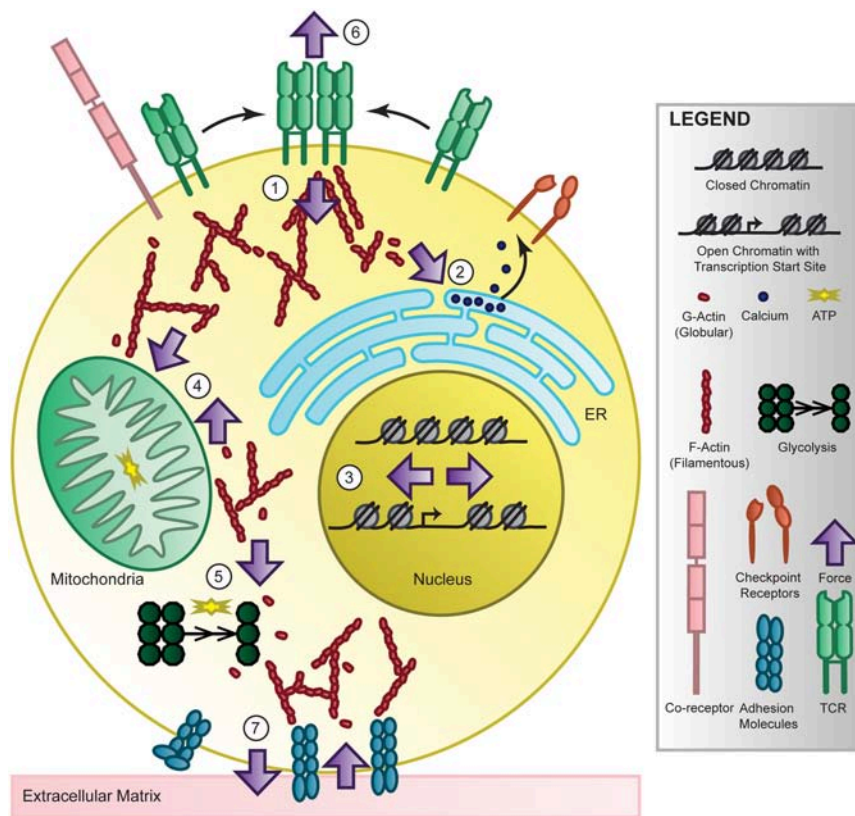


FIGURE 3 | T-cell mechanotransduction. T cells perceive environmental force cues such as ligand binding, substrate stiffness, and shear stress through their surface receptors and membrane structure. These forces are internalized and transmitted at the cytoplasmic side of the receptors (1) and by the cytoskeleton, converted into biochemical signaling, then propagated to organelles such as the endoplasmic reticulum (ER) where calcium is released (2), the nucleus where the chromatin landscape and transcription changes (3), the mitochondria (4) or cytoskeletal-bound glycolytic compartments (5) to change energy production or back out to the surface of the T cell at the immunological synapse (6) or extracellular matrix (7) to modulate adhesion and cytokine release. Though accumulating evidence links force to T cell recognition, signaling, differentiation, metabolism, and genetics in T cells and others, the mechanisms require further study. However, energy-dependent cytoskeletal rearrangement is most likely the key to providing future investigators direction for uncovering the molecular players of T-cell mechanotransduction and elucidating force-dependent function.

downstream signaling require further elucidation but most likely involve critical regulation and propagation by the cytoskeleton as accumulating evidence suggests [79, 111, 116, 117, 174]. Nevertheless, future studies should seek to define the force-mediated signaling pathway to determine how exactly external force is translated into biochemical energy to better understand T cell activation and function.

FUTURE DIRECTIONS

The interplay between mechanical forces and cellular functions was first suggested in early twenty century by D'Arcy Thompson who postulated a possible contribution of physical forces in controlling the size and shape of living organisms [175]. How cells sense and transduce biomechanical forces into biological signaling has since become an intensely studied field and the frontier of current cell biology. Nevertheless, T-cell mechanoimmunology is still in its infancy and understanding how T cells actively respond to varying biomechanical cues could lead to not only novel molecular insights in

mechanobiology but also fruitful discoveries to facilitate the emerging T cell-based therapies. Here we pose outstanding questions and likely directions of T-cell mechanobiology, depicted in Figure 3.

Force in T-Cell Selection and Differentiation

Although the field of T-cell immunology has started to realize the importance of mechanical forces in T-cell activation, very little is known about whether and how mechanical forces regulate T-cell selection and differentiation. It is known that T-cell selection and differentiation are controlled by chromatin landscape and transcription factors in T-cell programming [176]. It is also known that mechanical forces can regulate chromatin structures: changes in the mechanical properties of the perinuclear cytoskeleton, nuclear lamina and chromatin are critical for cellular responses and adaptation to external mechanical cues. It has been found that altered nuclear mechanics are associated with many human diseases [177, 178]. However, it is poorly understood how forces regulate T-cell selection and differentiation.

For T-cell selection, the recognition of self-pMHCs on thymic APCs is critical for determining the fate of developing T cells. Somewhat paradoxically, recognition of self can elicit diametrically opposed outcomes. According to the classical affinity model, weak interaction is required for cell survival (positive selection) while strong interaction causes cell death (negative selection). However, the strength of binding affinity that determines negative and positive selection is poorly defined, mainly described as weak or strong interactions. It is not clear which affinity value/threshold can lead to positive selection or negative selection. It also does not take into account the fact that positive and negative selection largely occur in discrete thymic microenvironments, namely the cortex and the medulla, respectively. In addition to biological cues, both compartments have different force loads, thereby providing very different biomechanical microenvironments that orchestrate a spatial and temporal segregation of thymocyte selection [179, 180].

For T-cell differentiation, it is generally thought that the cytokine environment plays a central role in cell fate determination and effector function. The distinctive differentiated states of the various T-cell subpopulations are determined largely by the set of transcription factors they express and the genes they transcribe [181]. Although we know that T cells migrate and differentiate into distinct subpopulations at different organs of the human body, where they encounter different antigens and are subjected to different mechanical forces, the importance of TCR binding strength and the environmental mechanical cues are mainly ignored or understudied. It has been found that the matrix elasticity controls stem cell fate [130], and it is not known whether and how the TCR binding strength and forces regulate T-cell differentiation. We have known that binding of the TCR to different pMHCs exhibits different interaction characteristics in T-cell selection and activation. However, the role of TCR binding force in determine T-cell differentiation and memory is much less understood. For example, why the majority of effector cells undergo apoptosis but only a small subset (5%) of T cells change to a memory phenotype after the clearance of infections. Besides cytokines, do other environmental cues like force play a role in this process?

To fully understand the mechanisms of T-cell selection and differentiation, in addition to existing biological and chemical cues, we need to link the mechanical forces with traditional biochemical cues under physiological settings using precise quantitative analyses, instead of only qualitative descriptions.

Force in T-Cell Metabolism and Genetics

In addition to aforementioned mechanotransduction mechanisms we discussed in this review, we believe that the integration of emerging research fields such as cellular metabolism and human genetics may significantly influence future T-cell mechanobiology investigations. Accumulating evidence strongly suggests the pervasive role of metabolism, the sum of biochemical reactions in living organisms that produce or consume energy, in regulating essentially every aspect of biological functions [182]. Core metabolic pathways such as glycolysis and oxidative phosphorylation (OXPHOS)

have been long recognized to provide energy source for cells to sustain life. In addition to molecule degradation for energy release (catabolism), cellular metabolism is the major control for complex macromolecules synthesis and biomass creation (anabolism) which are instrumental to lymphocyte signaling and functions. For instance, naïve T-cells primarily rely on OXPHOS and exhibit low anabolic capacity given limited need for *de novo* synthesis of DNA, lipids, and proteins while integrated signals from pre-TCR, Notch1, and CXCR4 can temporarily increase glycolysis and promote anabolic metabolism during times of T-cell proliferation in thymus. Metabolic reprogramming is equally important during effector T-cell differentiation/activation. TCR ligation, co-stimulation, and cytokine signaling have been shown to collectively induce metabolic remodeling of naïve T-cells to promote anabolic growth and biomass accumulation [183] necessary for clonal expansion of antigen-specific T cells. Notably, phenotypic switch to activated effector T-cells requires not only elevated OXPHOS but also aerobic glycolysis (Warburg effect), a biological process in which glucose is converted into lactate even in the presence of normal levels of oxygen. It is thought that although OXPHOS (which produces 32 ATP per molecule of glucose) can sufficiently supply the ATP demands during T-cell activation, aerobic glycolysis (which produces only 2 ATP per molecule of glucose) is necessary for redox balance (NAD^+/NADH) in the cell and the synthesis of metabolic intermediates important for cell growth and proliferation [184]. Moreover, emerging studies demonstrate that metabolic pathways and signal transduction are tightly regulated in a reciprocal fashion and strategic integration of metabolic and signaling cues allows cells to modulate key activities such as proliferation and differentiation depending on the metabolic status [185]. For instance, the cooperation between transcription factors and the chromatin landscape, important for the spatiotemporal control of gene expression programs and cell lineage determinations, are dynamically regulated by intermediate metabolites of cellular metabolic pathways. Acetyl-CoA and NAD^+ generated from oxidative metabolism are key substrates for histone acetyltransferase (HAT) and histone deacetylase (HDAC) which control histone acetylation/deacetylation and consequent chromatin remodeling [186] crucial for regulation of cytokine gene expression during T-cell differentiation and activation. Whether and how mechanical forces regulate immune cell metabolism (“Mechanoimmunometabolism”) remains virtually unexplored but few lines of evidence from non-immune cells may provide some possible molecular insights. First, fluid flow shear stress has been recently reported to be a critical regulator of endothelial metabolic reprogramming [146, 187]; unidirectional blood flow promotes endothelial OXPHOS while reduced shear stress increases glycolysis by stabilizing HIF-1 α . Second, Notch1 that is necessary for T-cell lineage commitment was recently identified as a fluid shear stress sensor during vasculogenesis [188] and in adult artery [189]. At the molecular level, Hu et al. reported that actin cytoskeleton remodeling induced by small GTPase Rac1 directly regulates glycolytic outputs in epithelial cells by freeing the low-activity state, actin-bound aldolase A [124].

Recent human genetics studies not only significantly advanced our knowledge in the genetic basis of complex diseases associated with T-cell functions but also provide a new avenue for future T-cell mechanotransduction investigations. Human genetic mutations have been well-established as major regulators of T-cell homeostasis and dysfunctions related to a variety of diseases such as multiple sclerosis, autoimmune disease, and acute lymphoblastic leukemia [190–192]. Although the putative role of genetic variants in regulating T-cell mechanosensing biology remains to be elucidated, recent investigations have suggested that genetic predisposition provides a previously unappreciated layer of regulatory control in cellular mechanosensing mechanisms. For instance, mutations in DPP homolog 4 (SMAD4) in humans is hypothesized to promote epithelial β 1-integrin mechanosignaling, leading to matricellular fibrosis, increased tissue tension, and tumor progress [193]. In addition, a genetic variant at phospholipid phosphatase 3 (PLPP3) implicated in coronary artery disease and ischemic stroke by genome-wide association studies (GWAS) has been shown to regulate endothelial mechanosensing responses to hemodynamic forces [194]. It remains poorly understood regarding the interplay between genetic predisposition and mechanosensing mechanisms converging on key T-cell functions, an area has tremendous potential to provide novel molecular insights in diagnosing and treating human genetic diseases associated with T-cell homeostasis and dysfunction.

Concluding Remarks

Major efforts of mechanotransduction investigations have been focusing on identifying putative mechanosensors and cellular

components in isolation. It remains poorly understood how the whole cell and entire tissue process and integrate this molecular scale information and further orchestrate physiologically relevant responses in the context of the multiscale architecture of our whole bodies. T cells serve as an ideal model system for an integrated approach to elucidate the dynamic interactions of individual components that operate at multiple spatiotemporal scales to mediate the cellular mechanosensing responses. Not only are T cells uniquely exposed to diverse external environments with distinct mechanical cues during their life cycle but major “-omics” techniques and systems biology approaches are becoming mature in T-cell investigations. These would allow investigators to monitor and record T-cell responses to mechanical perturbation at multiple levels of regulatory controls in a high-throughput fashion, providing novel molecular insights by which biological information flows from DNA to proteins to metabolites to cell structures to cell interactions in the context of T-cell mechanobiology.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by NIH grants R00AI106941 (JH) R21AI120010 (JH), R01HL138223 (YF), and R01HL136765 (YF), NSF Career Award 1653782 (JH), and NIH New Innovator Award DP2AI144245 (JH). DH is supported by NIH T32 EB009412.

REFERENCES

- Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol.* (2003) 4:835–42. doi: 10.1038/ni969
- Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood.* (2008) 112:1557–69. doi: 10.1182/blood-2008-05-078154
- Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. *Immunity.* (2011) 35:161–8. doi: 10.1016/j.immuni.2011.07.010
- Davila ML, Brentjens R, Wang X, Rivière I, Sadelain M. How do CARs work?: early insights from recent clinical studies targeting CD19. *Oncoimmunology.* (2012) 1:1577–83. doi: 10.4161/onci.22524
- Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. *Cancer Discov.* (2013) 3:388–98. doi: 10.1158/2159-8290.CD-12-0548
- Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med.* (2013) 5:177ra38. doi: 10.1126/scitranslmed.3005930
- Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med.* (2013) 368:1509–18. doi: 10.1056/NEJMoa1215134
- Korman AJ, Peggs KS, Allison JP. Checkpoint blockade in cancer immunotherapy. *Adv Immunol.* (2006) 90:297–339. doi: 10.1016/S0065-2776(06)90008-X
- Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* (2018) 359:1350–5. doi: 10.1126/science.aar4060
- Alam SM, Travers PJ, Wung JL, Nasholds W, Redpath S, Jameson SC, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature.* (1996) 381:616–20. doi: 10.1038/381616a0
- Huang J, Zarnitsyna VI, Liu B, Edwards LJ, Jiang N, Evavold BD, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature.* (2010) 464:932–6. doi: 10.1038/nature08944
- Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature.* (2002) 419:845–9. doi: 10.1038/nature01076
- Purbhoo MA, Irvine DJ, Huppa JB, Davis MM. T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol.* (2004) 5:524–30. doi: 10.1038/ni1058
- Huang J, Brameshuber M, Zeng X, Xie J, Li Q, Chien Y, et al. A single peptide-major histocompatibility complex ligand triggers digital cytokine secretion in CD4(+) T cells. *Immunity.* (2013) 39:846–57. doi: 10.1016/j.immuni.2013.08.036
- Li Q-J, Dinner AR, Qi S, Irvine DJ, Huppa JB, Davis MM, et al. CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse. *Nat Immunol.* (2004) 5:791–9. doi: 10.1038/ni1095
- Wang F, Beck-García K, Zorzin C, Schamel WWA, Davis MM. Inhibition of T cell receptor signaling by cholesterol sulfate, a naturally occurring derivative of membrane cholesterol. *Nat Immunol.* (2016) 17:844–50. doi: 10.1038/ni.3462
- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature.* (1998) 395:82–6. doi: 10.1038/25764

18. Lim TS, Mortellaro A, Lim CT, Hämmerling GJ, Ricciardi-Castagnoli P. Mechanical interactions between dendritic cells and T cells correlate with T cell responsiveness. *J Immunol.* (2011) **187**:258–65. doi: 10.4049/jimmunol.1100267
19. Jiang N, Huang J, Edwards LJ, Liu B, Zhang Y, Beal CD, et al. Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. *Immunity.* (2011) **34**:13–23. doi: 10.1016/j.immuni.2010.12.017
20. Huppa JB, Axmann M, Mörtelmaier MA, Lillemeier BF, Newell EW, Brameshuber M, et al. TCR-peptide-MHC interactions *in situ* show accelerated kinetics and increased affinity. *Nature.* (2010) **463**:963–7. doi: 10.1038/nature08746
21. Xiong Y, Kern P, Chang H, Reinherz E. T cell receptor binding to a pMHCII ligand is kinetically distinct from and independent of CD4. *J Biol Chem.* (2001) **276**:5659–67. doi: 10.1074/jbc.M009580200
22. Puech P-H, Nevoltris D, Robert P, Limozin L, Boyer C, Bongrand P. Force measurements of TCR/pMHC recognition at T cell surface. *PLoS ONE.* (2011) **6**:e22344. doi: 10.1371/journal.pone.0022344
23. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med.* (2012) **209**:1201–17. doi: 10.1084/jem.20112741
24. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol.* (2004) **173**:945–54. doi: 10.4049/jimmunol.173.2.945
25. Sheppard K-A, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett.* (2004) **574**:37–41. doi: 10.1016/j.febslet.2004.07.083
26. Liu B, Chen W, Evavold BD, Zhu C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell.* (2014) **157**:357–68. doi: 10.1016/j.cell.2014.02.053
27. Das DK, Feng Y, Mallis RJ, Li X, Keskin DB, Hussey RE, et al. Force-dependent transition in the T-cell receptor β -subunit allosterically regulates peptide discrimination and pMHC bond lifetime. *Proc Natl Acad Sci USA.* (2015) **112**:1517–22. doi: 10.1073/pnas.1424829112
28. Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell.* (2018) **174**:672–87 e27. doi: 10.1016/j.cell.2018.06.017
29. Lillemeier BF, Mörtelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol.* (2010) **11**:90–6. doi: 10.1038/ni.1832
30. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med.* (2005) **202**:1031–6. doi: 10.1084/jem.20051182
31. Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol.* (2005) **6**:1253–62. doi: 10.1038/ni1272
32. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity.* (2006) **25**:117–27. doi: 10.1016/j.immuni.2006.04.010
33. Razvag Y, Neve-Oz Y, Sajman J, Reches M, Sherman E. Nanoscale kinetic segregation of TCR and CD45 in engaged microvilli facilitates early T cell activation. *Nat Commun.* (2018) **9**:732. doi: 10.1038/s41467-018-03127-w
34. Chichili GR, Westmuckett AD, Rodgers W. T cell signal regulation by the actin cytoskeleton. *J Biol Chem.* (2010) **285**:14737–46. doi: 10.1074/jbc.M109.097311
35. Kim H-R, Mun Y, Lee K-S, Park Y-J, Park J-S, Park J-H, et al. T cell microvilli constitute immunological synaptosomes that carry messages to antigen-presenting cells. *Nat Commun.* (2018) **9**:3630. doi: 10.1038/s41467-018-06090-8
36. Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc Natl Acad Sci USA.* (2006) **103**:18992–7. doi: 10.1073/pnas.0609009103
37. Murphy K, Weaver C. *Janeway's Immunobiology*. 9th ed. New York, NY: Garland Science/Taylor & Francis Group, LLC (2017).
38. Davis MM, Boniface JJ, Reich Z, Lyons D, Hampl J, Arden B, et al. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol.* (1998) **16**:523–44. doi: 10.1146/annurev.immunol.16.1.523
39. Garcia KC, Teyton L, Wilson IA. Structural basis of T cell recognition. *Annu Rev Immunol.* (1999) **17**:369–97. doi: 10.1146/annurev.immunol.17.1.369
40. Davis SJ, Ikemizu S, Evans EJ, Fugger L, Bakker TR, van der Merwe PA. The nature of molecular recognition by T cells. *Nat Immunol.* (2003) **4**:217–24. doi: 10.1038/ni0303-217
41. Coombs D, Dushek O, van der Merwe PA. A review of mathematical models for T cell receptor triggering and antigen discrimination. In: Molina-Paris C, Lythe G, editors. *Mathematical Models and Immune Cell Biology*. New York, NY: Springer (2011). p. 25–45.
42. Kolawole EM, Andargachew R, Liu B, Jacobs JR, Evavold BD. 2D kinetic analysis of TCR and CD8 coreceptor for LCMV GP33 epitopes. *Front Immunol.* (2018) **9**:2348. doi: 10.3389/fimmu.2018.02348
43. Li K, Cheng X, Tilevik A, Davis SJ, Zhu C. *In situ* and *in silico* kinetic analyses of programmed cell death-1 (PD-1) receptor, programmed cell death ligands, and B7-1 protein interaction network. *J Biol Chem.* (2017) **292**:6799–809. doi: 10.1074/jbc.M116.763888
44. Wucherpfennig KW, Gagnon E, Call MJ, Huseby ES, Call ME. Structural biology of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. *Cold Spring Harb Perspect Biol.* (2010) **2**:a005140. doi: 10.1101/cshperspect.a005140
45. Kuhns MS, Badgandi HB. Piecing together the family portrait of TCR-CD3 complexes. *Immunol Rev.* (2012) **250**:120–43. doi: 10.1111/imr.12000
46. Birnbaum ME, Berry R, Hsiao Y-S, Chen Z, Shingu-Vazquez MA, Yu X, et al. Molecular architecture of the $\alpha\beta$ T cell receptor-CD3 complex. *Proc Natl Acad Sci USA.* (2014) **111**:17576–81. doi: 10.1073/pnas.1420936111
47. Call ME, Pyrdol J, Wiedmann M, Wucherpfennig KW. The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell.* (2002) **111**:967–79. doi: 10.1016/S0092-8674(02)01194-7
48. Laydon DJ, Bangham CRM, Asquith B. Estimating T-cell repertoire diversity: limitations of classical estimators and a new approach. *Philos Trans R Soc Lond B Biol Sci.* (2015) **370**:20140291. doi: 10.1098/rstb.2014.0291
49. Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, et al. An estimation of the number of cells in the human body. *Ann Hum Biol.* (2013) **40**:463–71. doi: 10.3109/03014460.2013.807878
50. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. *Cell.* (2002) **109**(Suppl.):S45–55. doi: 10.1016/S0092-8674(02)00675-X
51. Dustin ML, Bromley SK, Davis MM, Zhu C. Identification of self through two-dimensional chemistry and synapses. *Annu Rev Cell Dev Biol.* (2001) **17**:133–57. doi: 10.1146/annurev.cellbio.17.1.133
52. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science.* (1999) **285**:221–7. doi: 10.1126/science.285.5425.221
53. Margulies DH, Plaksin D, Khilko SN, Jelonek MT. Studying interactions involving the T-cell antigen receptor by surface plasmon resonance. *Curr Opin Immunol.* (1996) **8**:262–70. doi: 10.1016/S0952-7915(96)80066-5
54. Corr M, Slanetz AE, Boyd LF, Jelonek MT, Khilko S, al-Ramadi BK, et al. T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. *Science.* (1994) **265**:946–9. doi: 10.1126/science.8052850
55. Matsui K, Boniface JJ, Steffner P, Reay PA, Davis MM. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci USA.* (1994) **91**:12862–6. doi: 10.1073/pnas.91.26.12862
56. Krogsgaard M, Prado N, Adams EJ, He X, Chow D-C, Wilson DB, et al. Evidence that structural rearrangements and/or flexibility during TCR

- binding can contribute to T cell activation. *Mol Cell*. (2003) **12**:1367–78. doi: 10.1016/S1097-2765(03)00474-X
57. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. (1996) **274**:94–6. doi: 10.1126/science.274.5284.94
 58. Holmberg K, Mariathasan S, Ohteki T, Ohashi PS, Gascoigne NRJ. TCR binding kinetics measured with MHC class I tetramers reveal a positive selecting peptide with relatively high affinity for TCR. *J Immunol*. (2003) **171**:2427–34. doi: 10.4049/jimmunol.171.5.2427
 59. Laugel B, van den Berg HA, Gostick E, Cole DK, Wooldridge L, Boulter J, et al. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *J Biol Chem*. (2007) **282**:23799–810. doi: 10.1074/jbc.M700976200
 60. Huang J, Chen J, Chesla SE, Yago T, Mehta P, McEver RP, et al. Quantifying the effects of molecular orientation and length on two-dimensional receptor-ligand binding kinetics. *J Biol Chem*. (2004) **279**:44915–23. doi: 10.1074/jbc.M407039200
 61. Choudhuri K, Wiseman D, Brown MH, Gould K, van der Merwe PA. T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature*. (2005) **436**:578–82. doi: 10.1038/nature03843
 62. Choudhuri K, Parker M, Milicic A, Cole DK, Shaw MK, Sewell AK, et al. Peptide-major histocompatibility complex dimensions control proximal kinase-phosphatase balance during T cell activation. *J Biol Chem*. (2009) **284**:26096–105. doi: 10.1074/jbc.M109.039966
 63. Williams JG, Drugan JK, Yi GS, Clark GJ, Der CJ, Campbell SL. Elucidation of binding determinants and functional consequences of Ras/Raf-cysteine-rich domain interactions. *J Biol Chem*. (2000) **275**:22172–9. doi: 10.1074/jbc.M000397200
 64. Li J, Springer TA. Energy landscape differences among integrins establish the framework for understanding activation. *J Cell Biol*. (2018) **217**:397–412. doi: 10.1083/jcb.201701169
 65. Nordenfelt P, Elliott HL, Springer TA. Coordinated integrin activation by actin-dependent force during T-cell migration. *Nat Commun*. (2016) **7**:13119. doi: 10.1038/ncomms13119
 66. Li J, Springer TA. Integrin extension enables ultrasensitive regulation by cytoskeletal force. *Proc Natl Acad Sci USA*. (2017) **114**:4685–90. doi: 10.1073/pnas.1704171114
 67. Chen W, Zhu C. Mechanical regulation of T-cell functions. *Immunol Rev*. (2013) **256**:160–76. doi: 10.1111/immr.12122
 68. Axmann M, Huppa JB, Davis MM, Schütz GJ. Determination of interaction kinetics between the T cell receptor and peptide-loaded MHC class II via single-molecule diffusion measurements. *Biophys J*. (2012) **103**:L17–9. doi: 10.1016/j.bpj.2012.06.019
 69. O'Donoghue GP, Pielak RM, Smoligovets AA, Lin JJ, Groves JT. Direct single molecule measurement of TCR triggering by agonist pMHC in living primary T cells. *elife*. (2013) **2**:e00778. doi: 10.7554/eLife.00778
 70. Taylor MJ, Husain K, Gartner ZJ, Mayor S, Vale RD. A DNA-based T Cell receptor reveals a role for receptor clustering in ligand discrimination. *Cell*. (2017) **169**:108–19 e20. doi: 10.1016/j.cell.2017.03.006
 71. Klein LO. *Imaging Initial Events in T-Cell Activation: Ligand Binding and Signaling*. Stanford: Stanford University (2010).
 72. Ma Z, Discher DE, Finkel TH. Mechanical force in T cell receptor signal initiation. *Front Immunol*. (2012) **3**:217. doi: 10.3389/fimmu.2012.00217
 73. Ma Z, Finkel TH. T cell receptor triggering by force. *Trends Immunol*. (2010) **31**:1–6. doi: 10.1016/j.it.2009.09.008
 74. Kim ST, Takeuchi K, Sun Z-Y, Touma M, Castro CE, Fahmy A, et al. The alphabeta T cell receptor is an anisotropic mechanosensor. *J Biol Chem*. (2009) **284**:31028–37. doi: 10.1074/jbc.M109.052712
 75. Li Y-C, Chen B-M, Wu P-C, Cheng T-L, Kao L-S, Tao M-H, et al. Cutting edge: mechanical forces acting on T cells immobilized via the TCR complex can trigger TCR signaling. *J Immunol*. (2010) **184**:5959–63. doi: 10.4049/jimmunol.0900775
 76. Bashour KT, Gondarenko A, Chen H, Shen K, Liu X, Huse M, et al. CD28 and CD3 have complementary roles in T-cell traction forces. *Proc Natl Acad Sci USA*. (2014) **111**:2241–6. doi: 10.1073/pnas.1315606111
 77. Abadier M, Pramod AB, McArdle S, Marki A, Fan Z, Gutierrez E, et al. Effector and regulatory T cells roll at high shear stress by inducible tether and sling formation. *Cell Rep*. (2017) **21**:3885–99. doi: 10.1016/j.celrep.2017.11.099
 78. Hong J, Persaud SP, Horvath S, Allen PM, Evavold BD, Zhu C. Force-regulated *in situ* TCR-peptide-bound MHC class II kinetics determine functions of CD4+ T cells. *J Immunol*. (2015) **195**:3557–64. doi: 10.4049/jimmunol.1501407
 79. Feng Y, Brazin KN, Kobayashi E, Mallis RJ, Reinherz EL, Lang MJ. Mechanosensing drives acuity of $\alpha\beta$ T-cell recognition. *Proc Natl Acad Sci USA*. (2017) **114**:E8204–13. doi: 10.1073/pnas.1703559114
 80. Bell GI. Models for the specific adhesion of cells to cells. *Science*. (1978) **200**:618–27. doi: 10.1126/science.347575
 81. Dembo M, Torney DC, Saxman K, Hammer D. The reaction-limited kinetics of membrane-to-surface adhesion and detachment. *Proc R Soc Lond B Biol Sci*. (1988) **234**:55–83. doi: 10.1098/rspb.1988.0038
 82. Marshall BT, Long M, Piper JW, Yago T, McEver RP, Zhu C. Direct observation of catch bonds involving cell-adhesion molecules. *Nature*. (2003) **423**:190–3. doi: 10.1038/nature01605
 83. Thomas W. Catch bonds in adhesion. *Annu Rev Biomed Eng*. (2008) **10**:39–57. doi: 10.1146/annurev.bioeng.10.061807.160427
 84. Guo B, Guilford WH. Mechanics of actomyosin bonds in different nucleotide states are tuned to muscle contraction. *Proc Natl Acad Sci USA*. (2006) **103**:9844–9. doi: 10.1073/pnas.0601255103
 85. Akiyoshi B, Sarangapani KK, Powers AF, Nelson CR, Reichow SL, Arellano-Santoyo H, et al. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature*. (2010) **468**:576–9. doi: 10.1038/nature09594
 86. Sarangapani KK, Yago T, Klopocki AG, Lawrence MB, Fieger CB, Rosen SD, et al. Low force decelerates L-selectin dissociation from P-selectin glycoprotein ligand-1 and endoglycan. *J Biol Chem*. (2004) **279**:2291–8. doi: 10.1074/jbc.M310396200
 87. Thomas WE, Trintchina E, Forero M, Vogel V, Sokurenko EV. Bacterial adhesion to target cells enhanced by shear force. *Cell*. (2002) **109**:913–23. doi: 10.1016/S0092-8674(02)00796-1
 88. Yakovenko O, Sharma S, Forero M, Tchesnokova V, Aprikian P, Kidd B, et al. FimH forms catch bonds that are enhanced by mechanical force due to allosteric regulation. *J Biol Chem*. (2008) **283**:11596–605. doi: 10.1074/jbc.M707815200
 89. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci USA*. (1995) **92**:5042–6. doi: 10.1073/pnas.92.11.5042
 90. Rabinowitz JD, Beeson C, Lyons DS, Davis MM, McConnell HM. Kinetic discrimination in T-cell activation. *Proc Natl Acad Sci USA*. (1996) **93**:1401–5. doi: 10.1073/pnas.93.4.1401
 91. Goldstein B, Coombs D, Faeder JR, Hlavacek WS. Kinetic proofreading model. *Adv Exp Med Biol*. (2008) **640**:82–94. doi: 10.1007/978-0-387-09789-3_8
 92. Wooldridge L, Hutchinson SL, Choi EM, Lissina A, Jones E, Mirza F, et al. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHCI) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *J Immunol*. (2003) **171**:6650–60. doi: 10.4049/jimmunol.171.12.6650
 93. Harding S, Lipp P, Alexander DR. A therapeutic CD4 monoclonal antibody inhibits TCR-zeta chain phosphorylation, zeta-associated protein of 70-kDa Tyr319 phosphorylation, and TCR internalization in primary human T cells. *J Immunol*. (2002) **169**:230–8. doi: 10.4049/jimmunol.169.1.230
 94. Boniface JJ, Rabinowitz JD, Wülfing C, Hampl J, Reich Z, Altman JD, et al. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. *Immunity*. (1998) **9**:459–66.
 95. Woods M, Guy R, Waldmann H, Glennie M, Alexander DR. A humanised therapeutic CD4 mAb inhibits TCR-induced IL-2, IL-4, and IL-10 secretion and expression of CD25, CD40L, and CD69. *Cell Immunol*. (1998) **185**:101–13. doi: 10.1006/cimm.1998.1287
 96. Wyer JR, Willcox BE, Gao GF, Gerth UC, Davis SJ, Bell JI, et al. T cell receptor and coreceptor CD8 α bind peptide-MHC independently and with distinct kinetics. *Immunity*. (1999) **10**:219–25. doi: 10.1016/S1074-7613(00)80022-9

97. Gao GF, Rao Z, Bell JI. Molecular coordination of alphabeta T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands. *Trends Immunol.* (2002) **23**:408–13. doi: 10.1016/S1471-4906(02)02282-2
98. Huang J, Meyer C, Zhu C. T cell antigen recognition at the cell membrane. *Mol Immunol.* (2012) **52**:155–64. doi: 10.1016/j.molimm.2012.05.004
99. Huang J, Edwards LJ, Evavold BD, Zhu C. Kinetics of MHC-CD8 interaction at the T cell membrane. *J Immunol.* (2007) **179**:7653–62. doi: 10.4049/jimmunol.179.11.7653
100. Hong J, Ge C, Jothikumar P, Yuan Z, Liu B, Bai K, et al. A TCR mechanotransduction signaling loop induces negative selection in the thymus. *Nat Immunol.* (2018) **19**:1379–90. doi: 10.1038/s41590-018-0259-z
101. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* (1992) **11**:3887–95. doi: 10.1002/j.1460-2075.1992.tb05481.x
102. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol.* (2007) **19**:813–24. doi: 10.1093/intimm/dxm057
103. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest.* (2015) **125**:3384–91. doi: 10.1172/JCI80011
104. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science.* (2017) **355**:1428–33. doi: 10.1126/science.aaf1292
105. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science.* (2017) **355**:1423–7. doi: 10.1126/science.aaf0683
106. Zhao Y, Harrison DL, Song Y, Ji J, Huang J, Hui E. Antigen-presenting cell-intrinsic PD-1 neutralizes PD-L1 in cis to attenuate PD-1 signaling in T cells. *Cell Rep.* (2018) **24**:379–90 e6. doi: 10.1016/j.celrep.2018.06.054
107. Chaudhri A, Xiao Y, Klee AN, Wang X, Zhu B, Freeman GJ. PD-L1 binds to B7-1 only in cis on the same cell surface. *Cancer Immunol Res.* (2018) **6**:921–9. doi: 10.1158/2326-6066.CIR-17-0316
108. Kucik DF, Dustin ML, Miller JM, Brown EJ. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J Clin Invest.* (1996) **97**:2139–44. doi: 10.1172/JCI118651
109. Lub M, van Kooyk Y, van Vliet SJ, Figdor CG. Dual role of the actin cytoskeleton in regulating cell adhesion mediated by the integrin lymphocyte function-associated molecule-1. *Mol Biol Cell.* (1997) **8**:341–51. doi: 10.1091/mbc.8.2.341
110. Stewart MP, McDowall A, Hogg N. LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca²⁺-dependent protease, calpain. *J Cell Biol.* (1998) **140**:699–707. doi: 10.1083/jcb.140.3.699
111. Billadeau DD, Nolz JC, Gomez TS. Regulation of T-cell activation by the cytoskeleton. *Nat Rev Immunol.* (2007) **7**:131–43. doi: 10.1038/nri2021
112. Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen B-C, Gawden-Bone C, et al. Actin depletion initiates events leading to granule secretion at the immunological synapse. *Immunity.* (2015) **42**:864–76. doi: 10.1016/j.immuni.2015.04.013
113. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol.* (2006) **7**:803–9. doi: 10.1038/ni1369
114. Dustin ML, Shaw AS. Costimulation: building an immunological synapse. *Science.* (1999) **283**:649–50. doi: 10.1126/science.283.5402.649
115. Yu Y, Smoligovets AA, Groves JT. Modulation of T cell signaling by the actin cytoskeleton. *J Cell Sci.* (2013) **126**:1049–58. doi: 10.1242/jcs.098210
116. Thauland TJ, Hu KH, Bruce MA, Butte MJ. Cytoskeletal adaptivity regulates T cell receptor signaling. *Sci Signal.* (2017) **10**:eaah3737. doi: 10.1126/scisignal.aah3737
117. Basu R, Whitlock BM, Husson J, Le Floch A, Jin W, Oyler-Yaniv A, et al. Cytotoxic T cells use mechanical force to potentiate target cell killing. *Cell.* (2016) **165**:100–10. doi: 10.1016/j.cell.2016.01.021
118. Carisey AF, Mace EM, Saeed MB, Davis DM, Orange JS. Nanoscale dynamism of actin enables secretory function in cytolytic cells. *Curr Biol.* (2018) **28**:489–502 e9. doi: 10.1016/j.cub.2017.12.044
119. Huang J, Zeng X, Sigal N, Lund PJ, Su LF, Huang H, et al. Detection, phenotyping, and quantification of antigen-specific T cells using a peptide-MHC dodecamer. *Proc Natl Acad Sci USA.* (2016) **113**:E1890–7. doi: 10.1073/pnas.1602488113
120. Hui KL, Upadhyaya A. Dynamic microtubules regulate cellular contractility during T-cell activation. *Proc Natl Acad Sci USA.* (2017) **114**:E4175–83. doi: 10.1073/pnas.1614291114
121. Korn ED, Carlier MF, Pantaloni D. Actin polymerization and ATP hydrolysis. *Science.* (1987) **238**:638–44. doi: 10.1126/science.3672117
122. Liu Z, Zhang Y-W, Chang Y-S, Fang F-D. The role of cytoskeleton in glucose regulation. *Biochem Moscow.* (2006) **71**:476–80. doi: 10.1134/S0006297906050026
123. Araiza-Olivera D, Uribe-Carvajal S, Chiquete-Flix N, Rosas-Lemus M, Ruz-Granados G, Sampedro JG, et al. Metabolic optimization by enzyme-enzyme and enzyme-cytoskeleton associations. In: Bubulya P, editor. *Cell Metabolism - Cell Homeostasis and Stress Response*. London: InTech (2012). p. 101–14.
124. Hu H, Juvekar A, Lyssiotis CA, Lien EC, Albeck JG, Oh D, et al. Phosphoinositide 3-kinase regulates glycolysis through mobilization of aldolase from the actin cytoskeleton. *Cell.* (2016) **164**:433–46. doi: 10.1016/j.cell.2015.12.042
125. Robinson GA, Waddington KE, Pineda-Torra I, Jury EC. Transcriptional regulation of T-cell lipid metabolism: implications for plasma membrane lipid rafts and T-cell function. *Front Immunol.* (2017) **8**:1636. doi: 10.3389/fimmu.2017.01636
126. Trowbridge IS, Thomas ML. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol.* (1994) **12**:85–116. doi: 10.1146/annurev.iy.12.040194.000505
127. Cordoba S-P, Choudhuri K, Zhang H, Bridge M, Basat AB, Dustin ML, et al. The large ectodomains of CD45 and CD148 regulate their segregation from and inhibition of ligated T-cell receptor. *Blood.* (2013) **121**:4295–302. doi: 10.1182/blood-2012-07-442251
128. Guo X, Yan C, Li H, Huang W, Shi X, Huang M, et al. Lipid-dependent conformational dynamics underlie the functional versatility of T-cell receptor. *Cell Res.* (2017) **27**:505–25. doi: 10.1038/cr.2017.42
129. Jaalouk DE, Lammerding J. Mechanotransduction gone awry. *Nat Rev Mol Cell Biol.* (2009) **10**:63–73. doi: 10.1038/nrm2597
130. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* (2006) **126**:677–89. doi: 10.1016/j.cell.2006.06.044
131. O'Connor RS, Hao X, Shen K, Bashour K, Akimova T, Hancock WW, et al. Substrate rigidity regulates human T cell activation and proliferation. *J Immunol.* (2012) **189**:1330–9. doi: 10.4049/jimmunol.1102757
132. Porter JC, Hogg N. Integrin cross talk: activation of lymphocyte function-associated antigen-1 on human T cells alters alpha4beta1- and alpha5beta1-mediated function. *J Cell Biol.* (1997) **138**:1437–47. doi: 10.1083/jcb.138.6.1437
133. Steiner O, Coisne C, Cecchelli R, Boscacci R, Deutsch U, Engelhardt B, et al. Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium. *J Immunol.* (2010) **185**:4846–55. doi: 10.4049/jimmunol.0903732
134. Schreiber TH, Shinder V, Cain DW, Alon R, Sackstein R. Shear flow-dependent integration of apical and subendothelial chemokines in T-cell transmigration: implications for locomotion and the multistep paradigm. *Blood.* (2007) **109**:1381–6. doi: 10.1182/blood-2006-07-032995
135. Rosen J, Brown JD, De S, Sinanan M, Hannaford B. Biomechanical properties of abdominal organs *in vivo* and postmortem under compression loads. *J Biomech Eng.* (2008) **130**:021020. doi: 10.1115/1.2898712
136. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. *Nat Rev Mol Cell Biol.* (2014) **15**:802–12. doi: 10.1038/nrm3896
137. Kadow CE, Georges PC, Janmey PA, Beningo KA. Polyacrylamide hydrogels for cell mechanics: steps toward optimization and alternative uses. In: Wang YL, Discher DE, editors. *Cell Mechanics, Methods in Cell Biology*. San Diego, CA: Elsevier (2007). p. 29–46.
138. Trappmann B, Gautrot JE, Connelly JT, Strange DGT, Li Y, Oyen ML, et al. Extracellular-matrix tethering regulates stem-cell fate. *Nat Mater.* (2012) **11**:642–9. doi: 10.1038/nmat3339
139. Calia SR, Burdick JA. A practical guide to hydrogels for cell culture. *Nat Methods.* (2016) **13**:405–14. doi: 10.1038/nmeth.3839
140. Frangos JA, Eskin SG, McIntire LV, Ives CL. Flow effects on prostacyclin production by cultured human endothelial cells. *Science.* (1985) **227**:1477–9. doi: 10.1126/science.3883488

141. Reich KM, Frangos JA. Effect of flow on prostaglandin E2 and inositol trisphosphate levels in osteoblasts. *Am J Physiol.* (1991) **261**:C428–32. doi: 10.1152/ajpcell.1991.261.3.C428
142. Shyy YJ, Hsieh HJ, Usami S, Chien S. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci USA.* (1994) **91**:4678–82. doi: 10.1073/pnas.91.11.4678
143. Dewey CF, Bussolari SR, Gimbrone MA, Davies PF. The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng.* (1981) **103**:177–85. doi: 10.1115/1.3138276
144. Wu C, Huang R-T, Kuo C-H, Kumar S, Kim CW, Lin Y-C, et al. Mechanosensitive PPAP2B regulates endothelial responses to atherorelevant hemodynamic forces. *Circ Res.* (2015) **117**:e41–53. doi: 10.1161/CIRCRESAHA.117.306457
145. Dai G, Kaazempur-Mofrad MR, Kamm RD, Zhang Y, Vaughn S, Garcia-Cardena G, et al. Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-prone and atherosclerosis-protected regions of the human vasculature. *Cardiovasc Pathol.* (2004) **13**:26. doi: 10.1016/j.carpath.2004.03.070
146. Wu D, Huang R-T, Hamanaka RB, Krause M, Oh M-J, Kuo C-H, et al. HIF-1 α is required for disturbed flow-induced metabolic reprogramming in human and porcine vascular endothelium. *elife.* (2017) **6**:e25217. doi: 10.7554/eLife.25217
147. Woolf E, Grigorova I, Sagiv A, Grabovsky V, Feigelson SW, Shulman Z, et al. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat Immunol.* (2007) **8**:1076–85. doi: 10.1038/ni1499
148. Dahl JB, Lin J-MG, Muller SJ, Kumar S. Microfluidic strategies for understanding the mechanics of cells and cell-mimetic systems. *Annu Rev Chem Biomol Eng.* (2015) **6**:293–317. doi: 10.1146/annurev-chembioeng-061114-123407
149. Butt H-J, Cappella B, Kappl M. Force measurements with the atomic force microscope: technique, interpretation and applications. *Surf Sci Rep.* (2005) **59**:1–152. doi: 10.1016/j.surfrep.2005.08.003
150. Hu KH, Butte MJ. T cell activation requires force generation. *J Cell Biol.* (2016) **213**:535–42. doi: 10.1083/jcb.201511053
151. Moffitt JR, Chemla YR, Smith SB, Bustamante C. Recent advances in optical tweezers. *Annu Rev Biochem.* (2008) **77**:205–28. doi: 10.1146/annurev.biochem.77.043007.090225
152. Nieminen TA, du Preez-Wilkinson N, Stilgoe AB, Loke VLY, Bui AAM, Rubinsztein-Dunlop H. Optical tweezers: theory and modelling. *J Quant Spectrosc Radiat Transf.* (2014) **146**:59–80. doi: 10.1016/j.jqsrt.2014.04.003
153. Chen W, Zarnitsyna VI, Sarangapani KK, Huang J, Zhu C. Measuring receptor-ligand binding kinetics on cell surfaces: from adhesion frequency to thermal fluctuation methods. *Cell Mol Bioeng.* (2008) **1**:276–88. doi: 10.1007/s12195-008-0024-8
154. Zarnitsyna V, Zhu C. T cell triggering: insights from 2D kinetics analysis of molecular interactions. *Phys Biol.* (2012) **9**:045005. doi: 10.1088/1478-3975/9/4/045005
155. Chen W, Evans EA, McEver RP, Zhu C. Monitoring receptor-ligand interactions between surfaces by thermal fluctuations. *Biophys J.* (2008) **94**:694–701. doi: 10.1529/biophysj.107.117895
156. Ju L, Chen Y, Li K, Yuan Z, Liu B, Jackson SP, et al. Dual biomembrane force probe enables single-cell mechanical analysis of signal crosstalk between multiple molecular species. *Sci Rep.* (2017) **7**:14185. doi: 10.1038/s41598-017-13793-3
157. Sawicka A, Babataheri A, Dogniaux S, Barakat AI, Gonzalez-Rodriguez D, Hivroz C, et al. Micropipette force probe to quantify single-cell force generation: application to T-cell activation. *Mol Biol Cell.* (2017) **28**:3229–39. doi: 10.1091/mbc.e17-06-0385
158. Roca-Cusachs P, Conte V, Trepas X. Quantifying forces in cell biology. *Nat Cell Biol.* (2017) **19**:742–51. doi: 10.1038/ncb3564
159. Wang JH-C, Li B. Application of cell traction force microscopy for cell biology research. *Methods Mol Biol.* (2009) **586**:301–13. doi: 10.1007/978-1-60761-376-3_17
160. Legant WR, Miller JS, Blakely BL, Cohen DM, Genin GM, Chen CS. Measurement of mechanical tractions exerted by cells in three-dimensional matrices. *Nat Methods.* (2010) **7**:969–71. doi: 10.1038/nmeth.1531
161. Judokusumo E, Tabdanov E, Kumari S, Dustin ML, Kam LC. Mechanosensing in T lymphocyte activation. *Biophys J.* (2012) **102**:L5–7. doi: 10.1016/j.bpj.2011.12.011
162. Savinko T, Guenther C, Uotila LM, Lloret Asens M, Yao S, Tojkander S, et al. Filamin A is required for optimal T cell integrin-mediated force transmission, flow adhesion, and T cell trafficking. *J Immunol.* (2018) **200**:3109–16. doi: 10.4049/jimmunol.1700913
163. Schoen I, Hu W, Klotzsch E, Vogel V. Probing cellular traction forces by micropillar arrays: contribution of substrate warping to pillar deflection. *Nano Lett.* (2010) **10**:1823–30. doi: 10.1021/nl100533c
164. Liu Y, Galior K, Ma VP-Y, Salaita K. Molecular tension probes for imaging forces at the cell surface. *Acc Chem Res.* (2017) **50**:2915–24. doi: 10.1021/acs.accounts.7b00305
165. Liu Y, Blanchfield L, Ma VP-Y, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci USA.* (2016) **113**:5610–5. doi: 10.1073/pnas.1600163113
166. Albrecht C, Blank K, Lalic-Mülthaler M, Hirler S, Mai T, Gilbert I, et al. DNA: a programmable force sensor. *Science.* (2003) **301**:367–70. doi: 10.1126/science.1084713
167. Dutta PK, Zhang Y, Blanchard A, Ge C, Rushdi M, Weiss K, et al. Programmable multivalent DNA-origami tension probes for reporting cellular traction forces. *Nano Lett.* (2018) **18**:4803–11. doi: 10.1021/acs.nanolett.8b01374
168. Grashoff C, Hoffman BD, Brenner MD, Zhou R, Parsons M, Yang MT, et al. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature.* (2010) **466**:263–6. doi: 10.1038/nature09198
169. Love PE, Hayes SM. ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harb Perspect Biol.* (2010) **2**:a002485. doi: 10.1101/cshperspect.a002485
170. Horejsi V, Zhang W, Schraven B. Transmembrane adaptor proteins: organizers of immunoreceptor signalling. *Nat Rev Immunol.* (2004) **4**:603–16. doi: 10.1038/nri1414
171. Balagopalan L, Kortum RL, Coussens NP, Barr VA, Samelson LE. The linker for activation of T cells (LAT) signaling hub: from signaling complexes to microclusters. *J Biol Chem.* (2015) **290**:26422–9. doi: 10.1074/jbc.R115.665869
172. Joseph N, Reicher B, Barda-Saad M. The calcium feedback loop and T cell activation: how cytoskeleton networks control intracellular calcium flux. *Biochim Biophys Acta.* (2014) **1838**:557–68. doi: 10.1016/j.bbame.2013.07.009
173. Cinamon G, Shinder V, Alon R. Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat Immunol.* (2001) **2**:515–22. doi: 10.1038/88710
174. Kumari S, Curado S, Mayya V, Dustin ML. T cell antigen receptor activation and actin cytoskeleton remodeling. *Biochim Biophys Acta.* (2014) **1838**:546–56. doi: 10.1016/j.bbame.2013.05.004
175. Thompson DW. *On Growth and Form*. 2nd ed. Cambridge: Cambridge University Press (1942).
176. Rothenberg EV. The chromatin landscape and transcription factors in T cell programming. *Trends Immunol.* (2014) **35**:195–204. doi: 10.1016/j.it.2014.03.001
177. Stephens AD, Banigan EJ, Adam SA, Goldman RD, Marko JF. Chromatin and lamin A determine two different mechanical response regimes of the cell nucleus. *Mol Biol Cell.* (2017) **28**:1984–96. doi: 10.1091/mbc.e16-09-0653
178. Miroshnikova YA, Nava MM, Wickström SA. Emerging roles of mechanical forces in chromatin regulation. *J Cell Sci.* (2017) **130**:2243–50. doi: 10.1242/jcs.202192
179. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol.* (2014) **14**:377–91. doi: 10.1038/nri3667
180. Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. *Annu Rev Immunol.* (1995) **13**:93–126. doi: 10.1146/annurev.13.040195.000521
181. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol.* (2010) **28**:445–89. doi: 10.1146/annurev-immunol-030409-101212

182. DeBerardinis RJ, Thompson CB. Cellular metabolism and disease: what do metabolic outliers teach us? *Cell*. (2012) **148**:1132–44. doi: 10.1016/j.cell.2012.02.032
183. Ciofani M, Zúñiga-Pflücker JC. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol*. (2005) **6**:881–8. doi: 10.1038/ni1234
184. Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. *J Exp Med*. (2015) **212**:1345–60. doi: 10.1084/jem.20151159
185. Wellen KE, Thompson CB. A two-way street: reciprocal regulation of metabolism and signalling. *Nat Rev Mol Cell Biol*. (2012) **13**:270–6. doi: 10.1038/nrm3305
186. Schwartzman JM, Thompson CB, Finley LWS. Metabolic regulation of chromatin modifications and gene expression. *J Cell Biol*. (2018) **217**:2247–59. doi: 10.1083/jcb.201803061
187. Feng S, Bowden N, Fragiadaki M, Souilhol C, Hsiao S, Mahmoud M, et al. Mechanical activation of hypoxia-inducible factor 1 α drives endothelial dysfunction at atheroprone sites. *Arterioscler Thromb Vasc Biol*. (2017) **37**:2087–101. doi: 10.1161/ATVBAHA.117.309249
188. Fang JS, Coon BG, Gillis N, Chen Z, Qiu J, Chittenden TW, et al. Shear-induced notch-Cx37-p27 axis arrests endothelial cell cycle to enable arterial specification. *Nat Commun*. (2017) **8**:2149. doi: 10.1038/s41467-017-01742-7
189. Mack JJ, Mosqueiro TS, Archer BJ, Jones WM, Sunshine H, Faas GC, et al. NOTCH1 is a mechanosensor in adult arteries. *Nat Commun*. (2017) **8**:1620. doi: 10.1038/s41467-017-01741-8
190. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature*. (2012) **481**:157–63. doi: 10.1038/nature10725
191. Ueda H, Howson JMM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature*. (2003) **423**:506–11. doi: 10.1038/nature01621
192. International Multiple Sclerosis Genetics Consortium, Wellcome Trust Case Control Consortium 2, Sawcer S, Hellenthal G, Pirinen M, Spencer CCA, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature*. (2011) **476**:214–9. doi: 10.1038/nature10251
193. Laklai H, Miroshnikova YA, Pickup MW, Collisson EA, Kim GE, Barrett AS, et al. Genotype tunes pancreatic ductal adenocarcinoma tissue tension to induce matricellular fibrosis and tumor progression. *Nat Med*. (2016) **22**:497–505. doi: 10.1038/nm.4082
194. Krause MD, Huang R-T, Wu D, Shentu T-P, Harrison DL, Whalen MB, et al. Genetic variant at coronary artery disease and ischemic stroke locus 1p32.2 regulates endothelial responses to hemodynamics. *Proc Natl Acad Sci USA*. (2018) **115**:E11349–58. doi: 10.1073/pnas.1810568115

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dual Role of CD4 in Peripheral T Lymphocytes

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OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
Imperial College London,
United Kingdom

Reviewed by:

Cosima T. Baldari,
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Karin Schillbach,
University of Tübingen, Germany

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 31 October 2018

Accepted: 08 March 2019

Published: 02 April 2019

Citation:

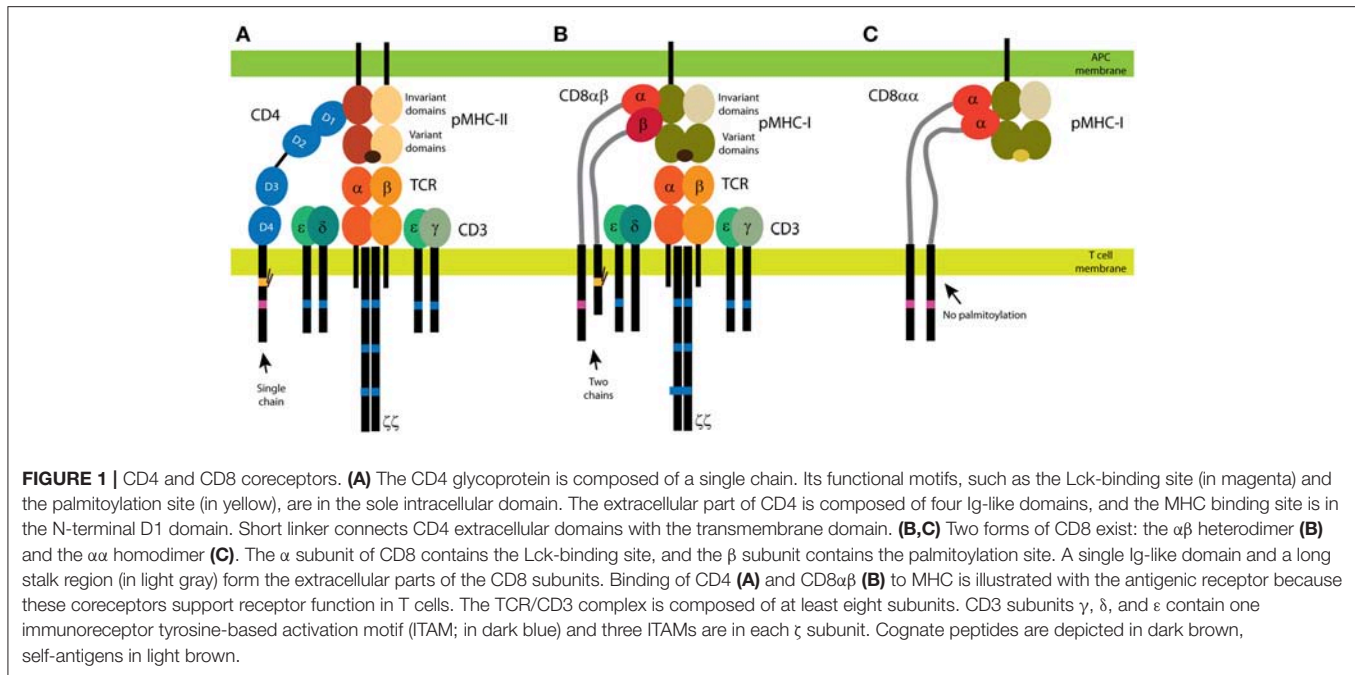
Glatzová D and Cebecauer M (2019)
Dual Role of CD4 in Peripheral T
Lymphocytes.
Front. Immunol. 10:618.
doi: 10.3389/fimmu.2019.00618

The interaction of T-cell receptors (TCRs) with self- and non-self-peptides in the major histocompatibility complex (MHC) stimulates crucial signaling events, which in turn can activate T lymphocytes. A variety of accessory molecules further modulate T-cell signaling. Of these, the CD4 and CD8 coreceptors make the most critical contributions to T cell sensitivity *in vivo*. Whereas, CD4 function in T cell development is well-characterized, its role in peripheral T cells remains incompletely understood. It was originally suggested that CD4 stabilizes weak interactions between TCRs and peptides in the MHC and delivers Lck kinases to that complex. The results of numerous experiments support the latter role, indicating that the CD4-Lck complex accelerates TCR-triggered signaling and controls the availability of the kinase for TCR in the absence of the ligand. On the other hand, extremely low affinity of CD4 for MHC rules out its ability to stabilize the receptor-ligand complex. In this review, we summarize the current knowledge on CD4 in T cells, with a special emphasis on the spatio-temporal organization of early signaling events and the relevance for CD4 function. We further highlight the capacity of CD4 to interact with the MHC in the absence of TCR. It drives the adhesion of T cells to the cells that express the MHC. This process is facilitated by the CD4 accumulation in the tips of microvilli on the surface of unstimulated T cells. Based on these observations, we suggest an alternative model of CD4 role in T-cell activation.

Keywords: T lymphocytes, CD4, TCR coreceptor, Lck, cell-cell adhesion, microvilli

INTRODUCTION

In vertebrates, T lymphocytes (also called T cells) continuously scan tissues for foreign antigens. On the surface of these cells, T-cell receptors (TCRs) recognize the antigens as short peptides bound to the major histocompatibility complex (MHC) on antigen-presenting cells (APCs; **Figure 1**). Thus, TCR-peptide-MHC (pMHC) pairs determine the specificity of the T cell-dependent immune response. However, several other surface receptors of T cells (e.g., CD2, CD4, CD5, CD8, CD28, and LFA-1) and of APCs (e.g., CD58, CTLA-4, and ICAM-1) can regulate the sensitivity and output of T cell responses. Of these receptors, CD4 and CD8 most critically contribute to the T cell function *in vivo* and thus are known as coreceptors of TCR. CD4 and CD8 share ligands with TCRs by binding to invariant segments of the MHC (**Figure 1**). As discussed below, CD4 and CD8 also contribute to T-cell development, homeostasis and antigenic response. However, the mechanisms behind these activities are not yet fully understood. This is especially true for CD4, which has extremely low affinity for its ligand but which is also essential in T-cell development and in the removal of pathogens during T cell-dependent immune responses.



In this work, we focus on dual role of CD4 in peripheral T cells. Contributions of CD4 to antigen-dependent TCR signaling are well-established. However, its antigen-independent function has not been studied in detail. After a brief introduction to the biochemistry of initial events, we focus on providing more in-depth insight into the spatio-temporal organization of signaling events in T cells so as to highlight the importance of nanoscopic localization of molecules. In later sections, we present and discuss the accumulated knowledge on function of CD4 in TCR signaling, with an emphasis on spatial organization of CD4 in T cells. Finally, we describe antigen-independent role of CD4 and speculate on its role in T-cell activation.

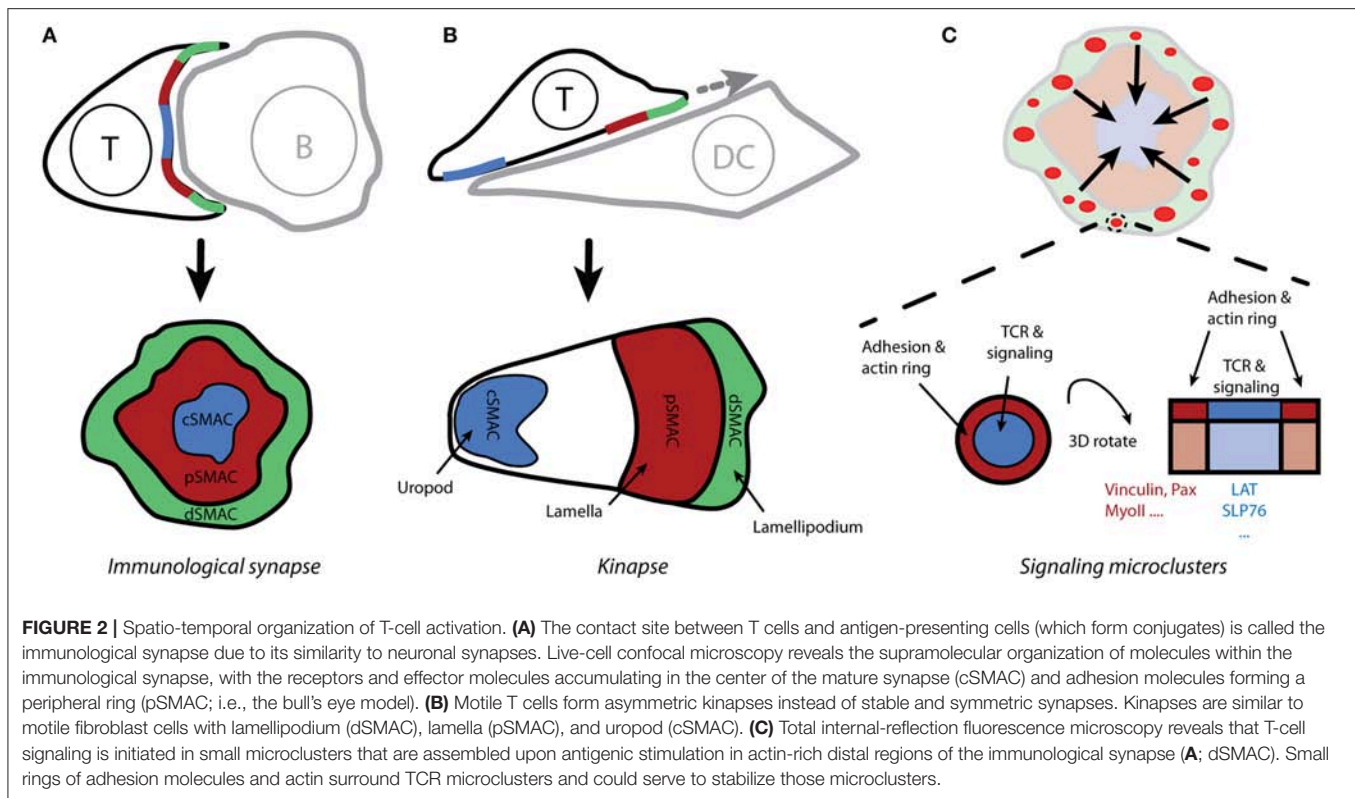
T CELLS AND ANTIGEN-INDUCED SIGNALING

T cells originate in bone-marrow haematopoietic stem cells. The progenitors of these cells migrate to the thymus, where thymocytes undergo a series of maturation and selection processes to complete the TCR expression and to avoid stimulation by self-antigens. This process, called thymic T cell development, gives rise to the peripheral pool of T cells, which mainly express $\alpha\beta$ TCR. Although 1–10% of T cells express $\gamma\delta$ TCR on their surface, these cells recognize non-peptidic antigens (1). This review focuses on peripheral $\alpha\beta$ T cells.

TCRs are heterodimers formed by the subunits α and β , each of which contains two extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain and a short intracellular tail that lacks any known structural or functional motif (Figure 1). The $\alpha\beta$ heterodimer forms a complex with the CD3 subunits (γ , δ , ϵ , ζ) for surface expression and full function

(Figure 1). The intracellular tails of CD3 subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs), which are involved in TCR-induced signaling. The TCR/CD3 complex lacks enzymatic activity. This distinguishes TCRs (and other immunoreceptors) from the receptors that directly stimulate downstream events upon binding to a ligand (e.g., receptor kinases).

Based on the current understanding of these processes, it is predicted that the interaction between TCRs and the pMHC is the first step toward antigen-induced T-cell activation. Consequently, early signaling events can be detected when Lck kinase phosphorylates ITAMs in the cytosolic tails of the CD3 subunits that are associated with TCR. Each ITAM contains two phosphorylated tyrosines, which serve as high-affinity docking sites for the tandem SH2 domains of ZAP-70 kinase. Lck also phosphorylates and binds ZAP-70 to induce its full activation (2). As Lck is bound to ZAP-70 via its SH2 domain, its open form provides a docking site (the SH3 domain) for the LAT adaptor protein. This leads to bridging between ZAP-70 and its substrates, LAT and SLP-76 (3). The ZAP-70 phosphorylation of the activating tyrosines on LAT forms a platform for the interactions of LAT with signaling molecules such as SLP-76, Grb2/Sos, PLC γ 1, and Vav1, and for the formation of a signalosome that regulates the downstream effector events associated with T-cell activation (4). Although the signaling pathways that are downstream of LAT have been thoroughly described, the initial events of the T-cell activation are still incompletely understood (5). Importantly, there is a lack of clarity regarding how and when Lck associates with the TCR-signaling complex. CD4 and CD8 potentially play important roles in this process because, in resting T cells, a large fraction of Lck is associated with these molecules (6, 7).



T-CELL ACTIVATION EVENTS: SPATIOTEMPORAL ORGANIZATION

After recognizing an antigen, T cells form tight contact with target cells. The instruction to stop the crawling of T cells comes from the interaction of TCRs with a cognate pMHC. This process, which is called inside-out signaling, enhances integrin affinity for its ligands [e.g., ICAM-1; (8, 9)]. Consequently, a site of extensive contact between T cells and APC forms. The contact site remains dynamic but is also highly organized in time and space. As such sites are reminiscent of the synapses formed between neurons they are named *immunological synapses* [IS; **Figure 2**; (10, 11)]. In addition to TCRs and pMHC, diverse stimulatory and inhibitory receptor-ligand pairs, as well as intracellular signaling molecules are localized in the IS during T-cell activation (4).

In the classical model of IS, the TCR receptor and signaling molecules accumulate in the center of a structure that is reminiscent of a bull's eye (**Figure 2A**). This area is called the central supramolecular activating cluster (cSMAC) and it is surrounded by an adhesive ring (LFA-1/ICAM-1) called a peripheral SMAC (pSMAC). The initial theory was that an IS functions as a stabilizing element, supporting sustained signaling via TCRs (12). Researchers challenged this concept after finding that T cells often lack a classical IS when conjugated with dendritic cells that are loaded with physiological levels of antigen (13). Several observations indicate that IS result from, rather than being a prerequisite for, T-cell signaling (14–17). It is thus evident that, at least in its early phase, the IS structure must be more dynamic than originally thought; this resulted in the

concept of *kinapses* (18). When forming kinapses (**Figure 2B**), migrating T cells decelerate upon stimulation but do not stop to form stable, symmetric synapses. The T-cell leading edge, which is reminiscent of the lamellipodium in motile fibroblasts, forms a structure for TCR signaling initiation, whereas adhesive molecules and a densely branched actin cytoskeleton accumulate in the lamella. Importantly, the signaling and the adhesive molecules are spatially segregated in both modes of the T cell-APC contact site: synapses and kinapses. Primary T cells mainly form kinapses when interacting with stimulatory cells or surfaces, both *in vivo* and *in vitro* (17, 19).

Whereas, intravital microscopy confirms the formation of organized contact sites between T cells and APCs *in vivo*, a better understanding of spatio-temporal organization of signaling events required new microscopy techniques (e.g., total internal-reflection fluorescence and super-resolution microscopy) and supported planar bilayers functionalized with activating (pMHC) and adhesive (ICAM-1) molecules (20, 21). Improved imaging conditions enabled the discovery of *signaling microclusters*. For instance, TCR microclusters are formed in the distal regions of the IS (**Figures 2A,C**) and represent the sites of signal initiation (22, 23). These microclusters are associated with essential signaling components such as Lck, ZAP-70 and LAT, but they exclude CD45 phosphatase, which can dephosphorylate ITAMs (2, 15, 23). Interestingly, TCR microclusters are mobile assemblies. In response to strong antigenic stimulation, the microclusters move centripetally from the periphery toward the center of the IS [**Figure 2C**; (11, 15, 22)]. It is unclear how these structures stabilize

during movement over several microns. The LFA-1/ICAM-1 micro-adhesive rings that surround microclusters, thus forming micro-synapses, may be a stabilizing factor [Figure 2C; (24)]. Of note, signaling microclusters gave way to *nanoclusters* due to improvements in microscopes, which provide more appropriate information about the size of these clusters: 100–500 nm (25–27).

The Varma group reported the existence of TCR microclusters in unstimulated T cells (28) and found that the number of TCR in microclusters remains constant upon stimulation. The LAT and Grb2 signaling molecules are associated with the pre-existing clusters, and CD45 is excluded from these structures even before the antigen stimulation (28). These results indicate that TCRs and effector molecules are pre-assembled in structures that are unresolvable using standard light microscopy [<300 nm; (29)]. Moreover, the data on the molecular organization of TCR microclusters are limited. Some researchers have reported the existence of TCR oligomers on the surface of T cells before antigen stimulation (30, 31), but others demonstrated that TCRs have random distribution and a monomeric character during ligand recognition (32–34). TCR assembly in higher-order structures then occurs upon stimulation (34). Importantly, pre-assembly of the receptor and the effector signaling molecules in higher-order structures could explain the rapid responsiveness of T cells (35). However, it is not clear whether these models authentically represent the T cells in the tissues of higher vertebrates. Current imaging technologies do not allow for high-resolution imaging of cellular structures in living animals.

T CELL CORECEPTORS

The previous sections focus on the essential molecules involved in T-cell activation and on descriptions of the morphological and molecular structures which were previously found to contribute to this process. These studies have usually investigated TCRs and downstream signaling molecules; they thus have provided little information about the involvement of coreceptors. The experiments have been often performed using stimulation with anti-CD3 or TCR antibodies, which overpass CD4 and CD8 coreceptors in the initial phase of signaling. Such stimulation activates T cells, as determined by the IL-2 production and increased expression of the CD25 and CD69 activation markers (36).

In vivo, CD4 and CD8 are essential for proper T cell development and thymic selection. These two coreceptors control the MHC specificity of selected thymocytes by limiting availability of Lck for TCR signaling in the absence of the ligand binding (6, 37). In peripheral T cells, the expression of coreceptors is mutually exclusive. CD4⁺ T cells primarily provide help for B lymphocytes and innate immune cells during infections, whereas most CD8⁺ T cells exhibit cytotoxicity toward virally infected or tumor cells. However, this definition is insufficient because the periphery contains many subsets of T cells with highly specific functions (38). In this review, we focus on general role of CD4 in T-cell activation, irrespective of cell type. We are aware that the coreceptor levels vary in T-cell subsets and that this can affect CD4 function (39). However, there is insufficient data to elaborate on specific function of CD4 in all

Box 1 | The CD4 and CD8 Coreceptors Are Structurally Diverse.

Even though both are called coreceptors, CD4 and CD8 have significantly different expression profiles and structures. CD8 is a dimer that occurs in two forms: the CD8 $\alpha\alpha$ homodimer and the CD8 $\alpha\beta$ heterodimer (Figure 1). It is predominantly expressed in a subset of T cells, but it can be found in some natural killer and dendritic cells (41–43). Little is known about the function of the CD8 $\alpha\alpha$ homodimer (44). The CD8 $\alpha\beta$ heterodimer supports TCR signaling when stimulated by antigens on MHC class I (45). In the heterodimer, two subunits with two intracellular tails can modulate TCR activity. Each subunit of CD8 also contains a single globular, Ig-like domain in the N-terminus; this domain is linked to the transmembrane domain via a long, flexible stalk. By contrast, CD4 comprises a single chain (Figure 1; (46)). Its single intracellular part defines all functions in the downstream signaling. Moreover, CD4 extracellular part is composed of four globular, Ig-like domains that are linked to the transmembrane domain only via a few amino-acid residues. Thus, CD4 extracellular part is extended further from the T-cell membrane and exhibits less flexibility, as compared to the extracellular part of CD8. Both CD4 and CD8 coreceptors can be palmitoylated and can bind Lck. In CD8, the palmitoylation site is in the β subunit (45), and the Lck-binding site is in the α subunit (47, 48). Both motifs are in single cytoplasmic tail of CD4 (Figure 1; (47–49)). These structural differences indicate that T cells use the specific properties of CD4 and CD8 to fine-tune their physiological roles.

CD4⁺ T-cell subsets. Sewell and colleagues reviewed CD8 and its function in T cells (40); please also see the direct comparison of CD4 and CD8 coreceptors in Box 1.

STRUCTURE AND FUNCTION OF CD4

Extracellular domain of CD4, which is responsible for the recognition of its ligands, is composed of four globular Ig-like domains (D1–D4; Figure 1). Whereas, the binding site for IL-16 is in the membrane-proximal D4 domain, the N-terminal D1 domain binds to a segment of the non-polymorphic β 2 domain of MHC class II (50, 51). Similarly, HIV (gp120) binds to D1 domain of CD4 (52). Important roles of CD4 in the life cycle of the HIV virus and in the activity of IL-16 in immune responses were reviewed recently (53, 54). To avoid the complexity of herein discussed processes, we focus on CD4 interaction with MHCII in the absence of other ligands.

The intracellular part is responsible for CD4 palmitoylation [residues 419 and 422 in human CD4 according to the UNIPROT numbering; (49)]. This reversible posttranslational modification is supposed to target proteins in lipid microdomains (55). It also contains a basic-rich motif (residues 423–427: sequence RHRRR) and a Lck-binding site (residues 445 and 447). The transmembrane domain of CD4 contains a conserved GGxxG motif, which was reported to mediate the dimerization of membrane proteins (56). However, such effect has not been confirmed for CD4. Rather, the mutation of this motif to GVxxL reduces the capacity of CD4 to enhance T cell sensitivity to weak antigens (57). This indicates that the importance of the CD4 transmembrane domain in T-cell activation but the molecular mechanism remains unknown.

The coreceptor CD4 is expressed in a subset of T cells, natural-killer (NK) cells, monocytes and macrophages. In macrophages and NK cells, CD4 plays a role in differentiation, migration and cytokine expression (58, 59). In T cells, it is involved in

thymic development and antigen recognition in the periphery (46). Although function of CD4 in the thymus is well-known, its role in the activation of peripheral T cells remains enigmatic. Originally, two models of CD4 function in peripheral T cells were suggested: 1) CD4 stabilizes the ternary complex of pMHC-TCR [**Model 1**; **Figure 3** (46)], and 2) CD4 recruits Lck kinase to the proximity of the TCR/CD3 complex in order to phosphorylate the ITAMs of CD3 molecules and initiate intracellular signaling during antigen-induced T cell activation [**Model 2**; **Figure 3**; (46, 60–62)].

The interaction of TCRs with pMHCII is CD4-independent. In some cases, as in the presence of a very strong agonist, this interaction can activate T cells (63). However, CD4 is required for the recognition of most antigens *in vivo*. The presence of the CD4 coreceptor enhances T cell sensitivity to antigens by 30- to 100-fold (64–67) and reduces by approximately tenfold the number of antigenic peptides on APCs that are required for sustained TCR signaling (68). Therefore, CD4 is often depicted as a part of the tightly assembled TCR receptor complex, along with agonist pMHCII (**Figure 3**, Model 1). However, the plasma membrane organization of the CD4-TCR-pMHC assembly remains unknown. In crystallographic studies of the quaternary complex (which comprises the extracellular domains of TCR, pMHCII and CD4), researchers have revealed a V-shaped arch that is created when TCR and CD4 bind simultaneously to the same pMHCII (51). This structure suggests that TCR bound to pMHCII forms one arm of the arch and that CD4 forms the other arm. The CD4-pMHCII contact site appears as the apex of this structure. The geometry of the interacting extracellular domains of pMHCII, TCR and CD4 supports the formation of the quaternary complex (69). However, the lack of the extracellular CD3 domains and other membrane components in the studied complex has led to speculations regarding the CD4-TCR-pMHC assembly under the physiological conditions of two interacting cells.

The Davis group suggested an alternative structure known as the “pseudo-dimer” model [**Model 1.2** in **Figure 3**; (70, 71)]. In this model, two TCR-pMHC pairs form a minimal signaling unit and CD4 bridges the two pairs by binding to MHCII, which contains an agonist (antigenic) peptide, as well as by associating with the TCR of the other TCR/MHCII pair, which contains endogenous (self) peptide (71). Importantly, in this model, they attempt to explain extreme sensitivity of CD4⁺ T cells by suggesting that endogenous peptides play a positive role in T-cell activation (70, 71). Most pMHCII on the surface of APCs contain peptides that are derived from endogenous proteins. Only very few antigenic peptides can be found on the MHCII of mature APCs (70, 72). Therefore, T cells must detect rare antigens in a sea of endogenous peptides by adjusting the TCR activation unit toward high sensitivity. However, T cells simultaneously have to distinguish small differences in affinities and/or the kinetics of TCR binding to agonist or self pMHCII (73). Recently, it was found that stimulatory TCR-pMHCII interaction involves numerous catch bonds; no such bonds exist for interactions that do not involve stimulation (74). This observation provides a new explanation for the numerous exceptions to the rule that 3D affinity of TCR for antigenic pMHCII is five- to seven-fold stronger than its affinity for ligands with endogenous peptides.

On the other hand, this finding does not explain how such minor differences in TCR-pMHC binding lead to opposite outputs in T cells. It has been predicted that CD4 would stabilize stimulatory (antigenic) but not homeostatic (self) TCR-pMHC interactions. It remains unclear how a molecule with extremely low 3D ($K_d > 2.5$ mM) and 2D ($K_d \sim 4800$ molecules/ μm^2) affinity for MHCII could achieve this, however (75). CD4 has a negligible effect on the TCR-pMHCII interaction (76, 77). On the other hand, CD4 forms a rather stable unit with Lck kinase (7), and the TCR/CD3 complex lacks enzymatic activity. Therefore, the signaling capacity and the ability to localize to MHCII-rich areas of the IS must determine CD4 function in T cells.

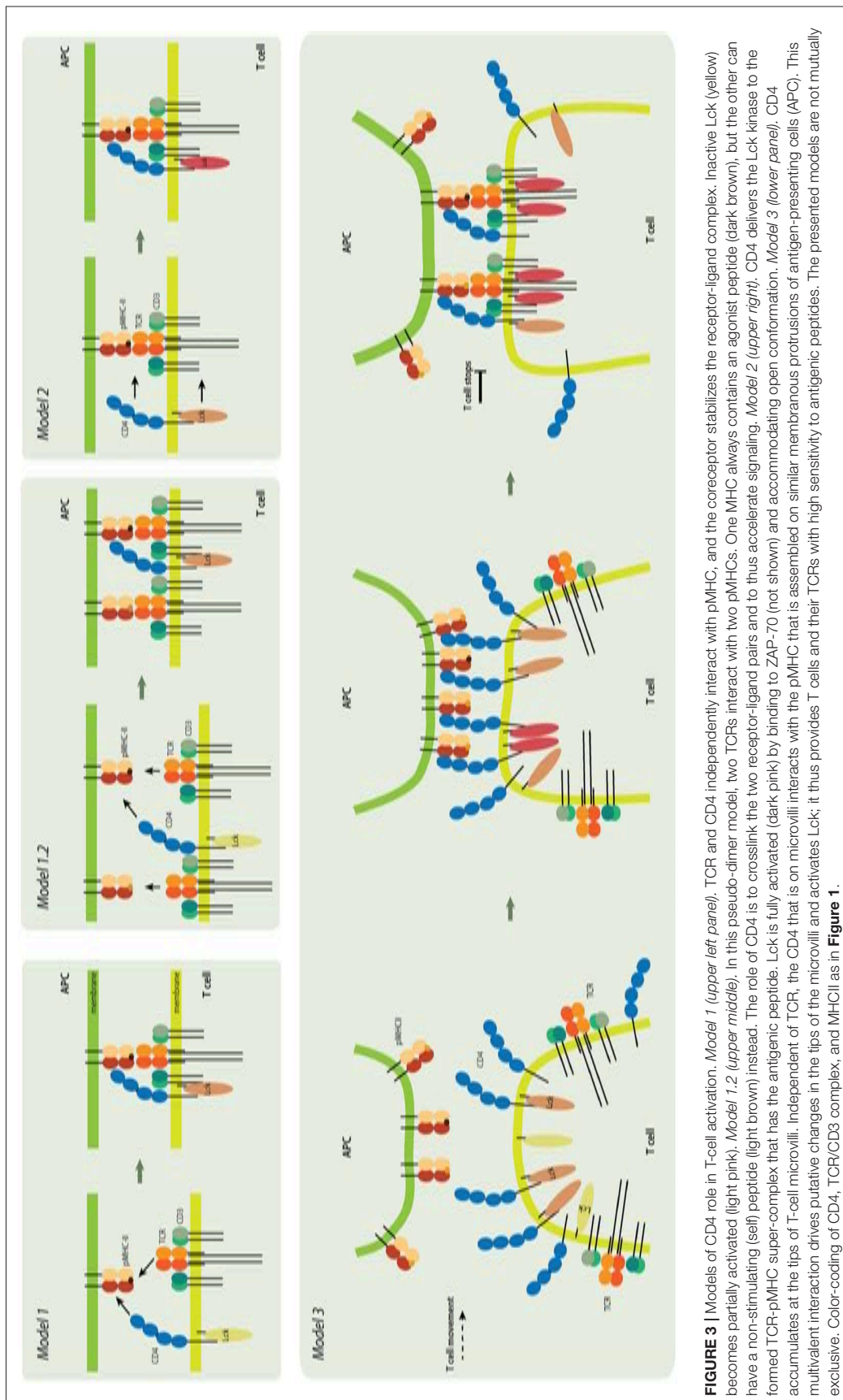
CD4 IN THE IMMUNOLOGICAL SYNAPSE

Varying levels of CD4 were reported to accumulate in the IS between T cells and the APCs that contain agonists (78–80). These discrepancies were probably caused by the employment of different experimental conditions and the sensitivity of applied imaging techniques. CD4 relocation to IS matches that of TCRs (78) in terms of timing, but CD4 may have faster kinetics (79). Whereas, TCRs and signaling molecules accumulate in the center of the IS upon strong stimulation (**Figure 2A**), CD4 was found to distribute evenly throughout the IS or preferentially locate to the periphery of the IS [>3 min; (78)]. Importantly, CD4 relocation to the contact site with MHCII-expressing cells is an antigen-independent process (79). Unlike with TCRs, the presence of antagonist does not prevent CD4 relocation toward the APC. These results and new observations from Kuhns and colleagues (81) indicate that CD4 moves in T cell membranes independent of TCR/CD3 complex and does not pre-associate with TCR in unstimulated cells.

At the plasma membrane, CD4 is strongly associated with Lck (7, 82). Therefore, CD4 localization to the IS results in Lck accumulation therein (68, 83). Lck shows a delayed association with the IS in CD4-knockout T cells, which in turn delays phosphorylation of the Lck activation site (residue Tyr₃₉₄ in mouse) and reduces CD4-knockout T cells responsiveness to antigens (83). On the other hand, phosphorylation of activatory tyrosine in Lck is not crucial to T-cell activation because anti-CD3 ϵ antibodies do not induce such phosphorylation but can stimulate T cells (82, 84, 85). This can be explained by the modest increase in Lck activity (two- to three-fold) upon phosphorylation of the activating tyrosine. CD4 thus delivers the crucial kinase to the site of TCR triggering and enables its full activation to maximize the sensitivity of T cells toward rare and weak antigens [**Model 2** of the CD4 function in T cells - **Figure 3**; (62)]. Lck kinase activity does not affect CD4 accumulation in the IS because the Src-family kinase inhibitor (PP1) does not prevent localization of CD4 to the contact site with APCs (79).

ANTIGEN-INDEPENDENT ROLE OF CD4 IN T CELLS

CD4 was originally described as an adhesion molecule that enhances the contact between T cells and APCs (86, 87). In their pillar work, Doyle and Strominger found a direct correlation



between the extent of cell-to-cell adhesion and the level of MHCII and CD4 expression. Using a monolayer of CD4-expressing fibroblasts and Raji B cell line, which expresses high levels of MHCII, they eliminated the possibility that TCR or other T cell-specific molecules are involved in the interaction (86); observing no adhesion of the cells that did not express MHCII to CD4⁺ cells, thus confirming the specificity of that interaction. However, using surface plasmon resonance assays, other researchers have shown that CD4 binds the MHCII molecule with an extremely low 3D affinity [see above; (75, 88)]. This is further supported by the results of 2D binding studies on MHCII-expressing cells and lipid bilayer-anchored extracellular domains of CD4 (as well as of CD2 to allow cell adhesion). In agreement with the adhesion studies (86), this binding is specific because the MHCII non-expressing cells did not bind to CD4 on supported planar bilayers (75). CD4 very weakly bound to MHCII (approaching the detection limit of the method) according to an adhesion frequency assay with micropipette-attached interacting cells (77). Therefore, it is unclear how CD4 facilitates adhesion between coreceptor- and MHCII-expressing cells.

The way to explain the ability of CD4-MHCII interaction to facilitate both cell-to-cell adhesion and the antigen-independent accumulation of coreceptors at contact sites with MHCII-expressing cells can be the organization of these molecules in higher-order structures. Multimerization enhances the avidity of the TCR-MHC interaction (89–91). Similarly, the multivalent interaction of CD4 and its ligand can provide this interaction with the appropriate strength. In this direction, it was suggested that palmitoylation targets CD4 to membrane lipid domains, called lipid rafts (55). Because the support for the existence of these domains in living cells remains inconclusive (92, 93), future studies must determine whether CD4 is associated with such entities and, if so, with what kinetics. Alternatively, CD4 can form large oligomers in unstimulated T cells (94). These oligomers must be disassembled upon stimulation because it is unlikely that such large structures are associated with the TCR/CD3 complex or with TCR microclusters. CD4 associates closely (< 5 nm distance, as determined by Förster resonance energy transfer, FRET) with the TCR/pMHC complex upon stimulation (68, 71, 79). Whereas, biochemical and functional data indicate the existence of CD4 dimers (or higher order oligomers) in unstimulated T cells (94, 95), direct observations of fixed or living cells using FRET has provided conflicting data (57, 96, 97). In these studies, FRET values are very low compared to the dimeric controls, which indicates either that only a small fraction of CD4 is oligomeric or that these structures are highly unstable. Moreover, the FRET-based characterization of CD4 oligomers may suffer from the limitations of this method which cannot distinguish between clustered molecules and oligomers, except only when a protein is assembled in a stable structure and when the appropriate data analysis methods are used (98). Such conditions have not yet been applied in studies of CD4 oligomerization. Thus, direct proof of CD4 oligomerization in living cells is still missing.

Another possible way to increase availability of CD4 for multivalent interactions is the formation of molecular clusters with a high density of coreceptors. Such CD4 nanoclusters

exist in both unstimulated and stimulated murine T-cell blasts (99) and unstimulated Jurkat T cells (100). We also previously found that clustering depends on the presence of intact CD4 extracellular domains and palmitoylation sites (100). Clustering in nanoscopic structures (average diameter of ~100 nm) allows for multivalent ligand binding and frequent rebinding (29), which can provide the CD4-MHC interaction with sufficient strength to stabilize the sites of the contact between CD4- and MHCII-expressing cells (86, 87). Shapes and molecular densities of CD4 clusters (99, 100) are similar to those of TCR and associated effector signaling molecules (25, 101). On the other hand, the 2D character of the applied analytical methods does not provide a full understanding of these structures.

More recently, TCRs and their effector molecules were found to accumulate in the tips of membrane protrusions that are reminiscent of *microvilli* (102, 103). Microvilli are finger-like plasma membrane protrusions with diameter ~100 nm; they are formed by cross-linked actin bundles that are tightly associated with membranes (**Figure 4A**). In sensory cells (e.g., hairy cells) or intestinal epithelial cells, these structures form extensive cell surfaces and accumulate selected receptors on their tips. The functions of microvilli in lymphocytes (**Figure 4B**) are less understood. Using scanning electron microscopy, microvilli were found to form primary contact sites with antigen-presenting dendritic cells (104, 105). The molecular details of this interaction have remained unknown until very recently (103). CD4 also accumulates in the microvilli of cultured T cells in a process that is regulated by the coreceptor association with Lck (106, 107). It is, therefore, possible that CD4 nanoclusters are indeed molecular assemblies of the coreceptor on the tips of the microvilli (**Figure 4C**). CD4 accumulation in the tips of the microvilli could explain its ability to facilitate adhesion between T cells and MHCII-positive cells.

Microvilli on dendritic cells interact with their counterparts on T cells during the antigen recognition (105). Biochemical and preliminary microscopy data indicate that MHCII accumulates in specialized membrane domains in a process that is regulated by members of the tetraspanins family, CD9 and CD63 (108–110). Tetraspanins CD9 and CD53 modulate the size and frequency of microvilli in leukocytes and epithelial cells (104, 111). However, it is unclear whether tetraspanins can enhance the sorting of MHCII to the tips of the microvilli; in addition, the organization of these structures on APCs must be characterized in the future.

CONCLUSIONS AND FUTURE PERSPECTIVES

The results of 30 years of research indicate that CD4 has a dual function in peripheral T cells (and potentially in thymocytes). Firstly, it interacts with its ligand in an antigen-independent manner so as to induce contact between T cells and MHCII-expressing cells (**Model 3; Figure 3**). Second, CD4 interacts with pMHCII-TCR in an antigen-dependent manner so as to deliver Lck kinase to the complex and thus enhance T cell sensitivity (**Model 2**). These two roles of CD4 do not have to be mutually exclusive. A direct role of CD4 in stabilizing

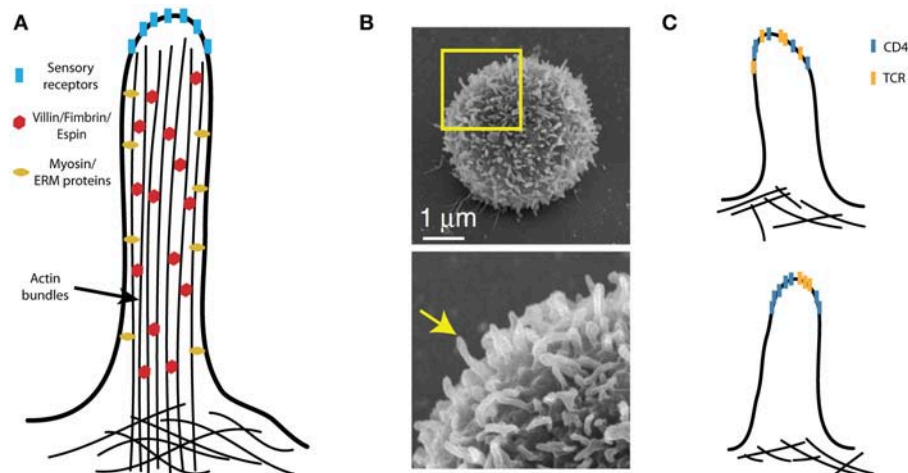


FIGURE 4 | Microvilli in T-cell activation. **(A)** A schematic illustration of a microvillus with villin, fimbrin and espin internally cross-linking compact actin bundles, which tightly fill the microvilli. The plasma membrane is also closely associated with the actin bundles via the ezrin, redoxin, and moesin (ERM) proteins; the dynamics of microvilli involve specialized myosins. The tips of the sensory (i.e., hairy) cell microvilli accumulate critical receptors. **(B)** The peripheral T cells are covered by finger-like protrusions that are reminiscent of microvilli on sensory cells. **(C)** Molecular organization of CD4 with respect to other T-cell signaling molecules (e.g., TCR) on microvilli remains unknown. These molecules may be randomly distributed (upper panel) or assembled into specific domains (lower panel). **(B)** is adapted from Kim et al. (104) licensed under Creative Commons **(CC BY 4.0)**.

the TCR-pMHCII interaction (**Model 1**) is not accepted any longer (62).

The antigen-independent function of CD4 is less understood. Its localization to microvilli (106) - as well as the evidence that the microvilli are the primary contacts between T cells and APCs (103–105) - indicates that CD4 can function as a scanning machinery, thus allowing T cells to select for cells that have MHCII on their surface. This may help to target TCRs toward the places with the highest MHC density and thus avoid interactions with cells that lack the ligand. The nanoscopic 3D organization of MHCII on APCs remains unknown. A full molecular anatomy of a synaptic vesicle (which was purified from neurons) indicates extreme protein density and reveals specific functional distribution of molecules in these structures (112). We believe that the creation of a similar model of microvillar tips on T cells and APCs will help to answer several intriguing questions regarding the initial phase of T-cell activation.

Importantly, the contact sites formed between T cells and APCs should also be explored using super-resolution techniques that have recently been adapted for living cells, including stimulated emission depletion (STED), super-resolution optical fluctuation imaging (SOFI) and lattice light sheet microscopy (103, 113, 114). Such studies are needed to confirm whether microvilli dominate the T cell-APC contact site and to determine the function of microvilli in T-cell activation. Other forms of membrane protrusions, such as filopodia and membrane ruffles, may also participate in this process. If CD4 scans the surface of the surrounding cells for MHCII-rich areas, it will be very important to determine whether such interactions

stimulate changes in the T-cell membrane topology or molecular architecture of T cell microvilli, as has been observed in mechanosensory cells (115). Such changes may predetermine the local environment that TCRs require for rapid but highly selective antigen-induced signaling.

CD4 is one of the most studied molecules in the human body. This is mainly because it facilitates the infection of T cells with HIV-1. On the other hand, its function in various subsets of peripheral T cells remains poorly understood. New technologies that enable high-detail imaging of cellular structures provide previously unexplored ways to resolve such long-neglected topics. Using these techniques can lead to a better understanding of multifaceted role of CD4 in peripheral T cells and, potentially, in other CD4-expressing cells.

AUTHOR CONTRIBUTIONS

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

FUNDING

The Czech Science Foundation funded this work (19-07043S).

ACKNOWLEDGMENTS

We would like to thank Silke Kerruth for providing a critical reading of the manuscript.

REFERENCES

- Garcillan B, Marin AV, Jimenez-Reinoso A, Briones AC, Munoz-Ruiz M, Garcia-Leon MJ, et al. gammadelta T Lymphocytes in the diagnosis of human T cell receptor immunodeficiencies. *Front Immunol.* (2015) 6:20. doi: 10.3389/fimmu.2015.00020
- Chakraborty AK, Weiss A. Insights into the initiation of TCR signaling. *Nat Immunol.* (2014) 15:798–807. doi: 10.1038/ni.2940
- Lo WL, Shah NH, Ahsan N, Horkova V, Stepanek O, Salomon AR, et al. Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT. *Nat Immunol.* (2018) 19:733–41. doi: 10.1038/s41590-018-0131-1
- Malissen B, Aguado E, Malissen M. Role of the LAT adaptor in T-cell development and Th2 differentiation. *Adv Immunol.* (2005) 87:1–25. doi: 10.1016/S0065-2776(05)87001-4
- Courtney AH, Lo WL, Weiss A. TCR Signaling: mechanisms of initiation and propagation. *Trends Biochem Sci.* (2018) 43:108–23. doi: 10.1016/j.tibs.2017.11.008
- Van Laethem F, Sarafova SD, Park JH, Tai X, Pobezinsky L, Guinter TI, et al. Deletion of CD4 and CD8 coreceptors permits generation of alphabetaT cells that recognize antigens independently of the MHC. *Immunity.* (2007) 27:735–50. doi: 10.1016/j.immuni.2007.10.007
- Ballek O, Valecka J, Manning J, Philipp D. The pool of preactivated Lck in the initiation of T-cell signaling: a critical re-evaluation of the Lck standby model. *Immunol Cell Biol.* (2015) 93:384–95. doi: 10.1038/icb.2014.100
- Dustin ML, Bromley SK, Kan Z, Peterson DA, Unanue ER. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc Natl Acad Sci USA.* (1997) 94:3909–13. doi: 10.1073/pnas.94.8.3909
- Drbal K, Angelisova P, Cerny J, Pavlistova D, Cebecauer M, Novak P, et al. Human leukocytes contain a large pool of free forms of CD18. *Biochem Biophys Res Commun.* (2000) 275:295–9. doi: 10.1006/bbrc.2000.3299
- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature.* (1998) 395:82–6. doi: 10.1038/25764
- Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science.* (1999) 285:221–7. doi: 10.1126/science.285.5425.221
- Dustin ML, Tseng SY, Varma R, Campi G. T cell-dendritic cell immunological synapses. *Curr Opin Immunol.* (2006) 18:512–6. doi: 10.1016/j.coi.2006.05.017
- Brossard C, Feuillet V, Schmitt A, Randriamampita C, Romao M, Raposo G, et al. Multifocal structure of the T cell - dendritic cell synapse. *Eur J Immunol.* (2005) 35:1741–53. doi: 10.1002/eji.200425857
- Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature.* (2004) 427:154–9. doi: 10.1038/nature02238
- Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity.* (2006) 25:117–27. doi: 10.1016/j.immuni.2006.04.010
- Friedman RS, Beemiller P, Sorensen CM, Jacobelli J, Krummel MF. (2010). Real-time analysis of T cell receptors in naive cells in vitro and in vivo reveals flexibility in synapse and signaling dynamics. *J Exp Med.* 207:2733–49. doi: 10.1084/jem.20091201
- Moreau HD, Lemaitre F, Garrod KR, Garcia Z, Lennon-Dumenil AM, Bousso P. Signal strength regulates antigen-mediated T-cell deceleration by distinct mechanisms to promote local exploration or arrest. *Proc Natl Acad Sci USA.* (2015) 112:12151–6. doi: 10.1073/pnas.1506654112
- Dustin ML. Cell adhesion molecules and actin cytoskeleton at immune synapses and kinapses. *Curr Opin Cell Biol.* (2007) 19:529–33. doi: 10.1016/j.ccb.2007.08.003
- Mayya V, Judokusumo E, Abu Shah E, Peel CG, Neiswanger W, Depoil D, et al. Durable Interactions of T Cells with T cell receptor stimuli in the absence of a stable immunological synapse. *Cell Rep.* (2018) 22:340–9. doi: 10.1016/j.celrep.2017.12.052
- Dustin ML. Supported bilayers at the vanguard of immune cell activation studies. *J Struct Biol.* (2009) 168:152–60. doi: 10.1016/j.jsb.2009.05.007
- Owen DM, Gaus K, Magee AI, Cebecauer M. Dynamic organization of lymphocyte plasma membrane: lessons from advanced imaging methods. *Immunology.* (2010) 131:1–8. doi: 10.1111/j.1365-2567.2010.03319.x
- Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med.* (2005) 202:1031–6. doi: 10.1084/jem.20051182
- Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol.* (2005) 6:1253–62. doi: 10.1038/ni1272
- Hashimoto-Tane A, Sakuma M, Ike H, Yokosuka T, Kimura Y, Ohara O, et al. Micro-adhesion rings surrounding TCR microclusters are essential for T cell activation. *J Exp Med.* (2016) 213:1609–25. doi: 10.1084/jem.20151088
- Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis M, et al. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat Immunol.* (2010) 11:90–6. doi: 10.1038/ni.1832
- Williamson DJ, Owen DM, Rossy J, Magenau A, Wehrmann M, Gooding JJ, et al. Pre-existing clusters of the adaptor Lat do not participate in early T cell signaling events. *Nat Immunol.* (2011) 12:655–62. doi: 10.1038/ni.2049
- Soares H, Henriques R, Sachse M, Ventimiglia L, Alonso MA, Zimmer C, et al. Regulated vesicle fusion generates signaling nanoterritories that control T cell activation at the immunological synapse. *J Exp Med.* (2013) 210:2415–33. doi: 10.1084/jem.20130150
- Crites TJ, Padhan K, Muller J, Krogsgaard M, Gudla PR, Lockett SJ, et al. TCR microclusters pre-exist and contain molecules necessary for TCR signal transduction. *J Immunol.* (2014) 193:56–67. doi: 10.4049/jimmunol.1400315
- Cebecauer M, Spitaler M, Serge A, Magee AI. Signalling complexes and clusters: functional advantages and methodological hurdles. *J Cell Sci.* (2010) 123:309–20. doi: 10.1242/jcs.061739
- Schamel WW, Arechaga I, Risueno RM, van Santen HM, Cabezas P, Risco C, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J Exp Med.* (2005) 202:493–503. doi: 10.1084/jem.20042155
- Kumar R, Ferez M, Swamy M, Arechaga I, Rejas MT, Valpuesta JM, et al. Increased sensitivity of antigen-experienced T cells through the enrichment of oligomeric T cell receptor complexes. *Immunity.* (2011) 35:375–87. doi: 10.1016/j.immuni.2011.08.010
- James JR, McColl J, Oliveira MI, Dunne PD, Huang E, Jansson A, et al. The T cell receptor triggering apparatus is composed of monovalent or monomeric proteins. *J Biol Chem.* (2011) 286:31993–2001. doi: 10.1074/jbc.M111.219212
- Brameshuber M, Kellner F, Rossboth BK, Ta H, Alge K, Sevcik E, et al. Monomeric TCRs drive T cell antigen recognition. *Nat Immunol.* (2018) 19:487–96. doi: 10.1038/s41590-018-0092-4
- Rossboth B, Arnold AM, Ta H, Platzer R, Kellner F, Huppa JB, et al. TCRs are randomly distributed on the plasma membrane of resting antigen-experienced T cells. *Nat Immunol.* (2018) 19:821–7. doi: 10.1038/s41590-018-0162-7
- Huse M, Klein LO, Girvin AT, Faraj JM, Li QJ, Kuhns MS, et al. Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. *Immunity.* (2007) 27:76–88. doi: 10.1016/j.immuni.2007.05.017
- Trickett A, Kwan YL. T cell stimulation and expansion using anti-CD3/CD28 beads. *J Immunol Methods.* (2003) 275:251–5. doi: 10.1016/S0022-1759(03)00010-3
- Van Laethem F, Tikhonova AN, Pobezinsky LA, Tai X, Kimura MY, Le Saout C, et al. Lck availability during thymic selection determines the recognition specificity of the T cell repertoire. *Cell.* (2013) 154:1326–41. doi: 10.1016/j.cell.2013.08.009
- Pennock ND, White JT, Cross EW, Cheney EE, Tamburini BA, Kedl R, et al. T cell responses: naive to memory and everything in between. *Adv Physiol Educ.* (2013) 37:273–83. doi: 10.1152/advan.00066.2013
- Itoh Y, Wang Z, Ishida H, Eichelberg K, Fujimoto N, Makino J, et al. Decreased CD4 expression by polarized T helper 2 cells contributes to suboptimal TCR-induced phosphorylation and reduced Ca²⁺ signaling. *Eur J Immunol.* (2005) 35:3187–95. doi: 10.1002/eji.200526064
- Cole DK, Laugel B, Clement M, Price DA, Wooldridge L, Sewell A, et al. The molecular determinants of CD8 co-receptor function. *Immunology.* (2012) 137:139–48. doi: 10.1111/j.1365-2567.2012.03625.x

41. Nakayama K, Nakayama K, Negishi I, Kuida K, Louie MC, Kanagawa O, et al. Requirement for CD8 beta chain in positive selection of CD8-lineage T cells. *Science*. (1994) 263:1131–3. doi: 10.1126/science.8108731
42. Gibbings DJ, Marcet-Palacios M, Sekar Y, Ng MC, Befus AD. CD8 alpha is expressed by human monocytes and enhances Fc gamma R-dependent responses. *BMC Immunol*. (2007) 8:12. doi: 10.1186/1471-2172-8-12
43. Schuster P, Thomann S, Werner M, Vollmer J, Schmidt B. A subset of human plasmacytoid dendritic cells expresses CD8alpha upon exposure to herpes simplex virus type 1. *Front Microbiol*. (2015) 6:557. doi: 10.3389/fmicb.2015.00557
44. Gangadharan D, Cheroutre H. The CD8 isoform CD8alphaalpha is not a functional homologue of the TCR co-receptor CD8alphabeta. *Curr Opin Immunol*. (2004) 16:264–70. doi: 10.1016/j.coi.2004.03.015
45. Arcaro A, Gregoire C, Boucheron N, Stoltz S, Palmer E, Malissen B, et al. Essential role of CD8 palmitoylation in CD8 coreceptor function. *J Immunol*. (2000) 165:2068–76. doi: 10.4049/jimmunol.165.4.2068
46. Janeway, C. A. Jr. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu Rev Immunol*. (1992) 10:645–74. doi: 10.1146/annurev.iy.10.040192.003241
47. Lin H, Hutchcroft JE, Andoniou CE, Kamoun M, Band H, Bierer B, et al. Association of p59(fyn) with the T lymphocyte costimulatory receptor CD2. Binding of the Fyn Src homology (SH) 3 domain is regulated by the Fyn SH2 domain. *J Biol Chem*. (1998) 273:19914–21. doi: 10.1074/jbc.273.31.19914
48. Kim PW, Sun ZY, Blacklow SC, Wagner G, Eck MJ. A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. *Science*. (2003) 301:1725–8. doi: 10.1126/science.1085643
49. Crise B, Rose JK. Identification of palmitoylation sites on CD4, the human immunodeficiency virus receptor. *J Biol Chem*. (1992) 267:13593–7.
50. König R, Huang LY, Germain RN. MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature*. (1992) 356:796–8. doi: 10.1038/356796a0
51. Yin Y, Wang XX, Mariuzza RA. Crystal structure of a complete ternary complex of T-cell receptor, peptide-MHC, and CD4. *Proc Natl Acad Sci USA*. (2012) 109:5405–10. doi: 10.1073/pnas.1118801109
52. Wang J-H, Meijers R, Xiong Y, Liu J-H, Sakihama T, Zhang R, et al. Crystal structure of the human CD4 N-terminal two-domain fragment complexed to a class II MHC molecule. *PNAS*. (2001) 98:10799–804. doi: 10.1073/pnas.191124098
53. Wilen CB, Tilton JC, Doms RW. HIV: cell binding and entry. *Cold Spring Harb Perspect Med*. (2012) 2:a006866. doi: 10.1101/cshperspect.a006866
54. Richmond J, Tuzova M, Cruikshank W, Center D. Regulation of cellular processes by interleukin-16 in homeostasis and cancer. *J Cell Physiol*. (2014) 229:139–47. doi: 10.1002/jcp.24441
55. Balamuth F, Brogdon JL, Bottomly K. CD4 raft association and signaling regulate molecular clustering at the immunological synapse site. *J Immunol*. (2004) 172:5887–92. doi: 10.4049/jimmunol.172.10.5887
56. Cymer F, Veerappan A, Schneider D. Transmembrane helix-helix interactions are modulated by the sequence context and by lipid bilayer properties. *Biochim Biophys Acta*. (2012) 1818:963–73. doi: 10.1016/j.bbame.2011.07.035
57. Parrish HL, Glassman CR, Keenen MM, Deshpande NR, Bronnmann MP, Kuhns M, et al. A transmembrane domain GGxxG motif in CD4 contributes to its Lck-independent function but does not mediate CD4 dimerization. *PLoS ONE*. (2015) 10:e0132333. doi: 10.1371/journal.pone.0132333
58. Bernstein HB, Plasterer MC, Schiff SE, Kitchen CM, Kitchen S, Zack J, et al. CD4 expression on activated NK cells: ligation of CD4 induces cytokine expression and cell migration. *J Immunol*. (2006) 177:3669–76. doi: 10.4049/jimmunol.177.6.3669
59. Zhen A, Krutzik SR, Levin BR, Kasparian S, Zack JA, Kitchen S, et al. CD4 ligation on human blood monocytes triggers macrophage differentiation and enhances HIV infection. *J Virol*. (2014) 88:9934–46. doi: 10.1128/JVI.00616-14
60. Straus DB, Weiss A. Genetic evidence for the involvement of the Lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*. (1992) 70:585–93. doi: 10.1016/0092-8674(92)90428-F
61. Straus DB, Weiss A. The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. *J Exp Med*. (1993) 178:1523–30. doi: 10.1084/jem.178.5.1523
62. Artyomov MN, Lis M, Devadas S, Davis MM, Chakraborty AK. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proc Natl Acad Sci U S A*. (2010) 107:16916–21. doi: 10.1073/pnas.1010568107
63. van der Merwe PA, Dushek O. Mechanisms for T cell receptor triggering. *Nat Rev Immunol*. (2011) 11:47–55. doi: 10.1038/nri2887
64. Marrack P, Endres R, Shimonkevitz R, Zlotnik A, Dialynas D, Fitch F, et al. The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. *J Exp Med*. (1983) 158:1077–91. doi: 10.1084/jem.158.4.1077
65. Hampl J, Chien YH, Davis MM. CD4 augments the response of a T cell to agonist but not to antagonist ligands. *Immunity*. (1997) 7:379–85. doi: 10.1016/S1074-7613(00)80359-3
66. Janeway CA, Chervonsky AV, Sant'Angelo D. T-cell receptors: is the repertoire inherently MHC-specific? *Curr Biol*. (1997) 7:R299–300. doi: 10.1016/S0960-9822(06)00142-4
67. Madrenas J, Chau LA, Smith J, Bluestone JA, Germain RN. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *Journal of Experimental Medicine*. (1997) 185:219–29. doi: 10.1084/jem.185.2.219
68. Li QJ, Dinner AR, Qi S, Irvine DJ, Huppa JB, Davis MM, et al. CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse. *Nat Immunol*. (2004) 5:791–9. doi: 10.1038/ni1095
69. Li Y, Yin Y, Mariuzza RA. Structural and biophysical insights into the role of CD4 and CD8 in T cell activation. *Front Immunol*. (2013) 4:206. doi: 10.3389/fimmu.2013.00206
70. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature*. (2002) 419:845–9. doi: 10.1038/nature01076
71. Krogsgaard M, Li QJ, Sumen C, Huppa JB, Huse M, Davis M, et al. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature*. (2005) 434:238–43. doi: 10.1038/nature03391
72. Bozzacco L, Yu H, Zebroski HA, Dengjel J, Deng H, Mojsos S, et al. Mass spectrometry analysis and quantitation of peptides presented on the MHC II molecules of mouse spleen dendritic cells. *J Proteome Res*. (2011) 10:5016–30. doi: 10.1021/pr200503g
73. Birnbaum ME, Mendoza JL, Sethi DK, Dong S, Glanville J, Dobbins J, et al. Deconstructing the peptide-MHC specificity of T cell recognition. *Cell*. (2014) 157:1073–87. doi: 10.1016/j.cell.2014.03.047
74. Sibener LV, Fernandes RA, Kolawole EM, Carbone CB, Liu F, McAfee D, et al. Isolation of a structural mechanism for uncoupling T cell receptor signaling from peptide-MHC binding. *Cell*. (2018) 174:672–87 e627. doi: 10.1016/j.cell.2018.06.017
75. Jonsson P, Southcombe JH, Santos AM, Huo J, Fernandes RA, McColl J, et al. Remarkably low affinity of CD4/peptide-major histocompatibility complex class II protein interactions. *Proc Natl Acad Sci U S A*. (2016) 113:5682–7. doi: 10.1073/pnas.1513918113
76. Huppa JB, Axmann M, Mortelmaier MA, Lillemeier BF, Newell EW, Brameshuber M, et al. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature*. (2010) 463:963–7. doi: 10.1038/nature08746
77. Hong J, Persaud SP, Horvath S, Allen PM, Evavold BD, Zhu C. Force-regulated in situ TCR-peptide-bound MHC class II kinetics determine functions of CD4+ T cells. *J Immunol*. (2015) 195:3557–64. doi: 10.4049/jimmunol.1501407
78. Krummel MF. Differential clustering of CD4 and CD3zeta during T cell recognition. *Science*. (2000) 289:1349–52. doi: 10.1126/science.289.5483.1349
79. Zal T, Zal MA, Gascoigne NR. Inhibition of T cell receptor-coreceptor interactions by antagonist ligands visualized by live FRET imaging of the T-hybridoma immunological synapse. *Immunity*. (2002) 16:521–34. doi: 10.1016/S1074-7613(02)00301-1
80. Kao H, Lin J, Littman DR, Shaw AS, Allen PM. Regulated movement of CD4 in and out of the immunological synapse. *J Immunol*. (2008) 181:8248–57. doi: 10.4049/jimmunol.181.12.8248
81. Glassman CR, Parrish HL, Deshpande NR, Kuhns MS. The CD4 and CD3deltaepsilon Cytosolic Juxtamembrane Regions Are Proximal within

- a Compact TCR-CD3-pMHC-CD4 Macrocomplex. *J Immunol.* (2016) 196:4713–22. doi: 10.4049/jimmunol.1502110
82. Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T-cell surface-antigens are associated with the internal membrane tyrosine-protein kinase P56Lck. *Cell.* (1988) 55:301–8. doi: 10.1016/0092-8674(88)90053-0
83. Holdorf AD, Lee KH, Burack WR, Allen PM, Shaw AS. Regulation of Lck activity by CD4 and CD28 in the immunological synapse. *Nat Immunol.* (2002) 3:259–64. doi: 10.1038/ni761
84. Danielian S, Fagard R, Alcover A, Acuto O, Fischer S. The lymphocyte-specific protein tyrosine kinase p56lck is hyperphosphorylated on serine and tyrosine residues within minutes after activation via T cell receptor or CD2. *Eur J Immunol.* (1989) 19:2183–9. doi: 10.1002/eji.1830191202
85. Soula M, Rothhut B, Camoin L, Guillaume JL, Strosberg D, Vorherr T, et al. Anti-CD3 and phorbol ester induce distinct phosphorylated sites in the SH2 domain of p56lck. *J Biol Chem.* (1993) 268:27420–7.
86. Doyle C, Strominger JL. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature.* (1987) 330:256–9. doi: 10.1038/330256a0
87. Kinch MS, Strominger JL, Doyle C. Cell-adhesion mediated by Cd4 and Mhc class-II proteins requires active cellular processes. *J Immunol.* (1993) 151:4552–61.
88. Hoerter JA, Brzostek J, Artyomov MN, Abel SM, Casas J, Rybakina V, et al. Coreceptor affinity for MHC defines peptide specificity requirements for TCR interaction with coagonist peptide-MHC. *J Exp Med.* (2013) 210:1807–21. doi: 10.1084/jem.20122528
89. Altman JD, Moss PAH, Goulder PJR, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science.* (1996) 274:94–6. doi: 10.1126/science.274.5284.94
90. Cochran JR, Cameron TO, Stern LJ. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity.* (2000) 12:241–50. doi: 10.1016/S1074-7613(00)80177-6
91. Cebecauer M, Guillaume P, Hozak P, Mark S, Everett H, Schneider P, et al. Soluble MHC-peptide complexes induce rapid death of CD8+ CTL. *J Immunol.* (2005) 174:6809–19. doi: 10.4049/jimmunol.174.11.6809
92. Sevcik E, Schutz GJ. With or without rafts? alternative views on cell membranes. *Bioessays.* (2015) 38:129–39. doi: 10.1002/bies.201500150
93. Bernardino de la Serna J, Schutz GJ, Eggeling C, Cebecauer M. There is no simple model of the plasma membrane organization. *Front Cell Develop Biol.* (2016) 4:106. doi: 10.3389/fcell.2016.00106
94. Sakihama T, Smolyar A, Reinherz EL. Oligomerization of CD4 is required for stable binding to class II major histocompatibility complex proteins but not for interaction with human immunodeficiency virus gp120. *Proc Natl Acad Sci USA.* (1995) 92:6444–8. doi: 10.1073/pnas.92.14.6444
95. Lynch GW, Sloane AJ, Raso V, Lai A, Cunningham AL. Direct evidence for native CD4 oligomers in lymphoid and monocytoid cells. *Eur J Immunol.* (1999) 29:2590–602. doi: 10.1002/(SICI)1521-4141(199908)29:08<2590::AID-IMMU2590>3.0.CO;2-R
96. Matthias LJ, Yam PT, Jiang XM, Vandegraaff N, Li P, Poumbourios P, et al. Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1. *Nat Immunol.* (2002) 3:727–32. doi: 10.1038/ni815
97. Moldovan M-C, Yachou A, Levesque K, Wu H, Hendrickson WA, Cohen EA, et al. CD4 dimers constitute the functional component required for T cell activation. *J Immunol.* (2002) 169:6261–8. doi: 10.4049/jimmunol.169.11.6261
98. King C, Sarabipour S, Byrne P, Leahy DJ, Hristova K. The FRET signatures of noninteracting proteins in membranes: simulations and experiments. *Biophys J.* (2014) 106:1309–17. doi: 10.1016/j.bpj.2014.01.039
99. Roh KH, Lillemeier BF, Wang F, Davis MM. The coreceptor CD4 is expressed in distinct nanoclusters and does not colocalize with T-cell receptor and active protein tyrosine kinase p56lck. *Proc Natl Acad Sci USA.* (2015) 112:E1604–13. doi: 10.1073/pnas.1503532112
100. Lukes T, Glatzová D, Kvalová Z, Levet F, Benda A, Letschert S, et al. Quantifying protein densities on cell membranes using super-resolution optical fluctuation imaging. *Nat Commun.* (2017) 8:1731. doi: 10.1038/s41467-017-01857-x
101. Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. *Nat Immunol.* (2013) 14:82–9. doi: 10.1038/ni.2488
102. Jung Y, Riven I, Feigelson SW, Kartvelishvili E, Tohya K, Miyasaka M, et al. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proc Natl Acad Sci USA.* (2016) 113:E5916–24. doi: 10.1073/pnas.1605399113
103. Cai E, Marchuk K, Beemiller P, Beppler C, Rubashkin MG, Weaver VM, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science.* (2017) 356:eaa13118. doi: 10.1126/science.aal3118
104. Kim HR, Mun Y, Lee KS, Park YJ, Park JS, Park JH, et al. T cell microvilli constitute immunological synapses that carry messages to antigen-presenting cells. *Nat Commun.* (2018) 9:3630. doi: 10.1038/s41467-018-06090-8
105. Fisher PJ, Bulur PA, Vuk-Pavlovic S, Prendergast FG, Dietz AB. Dendritic cell microvilli: a novel membrane structure associated with the multifocal synapse and T-cell clustering. *Blood.* (2008) 112:5037–45. doi: 10.1182/blood-2008-04-149526
106. Foti M, Phelouzat MA, Holm A, Rasmusson BJ, Carpentier JL. p56Lck anchors CD4 to distinct microdomains on microvilli. *Proc Natl Acad Sci USA.* (2002) 99:2008–13. doi: 10.1073/pnas.042689099
107. Steffens CM, Hope TJ. Localization of CD4 and CCR5 in living cells. *J Virol.* (2003) 77:4985–91. doi: 10.1128/JVI.77.8.4985-4991.2003
108. Unternaehrer JJ, Chow A, Pypaert M, Inaba K, Mellman I. The tetraspanin CD9 mediates lateral association of MHC class II molecules on the dendritic cell surface. *Proc Natl Acad Sci USA.* (2007) 104:234–9. doi: 10.1073/pnas.0609665104
109. Khandelwal S, Roche PA. Distinct MHC class II molecules are associated on the dendritic cell surface in cholesterol-dependent membrane microdomains. *J Biol Chem.* (2010) 285:35303–10. doi: 10.1074/jbc.M110.147793
110. Rocha-Perugini V, Martinez Del Hoyo G, Gonzalez-Granado J, Ramirez-Huesca, MM, Zorita V, Rubinstein E, et al. CD9 Regulates Major Histocompatibility Complex Class II Trafficking in Monocyte-Derived Dendritic Cells. *Mol Cell Biol.* (2017) 37:e00202-17. doi: 10.1128/MCB.00202-17
111. Singethan K, Muller N, Schubert S, Lutge D, Kremmentsov DN, Khurana SR, et al. CD9 clustering and formation of microvilli zippers between contacting cells regulates virus-induced cell fusion. *Traffic.* (2008) 9:924–35. doi: 10.1111/j.1600-0854.2008.00737.x
112. Takamori S, Holt M, Stenius K, Lemke EA, Grønborg M, Riedel D, et al. Molecular anatomy of a trafficking organelle. *Cell.* (2006) 127:831–46. doi: 10.1016/j.cell.2006.10.030
113. Deschout H, Lukes T, Sharipov A, Szlag D, Feletti L, Vandenberg W, et al. Complementarity of PALM and SOFI for super-resolution live-cell imaging of focal adhesions. *Nat Commun.* (2016) 7:13693. doi: 10.1038/ncomms13693
114. Fritzsche M, Fernandes RA, Chang VT, Colin-York H, Clausen MP, Felce JH, et al. Cytoskeletal actin dynamics shape a ramifying actin network underpinning immunological synapse formation. *Sci Adv.* (2017) 3:e1603032. doi: 10.1126/sciadv.1603032
115. Lange K. Fundamental role of microvilli in the main functions of differentiated cells: Outline of an universal regulating and signaling system at the cell periphery. *J Cell Physiol.* (2011) 226:896–927. doi: 10.1002/jcp.22302

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 26 October 2018

Accepted: 12 March 2019

Published: 05 April 2019

Citation:

Pereira PM, Albrecht D, Culley S,
Jacobs C, Marsh M, Mercer J and
Henriques R (2019) Fix Your
Membrane Receptor Imaging: Actin
Cytoskeleton and CD4 Membrane
Organization Disruption by Chemical
Fixation. *Front. Immunol.* 10:675.
doi: 10.3389/fimmu.2019.00675

Fix Your Membrane Receptor Imaging: Actin Cytoskeleton and CD4 Membrane Organization Disruption by Chemical Fixation

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Single-molecule localization microscopy (SMLM) techniques allow near molecular scale resolution (~ 20 nm) as well as precise and robust analysis of protein organization at different scales. SMLM hardware, analytics and probes have been the focus of a variety of studies and are now commonly used in laboratories across the world. Protocol reliability and artifact identification are increasingly seen as important aspects of super-resolution microscopy. The reliability of these approaches thus requires in-depth evaluation so that biological findings are based on solid foundations. Here we explore how different fixation approaches that disrupt or preserve the actin cytoskeleton affect membrane protein organization. Using CD4 as a model, we show that fixation-mediated disruption of the actin cytoskeleton correlates with changes in CD4 membrane organization. We highlight how these artifacts are easy to overlook and how careful sample preparation is essential for extracting meaningful results from super-resolution microscopy.

Keywords: super-resolution imaging, CD4, actin cortex, fixation, artefact analysis

INTRODUCTION

Super-resolution microscopy is a fundamental tool for exploring and understanding nanoscale biological assemblies. Single-molecule localization microscopy (SMLM) techniques in particular, such as photoactivated localization microscopy (PALM) (1) and stochastic optical reconstruction microscopy (STORM) (2), are the optical imaging gold standards to study membrane protein organization (3). SMLM techniques provide high spatial resolution (~ 20 nm) and allow for statistical, nonbiased analysis of membrane protein nanoscale organizations (1, 2, 4, 5). Thereby, super-resolution microscopy has provided new views on the organization of membrane receptors, from immune sensing to pathogen engagement (6). The organization of receptors into micro- and nanoclusters at the plasma membrane is a common feature and an important regulatory mechanism for cell signaling and activation (7–12). Thus, analyzing the nanoscale level organization of these molecules is critical to understand basic regulation of cellular signaling but also to understand the function of these proteins in disease. For example, CD4 plays an important role in immune cell activation through its ability to enhance T-cell receptor

(TCR)-mediated signaling by binding to the antigen-presenting major histocompatibility complex II (MHCII) (13). Besides its importance in immune signaling, CD4 is also the primary cellular receptor for human immunodeficiency viruses (HIV) (13, 14). The importance of super-resolution in the study of membrane receptor organization and function cannot be overstated. A recent example is the characterization of the spatiotemporal dynamics and stoichiometry of the interactions between CD4 (and co-receptors) and HIV-1 in the context of viral entry, impossible to achieve without molecular imaging approaches (14).

A key component of membrane organization is the actin cytoskeleton (15, 16). The actin cortex underlies the plasma membrane and interacts with both lipids and membrane proteins, functioning as a dynamic scaffold providing support and force for the continuous remodeling of membrane receptor organization (17–19). It is not surprising that the actin cytoskeleton has been the subject of a considerable number of studies in a variety of biological settings, from viral engagement to axon organization using (super-resolution) microscopy (17, 20, 21).

The increased resolution and detailed analytic information provided by SMLM requires rigorous scrutiny of collected data (22–24). The succession of steps from the native organization of a receptor in the plasma membrane to the final super-resolution image can be significantly influenced by artifacts, particularly if imaging requires chemical fixation (22–24). Ideally, chemical fixation preserves the macroscopic structure of the sample as well as the native nanoscale organization of target proteins. However, true preservation at the subcellular level is not trivial, as known from electron microscopy studies (25, 26). Furthermore, chemical fixation does not immediately immobilize membrane-associated proteins (27). Thus, given the increase in resolution afforded by super-resolution microscopy, the effect of fixation has been the focus of several recent studies (22–24). Importantly, there are multiple chemical fixation methods, differing by the fixative used (e.g., paraformaldehyde, glutaraldehyde, glyoxal or methanol), the buffer composition (e.g., phosphate buffered saline, cytoskeleton stabilizing buffer or PIPES-EGTA-magnesium buffer), and physical conditions (temperature and duration) (22–24, 28–30). There is, at this stage, no standardized sample preparation protocol to study membrane protein organization. Moreover, to the best of our knowledge, there is no correlative study to understand how, in the same cells, fixation-induced changes in the actin cytoskeleton may affect membrane protein organization.

Here, we analyze how the morphology of the actin cytoskeleton changes with different chemical fixation protocols and how these changes correlate with the membrane organization of the membrane receptor CD4 (**Figure 1**). We show that conditions that have detrimental effects on cytoskeleton organization correlate with changes in the membrane organization of CD4. We suggest that careful sample preparation and handling during all steps leading to the final image is essential for all scientists.

Suboptimal Fixation Protocols Affect the Actin Cytoskeleton and CD4 Membrane Organization Differently

To understand the effect of suboptimal actin fixation protocols on CD4 membrane organization we correlated live-cell and fixed-cell actin and CD4 organization using NanoJ-Fluidics (31) (**Figure 2A**) and Structured Illumination Microscopy (SIM) (32). We imaged actin in live COS7 cells with an utrophin domain (UtrCH-GFP) (33) probe and CD4 tagged with TagRFP-T. We performed chemical fixation using three different chemical fixation protocols, 4% paraformaldehyde (PFA) in PBS at 23°C, 4% PFA in PEM (23) at 4°C or at 37°C (**Figures 2B–D**). Subsequently, using NanoJ-SQUIRREL (22), to compare the live-cell vs. fixed-cell organization of actin and CD4, we were able to identify the effects of the suboptimal (4% PFA in PBS at 23°C and 4% PFA in PEM at 4°C) fixation protocols on these targets and compare with the optimal protocol (37°C 4% PFA in PEM) (23). As expected (23, 24), using PBS we observed a loss of protrusive actin-based structures and actin stress fibers appear to be disassembled or disrupted (**Figure 2B**). The fixation resulted in an almost indiscernible actin cytoskeleton, which translates to a NanoJ-SQUIRREL error map exhibiting strong artifacts (**Figure 2B**). Using PEM buffer, more suited for actin preservation (23), but at a suboptimal temperature (4°C), we see less of the aforementioned defects on the actin organization (**Figure 2C**). Pre-warming the PFA-containing PEM buffer to 37°C yielded a similar difference between live and fixed sample as measured by NanoJ-SQUIRREL (**Figure 2D**). Regardless of the fixation approach we did not see an effect on CD4 membrane organization, quantified on the error maps where most of the differences are due to vesicle motion during fixation (**Figures 2B–D**).

The Fixation Protocol Influences CD4 Cluster Size and Cluster Density at the Cell Surface

To ascertain if CD4 membrane organization was correlated with fixation-mediated actin cytoskeleton disruption we repeated the live-to-fixed cell correlation using SMLM and PEM with different fixation temperatures. PEM is an ideal buffer for actin preservation (23), and the range of temperatures provide different fixation efficiencies, with decreasing efficiency from 37°C (ideal) to 23°C (intermediate) to 4°C (lowest efficiency). We took advantage of the versatile NanoJ-Fluidics (31) framework to correlate live and fixed cell imaging of COS7 cells (**Figure 3A**). As expected, regardless of the fixation strategy we obtain a fairly homogeneous distribution of CD4 on the surface of COS7 cells (**Figure 3B**) at an in-cell high-resolution [43–50 nm by FRC (22)]. To further explore the nature of the CD4 organization we used SR-Tesseler (4) to determine if the cluster sizes and cluster density of CD4 would change depending on the fixation approach (**Figure 3C**). Interestingly, despite the little changes observed by SIM (**Figure 2**), both CD4 cluster size and cluster density changed with the fixation approach. Whereas, the mean CD4 cluster size in ideal conditions (PEM buffer at 37°C)

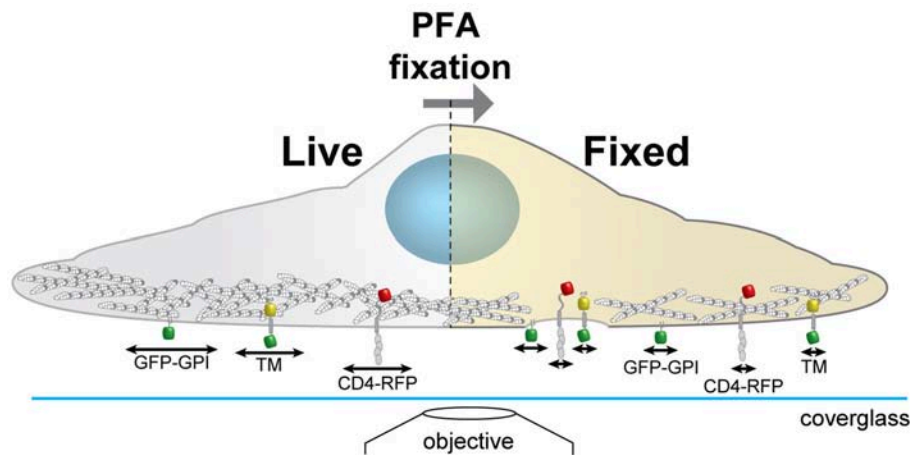


FIGURE 1 | Schematics of the experimental workflow to correlate actin morphology with CD4 membrane organization. We analyse on the same cells how the actin cytoskeleton morphology changes with different chemical fixation protocols and how this correlates with the membrane organization and mobility of CD4. Cortical actin (white and gray circles); arrows represent protein mobility; GPI anchored GFP (GFP-GPI); artificial transmembrane protein with cytosolic and extracellular domains (mHoneydew and YFP, respectively - TM); CD4 fused to TagRFP-T (CD4-RFP).

is 59 nm, reducing the temperature to 23 or 4°C is enough to change CD4 organization, increasing the mean cluster size to 65 nm ($p < 0.001$), albeit these differences are likely not biologically relevant (see section Discussion). The fixation conditions also influence the CD4 cluster density in COS7 cells, with densities of 1.3 clusters/ μm^2 at 37°C, 1.8 clusters/ μm^2 at 23°C, and 3.8 clusters/ μm^2 at 4°C.

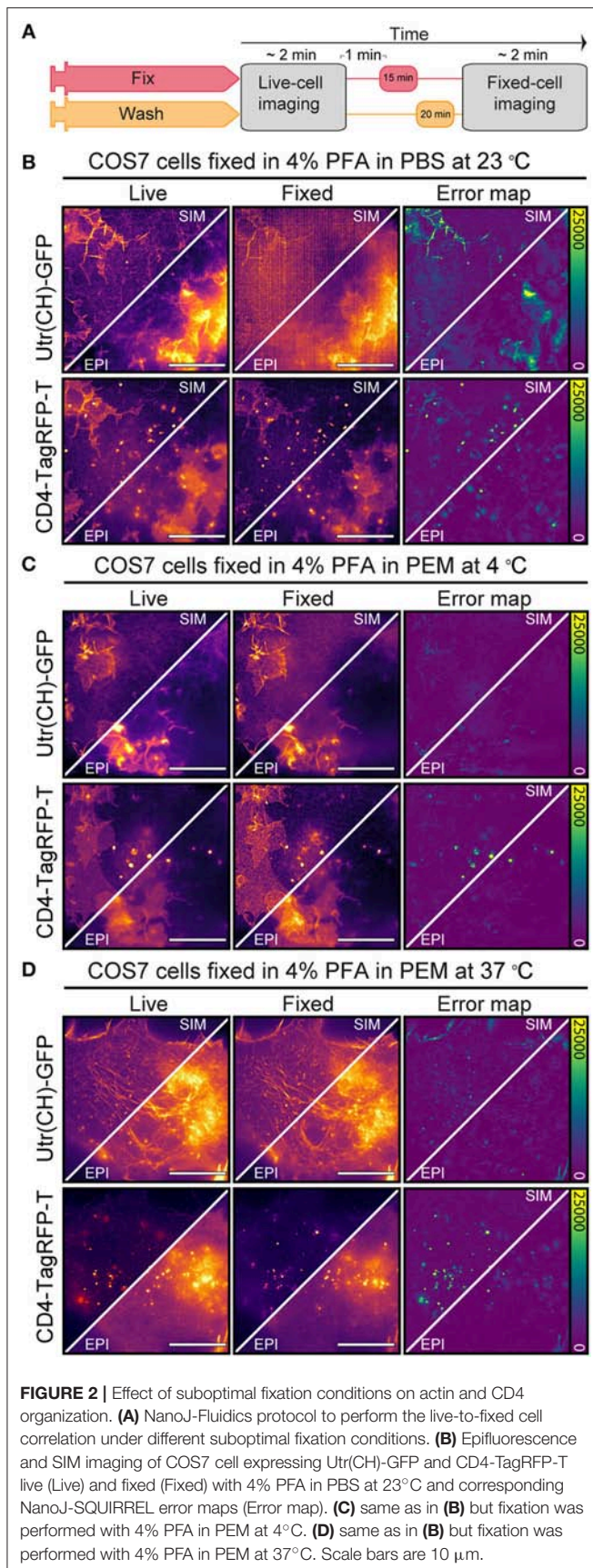
Fixation-Induced CD4 Reorganization Correlates With Actin Cytoskeleton Preservation

We posited that fixation-induced changes in CD4 organization could be related to disruption of the actin cytoskeleton (Figure 2). To determine if the actin cytoskeleton was affected we compared the actin organization in the cells pre- and post-fixation (Figure 3D). We observed a disruption of the actin cytoskeleton at 23 and 4°C when compared with fixation at 37°C (Figure 3D). Independent of the fixation condition the post-fixation actin organization is different from the live-cell actin organization (Figure 3D yellow arrowheads). With the decrease in fixation temperature there is a step-wise decrease in the fidelity of the fixed-cell actin structure in relation to the one observed in live-cells. At lower fixation temperatures, actin filaments disappear and there are gaps in the actin structure, possibly related to cell detachment from the substrate or actin cytoskeleton disruption (Figure 3D red arrowheads). These artifacts are less prevalent in cells fixed under conditions that preserve the actin cytoskeleton structure.

CD4 Membrane Reorganization Is not Related to Fixation-Induced Cell Membrane Disruption

The difference in membrane receptor organization could be the result of the dependence of fixation efficiency on

temperature. Employing our live-to-fix approach, we sought to determine how quickly the addition of PFA-containing PEM buffer immobilizes membrane associated proteins (Figure 4). An artificial transmembrane protein with a ~30 kDa cytosolic and extracellular domain (mHoneydew and YFP, respectively) was expressed in COS7 cells and individual proteins tracked with uPAINT (34), i.e., by adding low concentration (~20 pMol) of Atto647N labeled anti-GFP nanobodies (Chromotek) to the medium (Figure 4B, first panel). Diffusion coefficients based on particle velocity were $0.27 \pm 0.06 \mu\text{m}^2/\text{s}$ (mean). Exchanging the cell culture medium with 37°C pre-warmed 4% PFA in PEM immediately reduced the diffusion speed of transmembrane proteins (Figure 4B, middle panel, arrow) and, after 10 min fixation, 97% of proteins were immobilized ($D < 0.05 \mu\text{m}^2/\text{s}$) (Figure 4C). Addition of cold (4°C) 4% PFA in PEM had similar effects on measured diffusion coefficients and mobility (Figure 4C). Next, we tested if the same was true for a GPI-anchored protein that lacks any cytosolic domain that might interact directly with the cytoskeleton. GPI-anchored GFP was tracked via anti-GFP nanobodies. Addition of warm (37°C) or cold (4°C) 4% PFA in PEM buffer to live cells reduced the mobility of tracked individual particles without immobilizing them completely (Figure 4D, middle panel, arrow). In contrast to the transmembrane probe, only some particles were immobilized after fixation for 10 min. The reduction of diffusion coefficients as measured by velocity or mean square displacement (Figure 4E, left and middle panel) was not significantly different based on temperature. The mobile fraction was reduced to 36 and 32% (mean) after warm and cold fixation, respectively. Thus, changes in diffusive behavior were more dependent on the type of membrane protein tracked, rather than the fixation conditions (Figures 4C,E), which is in agreement with previous publications (27). However, even with only 4% PFA and without any cross-linking fixatives, we observed a rapid immobilization of transmembrane proteins that would



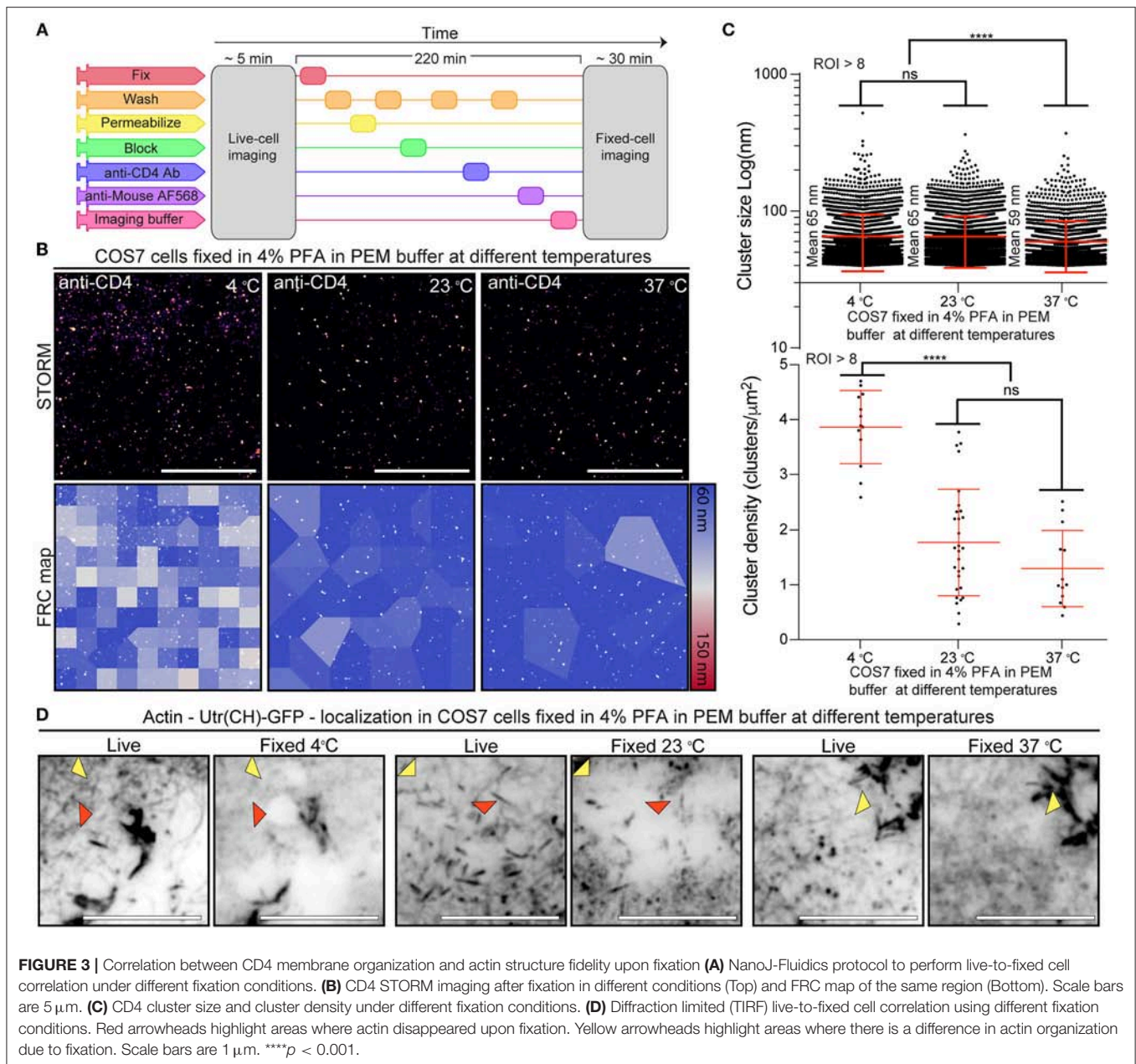
prevent artificial clustering by subsequent antibody labeling approaches. Comparison of trajectories during the 10 s before and after addition of the chemical fixative showed an immediate shift of the histogram of diffusion coefficients determined via MSD toward lower values (**Figure 4F**). While there was no striking difference between pre-warmed and ice-cold fixative, we observed a trend toward a faster decrease in mobility at the higher temperature.

Chemical Fixation Immediately Stops Cellular Motion

We determined how long cellular processes such as the motion of intracellular vesicles or lamellipodia persist during chemical fixation with PFA and whether this process was temperature dependent. Live COS7 cells were imaged in phase-contrast (**Figure 5A**). Upon exchange of the medium with 4% PFA pre-warmed to 37°C all cellular motion stopped immediately as determined by correlating images with the previous frame for the entire field of view (**Figure 5B**) or selected regions (**Figures 5C,D** and **Supplementary Movies 1, 2**). The plateaus in correlations pre- and post PFA addition correspond to cellular motion and noise during imaging. Chemical fixation with ice-cold 4% PFA inhibited cellular motion equally fast (**Figures 5E–G**). The increased fluctuations in correlations (**Figures 5F,G**) were caused by a shift in the focal plane, also observed in the image sequence (**Figure 5** and **Supplementary Movies 3, 4**).

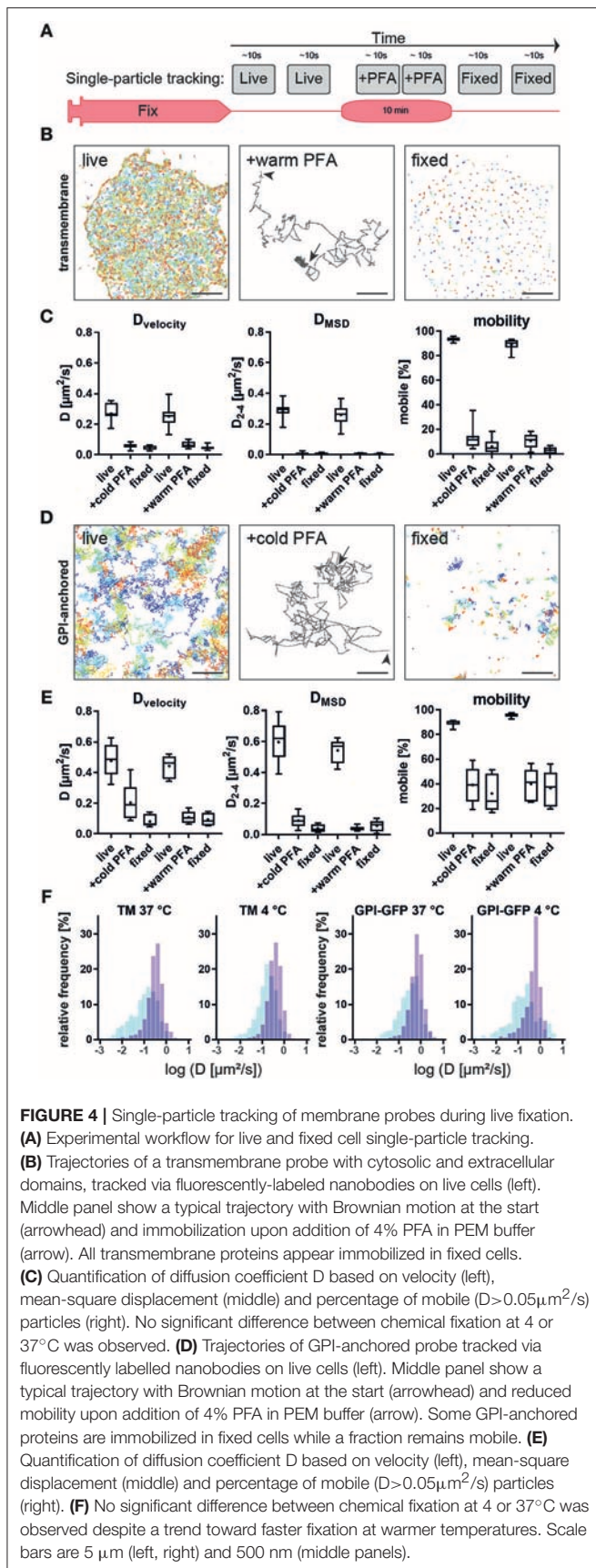
Discussion

The super-resolution revolution in optical microscopy offers even inexperienced users up to 10-fold increased resolution on commercial systems that have become commonly available through imaging facilities. However, established sample preparation protocols that were previously acceptable may be inadequate for super-resolution microscopy, as the inaccuracies are no longer masked by the diffraction limit. While the importance of careful sample preparation is readily accepted, its assessment remains challenging. Neglecting to recognize this cost associated with increased resolution could render imaging results useless or worse might incorrectly inform researchers about a biological system. To demonstrate sample preparation inadequacies in imaging regimes, we took advantage of NanoJ-Fluidics (31) and NanoJ-SQUIRREL (22) to compare the pre- and post-fixation actin structures and CD4 cellular organization, in the same cells. We asked what would be the influence of chemical fixation using different imaging regimes with increasing resolution (TIRF, SIM and SMLM) by correlating pre- and post-fixation images. The actin cytoskeleton acts as a supporting scaffold that orchestrates the organization of the plasma membrane (35, 36). However, while actin filaments are strongly affected by chemical fixation conditions, the plasma membrane itself is affected to a lesser extent. Chemical fixation is usually fast and even a simple protocol can achieve structural preservation of the organization of transmembrane proteins in the plasma membrane. Despite the availability of chemical fixation protocols that preserve the actin cytoskeleton, the predominant approach for studying protein organization is fixation with 4% PFA in PBS. Our data suggests this is insufficient



to produce reliable imaging data on receptor distributions for imaging modalities that break the diffraction limit. The chemical fixation protocol used was shown to play a crucial role on the introduction of artifacts. We applied SQUIRREL, a recently developed quality metric tool (22), to quantify how much cytoskeletal structures are distorted by chemical fixation at exemplary conditions. Our approach is widely applicable to determine the impact of any fixation protocol beyond those tested. Of course, a correlation between pre- and postfixation structures is required which, albeit greatly facilitated by NanoJ-Fluidics (31), is still a time-consuming quality control approach. However, in our opinion, the benefit of increased confidence in light microscopy data is worth the added effort. The increase

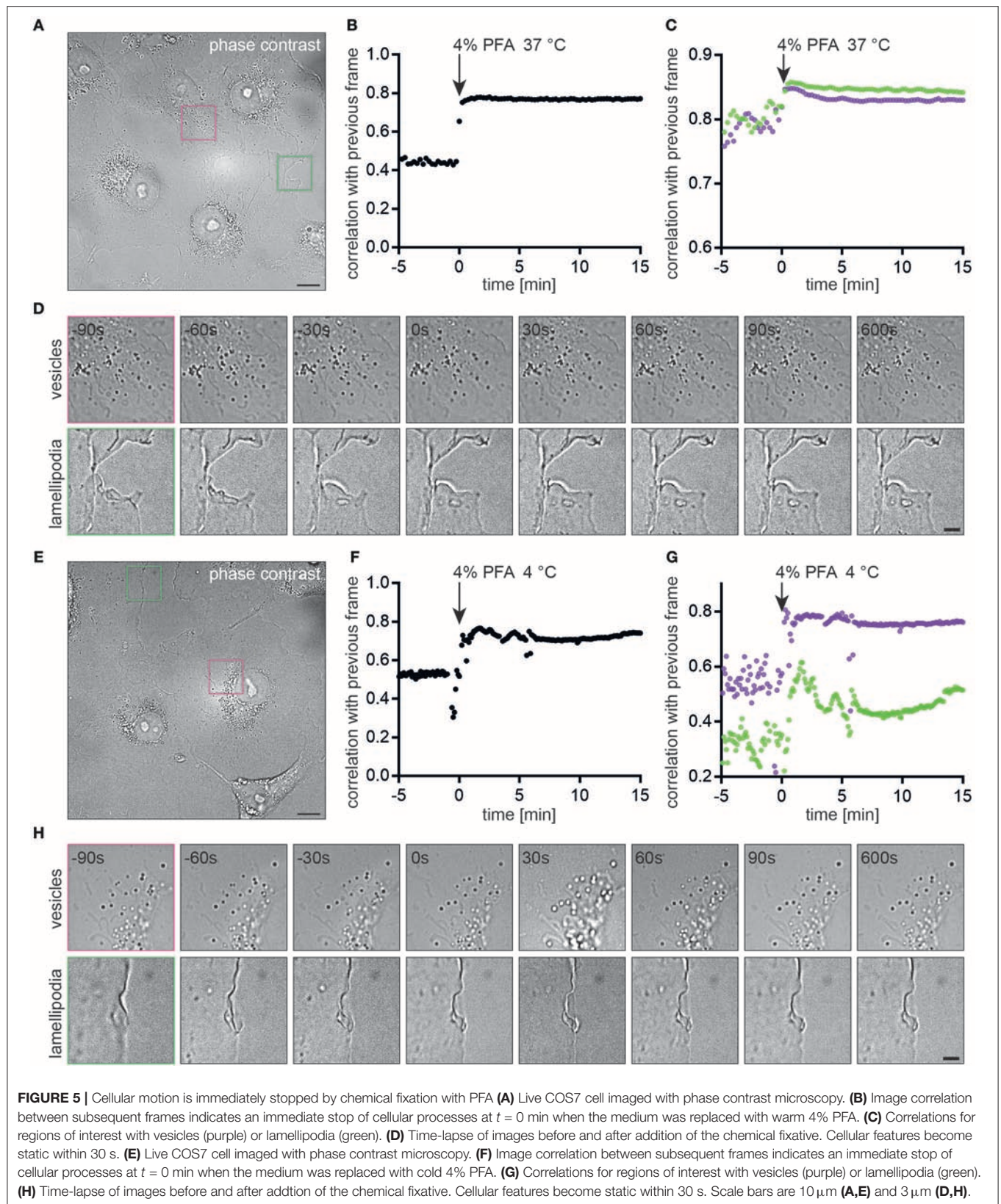
in cluster size and density we observed could be due to: (1) disruption of the actin cytoskeleton organization that could affect to CD4 membrane organization via protein-protein interaction; (2) fixation-induced changes in membrane properties, which would cause artificial reorganization of membrane proteins; (3) a combination of both factors. Using super-resolution microscopy we could show that the changes in CD4 organization coincided with a disrupted actin cytoskeleton profile. The cluster size in optimal conditions suggests CD4 may be organized in dimers (as seen by the mean cluster size of ~60 nm), which is consistent with its suggested capacity to homo-dimerize, a process that may increase the avidity of its binding to MHCII (37). The differences observed between temperatures regarding CD4 cluster size are



negligible (~ 6 nm) and likely related to the high number of data points skewing the statistical analysis. This is something the reader should always have in mind when analyzing statistical significance, as in this case the ~ 6 nm is significantly below the resolution our setup can provide and within the linker error introduced by using antibodies (~ 10 nm). The cluster density suggests a homogeneous distribution consistent with COS7 non-native CD4 expression. It is important to highlight that the considerable differences in cluster density are in a system where CD4 does not normally exist, hence lacking the regulatory machinery or native interactions that may normally regulate CD4 distribution. Presumably, the observed differences would be more striking in CD4-positive immune cells where CD4 is linked to p56/LCK (38). Interestingly, the degree of actin cytoskeleton disruption is consistent with the extent of the changes we observe in CD4 membrane organization. After chemical fixation at 4°C we observed almost complete disruption whereas at 23°C the cell displays a mixture of regions with disrupted and non-disrupted actin structures. This suggests that despite CD4 not existing in COS7 cells in native conditions, CD4 organization may be affected by the structure of the dense actin cortex (possibly through its cytoplasmic domain). Consequently, inadequate actin chemical fixation regimes can affect CD4 membrane organization and influence the biological information extracted from SMLM CD4 analysis. Challenging fields, such as the spatial distribution of immunomodulatory receptors require rigorous controls. For example, actin cytoskeleton dynamics affect clustering in immunological synapses (39, 40). Our approach could be employed to quantify the effects of actin perturbing drugs used on these cells.

We cannot exclude that membrane disruption and reorganization (such as membrane permeabilization or steep temperature mismatches between live-cell and fixation buffers, respectively) also plays a role in exacerbating the differences we observe. The importance of membrane composition and organization for surface protein distribution is well-known (41–43). Nonetheless, an indirect actin-related effect cannot be disregarded. The link between membrane composition and actin regulation is also recognized (44). For example, it is known that the pool of actin monomers is modulated by phosphoinositides (45, 46), or that alterations in the levels of cholesterol can change the membrane-cytoskeleton adhesion properties (47). However, our objective is to inform the reader on the possible outcomes that common sample preparation approaches (as multi-target IF or the use of intracellular epitopes, or different fixation temperatures) may have. If possible, cell membrane permeabilization and steep temperature changes should be avoided for their effect on the sample. Additionally, thermal drift affecting the optical system may reduce image quality or introduce artifacts.

These results are further supported by single-particle tracking experiments. Single-particle tracking of transmembrane proteins and a GPI-anchored protein showed that the size and orientation in the plasma membrane was more important than fixation conditions. GPI-anchored proteins that reside in the outer leaflet of the plasma membrane with only indirect interaction with the submembrane cytoskeleton (48) remain largely mobile in



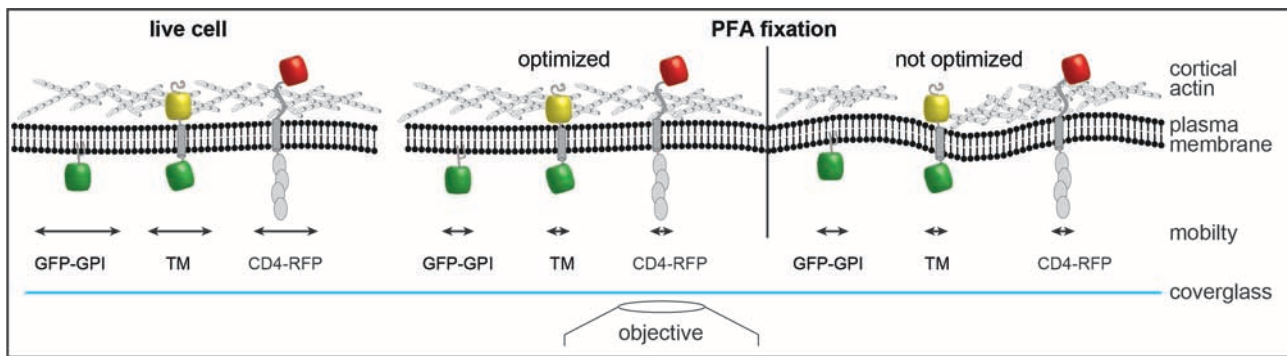


FIGURE 6 | Model of changes induced by chemical fixation on membrane architecture. Optimized fixation with PFA preserves the cortical actin cytoskeleton structure in a state resembling live imaging and rapidly stops diffusion of transmembrane proteins. Suboptimal fixation conditions induce deformations of membrane and cytoskeleton and could thereby introduce artifacts. While the mobility of membrane probes is reduced similarly to optimized chemical fixation the overall organization could be altered due to interruptions of the cytoskeleton. GPI anchored GFP (GFP-GPI); artificial transmembrane protein with cytosolic and extracellular domains (mHoneydew and YFP, respectively - TM); CD4 fused to TagRFP-T (CD4-RFP).

ideal actin-preserving conditions. Any distribution or clustering analysis must rule out post-fixation aggregation, e.g., by the use of single-binders such as nanobodies. This is in agreement with STED and FRAP data showing that appropriate fixation is critical for imaging of microclusters (49). In contrast, transmembrane proteins with a cytosolic domain such as CD4 or our artificial transmembrane probe are quickly immobilized, indicating an interaction with the submembrane cytoskeleton. During single-particle tracking of membrane probes at 45 Hz, we observed a trend toward faster immobilization in the first few seconds after addition of the pre-warmed chemical fixative. During phase contrast imaging at 0.066 Hz cellular motion was halted within 30 s for both conditions tested. The increased fluctuations in correlation analysis after addition of ice-cold fixative was likely due to thermal effects on sample structure and microscope optics and not diffusion or reaction rate of the fixative. Our observation that CD4 membrane organization is affected by poor actin chemical fixation should serve as a cautionary tale for sample preparation approaches to study membrane proteins. Optimal fixation approaches preserve the cortical actin cytoskeleton structure and the organization of transmembrane proteins in a near-native state (**Figure 6**). Conversely, suboptimal fixation conditions induce deformations of membrane and cytoskeleton that can result in artifacts that can influence the organization of membrane proteins, such as CD4 (**Figure 6**). Although, we and others (40, 50–54) suggest that the actin cytoskeleton, protein-protein interactions and the physiological context (e.g., temperature) are important for membrane proteins organization, many studies using SMLM focus on imaging unknown structures and distributions of proteins that do not have a known organization. It is important to highlight that this work does not intend to suggest a direct correlation between the actin cytoskeleton and CD4 surface organization (or other surface proteins). Rather that when performing essential protocol optimization, preservation of the overall cellular structure and physiological context should be a priority. This work also aims

to highlight that there are already established protocols that serve as excellent starting points (23, 24, 29, 30), hardware that permits the optimization of such protocols to be streamlined (31, 55) and tools that allow for seamless analysis of possible bottlenecks (22, 55). In conclusion, to extract the most from SMLM experiments it is essential to use reliable and repeatable imaging protocols that preserve, as much as possible, the overall cellular structure.

METHODS

Cell Lines

COS7 cells were cultured in phenol-red free DMEM (Gibco) supplemented with 2 mM GlutaMAX (Gibco), 50 U/ml penicillin, 50 µg/ml streptomycin (Penstrep, Gibco) and 10% fetal bovine serum (FBS; Gibco). Cells were grown at 37°C in a 5% CO₂ humidified incubator. Cell lines have not been authenticated.

Plasmids

The plasmid expressing the calponin homology domain of utrophin fused to GFP (GFP-UtrCH) was a gift from William Bement (33) (Addgene plasmid #26737). The plasmid expressing the cluster of differentiation 4 (CD4) fused to TagRFP-T was constructed for this study by fusing the CD4 (56) and TagRFP-T (57, 58) genes by overlapping PCR, with a 10 amino-acid linker (GGGGSGGGGS) encoded in the overlap primers, and cloning the resulting fragment into pcDNA3.1+ (Thermo Fisher Scientific) using HindIII and XhoI restriction enzymes (Promega). This plasmid is available from Addgene (Addgene plasmid #119238). The plasmid expressing GPI-GFP was a kind gift from Ari Helenius. The plasmid expressing the artificial transmembrane probe was constructed based on Patrick Keller's L-YFP-GT46 (59) by adding the beta-barrel fluorophore mHoneydew on the cytosolic side to increase size (60).

Live-to-Fixed Super-Resolution Imaging

The NanoJ-Fluidics syringe pump array was installed on a Zeiss Elyra PS.1 microscope equipped with 405, 488, 561, and 642 nm lasers (50, 200, 200, and 160 mW at the optical fiber output). All steps after cell transfection were performed on the microscope, using NanoJ-Fluidics (31, 61). COS7 cells (kind gift from Dr. A. Saiardi) were seeded on ultraclean (62) 25 mm diameter thickness 1.5H coverslips (Marienfeld) at a density of $0.3\text{--}0.9 \times 10^5$ cells/cm². One day after splitting, cells were transfected with UtrCH-GFP and pCD4-TagRFP-T using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's recommendations. Cells were imaged 1–2 days post transfection in culture medium using an Attotfluor cell chamber (ThermoFisher), covered with the lid of a 35 mm dish (ThermoFisher), that was kept in place using black non-reflective aluminum tape (T205-1.0 AT205, THORLABS).

Cells were fixed at 4, 23, or 37°C for 15 min with freshly prepared 4% paraformaldehyde (PFA) in the cytoskeleton-preserving buffer “PIPES-EGTA-Magnesium” (PEM: 80 mM PIPES pH 6.8, 5 mM EGTA, 2 mM MgCl₂) (23) or at 23°C for 15 min with 4% PFA in Phosphate Buffer Saline (PBS: 0.14 M NaCl, 10 mM NaH₂PO₄, 10 mM Na₂HPO₄).

For stained cells (**Figure 2**), after fixation cells were permeabilised (PEM with 0.25% Triton-X-100) for 20 min (at 23°C), blocked with blocking buffer [5% Bovine Serum Albumin (BSA) in PEM] for 30 min (at 23°C), and stained with anti-CD4 mAb (OKT4, 6 µg/ml) for 60 min (at 23°C), followed by anti-mouse Alexa Fluor 568 secondary Ab (Molecular Probes) for 60 min (at 23°C).

Structured Illumination Microscopy (SIM) imaging was performed using Plan-Apochromat 63x/1.4 oil DIC M27 objective, in a Zeiss Elyra PS.1 microscope (Zeiss). Images were acquired using 5 phase shifts and 3 grid rotations with the 561 and 488 nm lasers (at 5–10% of maximum output), and filter set 4 (1,851–248, Zeiss). Images were acquired using a sCMOS (pco.edge sCMOS) camera.

Total Internal Reflection Fluorescence (TIRF) imaging of live COS7 cells was performed at 37°C and 5% CO₂ on a Zeiss Elyra PS.1 microscope with 488 nm and 561 nm laser illumination at 0.5% of maximum output. A 100x TIRF objective (Plan-APOCHROMAT 100x/1.46 Oil, Zeiss) with additional 1.6x magnification was used to collect fluorescence onto an EMCCD camera (iXon Ultra 897, Andor), yielding a pixel size of 100 nm. TIRF STORM imaging of anti-CD4 Alexa Fluor 568 in fixed cells was performed on the same system. 50,000 frames were acquired with 33 ms exposure and 561 nm laser illumination at maximum output power with 405 nm pumping when required (0.5–1% of maximum output when the blinking density was below 1 particle/µm²). STORM imaging was performed in GLOX buffer (150 mM Tris, pH 8, 1% glycerol, 1% glucose, 10 mM NaCl, 1% β-mercaptoethanol, 0.5 mg/ml glucose oxidase, 40 µg/ml catalase). Single-particle tracking was performed in medium at 37°C and 5% CO₂ on a Zeiss Elyra PS.1 microscope in TIRF mode by acquiring 250/500 frames at 45 FPS with 642 nm laser illumination at 5% of maximum output. Live fixation during phase contrast imaging was performed in medium at 37°C and 5% CO₂ on a Zeiss Elyra PS.1 microscope at 0.066

FPS with white LED illumination. For live-fixation, medium was replaced by either ice-cold or 37°C pre-warmed 4% PFA in PEM buffer.

Image Reconstruction and Analysis

For **Figure 2** images were processed using the ZEN software (2012, version 8.1.6.484, Zeiss). For channel alignment, a multi-colored bead slide was imaged using the same image acquisition settings. For STORM datasets localizations were detected and rendered using ThunderSTORM (63) with default settings. Fourier Ring Correlation (FRC) values were obtained using NanoJ-SQUIRREL after reconstruction of original data separated into two different stacks composed of odd or even images (22). NanoJ-SQUIRREL and ThunderSTORM are available in Fiji (64). Statistical analysis (ordinary one-way ANOVA) was performed using Prism7 (GraphPad). Single-particle tracking data was analyzed using Trackmate (65) in Fiji and MSDanalyzer (66) in MATLAB (Mathworks). Images sequences for movies were bleach corrected (Fiji) and drift corrected (NanoJ).

Cross-correlation analysis was performed to analyse the stability of samples pre- and post-fixation. Analysis was performed using a custom-written plugin for Fiji (64) using tools from the NanoJ-Core software package (55). Phase contrast images were first drift-corrected using the drift correction functionality of NanoJ-Core. A normalized 2D cross-correlation matrix (CCM) was calculated between each frame of the image series and the frame immediately preceding it. The peak intensity in the CCM indicates the similarity between the two images, where a value of 1.0 indicates perfect similarity between the images. The plugin for this analysis is including in the latest release of the NanoJ-Core software package as “Similarity Evolution”.

AUTHOR CONTRIBUTIONS

These contributions follow the contributor roles taxonomy guidelines (<https://casrai.org/credit/>). PP, DA, CJ, and RH: conceptualization; PP, DA, SC, and CJ: data curation, formal analysis, visualization; PP, DA, and CJ: investigation, methodology, writing original draft; PP, DA, SC, CJ, JM, MM, and RH: resources, validation, writing, review, and editing; PP, DA, JM, MM, and RH: funding acquisition, supervision; PP, DA, and RH: project administration.

FUNDING

This work was funded by grants from the UK Biotechnology and Biological Sciences Research Council (BB/M022374/1; BB/P027431/1; BB/R000697/1; BB/S507532/1) (RH, PP, and CJ); The UK Medical Research Council (MR/K015826/1) (RH, JM, and MM); The Wellcome Trust (203276/Z/16/Z) (RH); the MRC Programme Grant (MC_UU12018/7) (JM); The European Research Council (649101-UbiProPox) (JM); The MRC Programme Grant (MC_U12016/1) (MM); DA has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie

Skłodowska-Curie grant agreement No. 750673. CJ was funded by a Commonwealth scholarship, funded by the UK government.

ACKNOWLEDGMENTS

We thank Dr. Christophe Leterrier at the Neuropathophysiology Institute (INP, CNRS-Aix Marseille University UMR 7051) for critical reading and advice. We thank the MRC-LMCB light

microscopy facility for the equipment maintenance and users training. This manuscript was released as a pre-print on biorxiv (<https://doi.org/10.1101/450635>).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00675/full#supplementary-material>

REFERENCES

- Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacio JS, et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. (2006) 313:1642–5. doi: 10.1126/science.1127344
- Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods*. (2006) 3:793. doi: 10.1038/nmeth929
- Nicovich PR, Owen DM, Gaus K. Turning single-molecule localization microscopy into a quantitative bioanalytical tool. *Nat Protoc*. (2017) 12:453. doi: 10.1038/nprot.2016.166
- Levet F, Hosy E, Kechkar A, Butler C, Beghin A, Choquet D, et al. SR-Tesseler: a method to segment and quantify localization-based super-resolution microscopy data. *Nat Methods*. (2015) 12:1065. doi: 10.1038/nmeth.3579
- Gray R, Albrecht D, Beerli C, Cohen G, Henriques R, Mercer J. Nanoscale polarization of the vaccinia virus entry fusion complex drives efficient fusion. *bioRxiv*. (2018) 360073. doi: 10.1101/360073
- Fernández-Suárez M, Ting AY. Fluorescent probes for super-resolution imaging in living cells. *Nat Rev Mol Cell Biol*. (2008) 9:929. doi: 10.1038/nrm2531
- Lopes FB, Bálint S, Valvo S, Felce JH, Hessel EM, Dustin ML, et al. Membrane nanoclusters of FcγRI segregate from inhibitory SIRPα upon activation of human macrophages. *J Cell Biol*. (2017) 216:1123–41. doi: 10.1083/jcb.201608094
- Saka SK, Honigsmann A, Eggeling C, Hell SW, Lang T, Rizzoli SO. Multi-protein assemblies underlie the mesoscale organization of the plasma membrane. *Nat Commun*. (2014) 5:4509. doi: 10.1038/ncomms5509
- Lee J, Sengupta P, Brzustowski J, Lippincott-Schwartz J, Pierce SK. The nanoscale spatial organization of B-cell receptors on immunoglobulin M- and G-expressing human B-cells. *Mol Biol Cell*. (2017) 28:511–23. doi: 10.1091/mbc.e16-06-0452
- Soares H, Henriques R, Sachse M, Ventimiglia L, Alonso MA, Zimmer C, et al. Regulated vesicle fusion generates signaling nanoterritories that control T cell activation at the immunological synapse. *J Exp Med*. (2013) 210:2415–33. doi: 10.1084/jem.20130150
- Silva JG, Martins NP, Henriques R, Soares H. HIV-1 Nef impairs the formation of calcium membrane territories controlling the signaling nanoarchitecture at the immunological synapse. *J Immunol*. (2016) 197:4042–52. doi: 10.4049/jimmunol.1601132
- Huttunen M, Turkki P, Mäki A, Paavola L, Ruusuvaara P, Marjomäki V. Echovirus 1 internalization negatively regulates epidermal growth factor receptor downregulation. *Cell Microbiol*. (2017) 19:e12671. doi: 10.1111/cmi.12671
- Love PE, Hayes SM. ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harb Perspect Biol*. (2010) 2:a002485. doi: 10.1101/cshperspect.a002485
- Iliopoulou M, Nolan R, Alvarez L, Watanabe Y, Coomer CA, Jakobsdottir GM, et al. A dynamic three-step mechanism drives the HIV-1 pre-fusion reaction. *Nat Struct Mol Biol*. (2018) 25:814. doi: 10.1038/s41594-018-0113-x
- Mattila PK, Batista FD, Treanor B. Dynamics of the actin cytoskeleton mediates receptor cross talk: an emerging concept in tuning receptor signaling. *J Cell Biol*. (2016) 212:267–80. doi: 10.1083/jcb.201504137
- Kraft ML. Plasma membrane organization and function: moving past lipid rafts. *Mol Biol Cell*. (2013) 24:2765–8. doi: 10.1091/mbc.e13-03-0165
- Comrie WA, Burkhardt JK. Action and traction: cytoskeletal control of receptor triggering at the immunological synapse. *Front Immunol*. (2016) 7:68. doi: 10.3389/fimmu.2016.00068
- Mooren OL, Galletta BJ, Cooper JA. Roles for actin assembly in endocytosis. *Ann Rev Biochem*. (2012) 81:661–86. doi: 10.1146/annurev-biochem-060910-094416
- Trimble WS, Grinstein S. Barriers to the free diffusion of proteins and lipids in the plasma membrane. *J Cell Biol*. (2015) 208:259–71. doi: 10.1083/jcb.201410071
- Sahl SJ, Hell SW, Jakobs S. Fluorescence nanoscopy in cell biology. *Nat Rev Mol Cell Biol*. (2017) 18:685. doi: 10.1038/nrm.2017.71
- Stradal TE, Schelhaas M. Actin dynamics in host-pathogen interaction. *FEBS Lett*. 592:3658–69. doi: 10.1002/1873-3468.13173
- Culley S, Albrecht D, Jacobs C, Pereira PM, Leterrier C, Mercer J, et al. Quantitative mapping and minimization of super-resolution optical imaging artifacts. *Nat Methods*. (2018) 15:263. doi: 10.1038/nmeth.4605
- Leyton-Puig D, Kedziora KM, Isogai T, van den Broek B, Jalink K, Innocenti M. Pfa fixation enables artifact-free super-resolution imaging of the actin cytoskeleton and associated proteins. *Biol Open*. (2016) 5:1001–9. doi: 10.1242/bio.019570
- Whelan DR, Bell TD. Image artifacts in single molecule localization microscopy: why optimization of sample preparation protocols matters. *Sci Rep*. (2015) 5:7924. doi: 10.1038/srep07924
- Zhang Y, Huang T, Jorgens DM, Nickerson A, Lin LJ, Pelz J, et al. Quantitating morphological changes in biological samples during scanning electron microscopy sample preparation with correlative super-resolution microscopy. *PLoS ONE*. (2017) 12:e0176839. doi: 10.1371/journal.pone.0176839
- Small J. Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J Cell Biol*. (1981) 91:695–705. doi: 10.1083/jcb.91.3.695
- Tanaka KA, Suzuki KG, Shirai YM, Shibutani ST, Miyahara MS, Tsuboi H, et al. Membrane molecules mobile even after chemical fixation. *Nat Methods*. (2010) 7:865. doi: 10.1038/nmeth.f.314
- Xu K, Zhong G, Zhuang X. Actin, spectrin, and associated proteins form a periodic cytoskeletal structure in axons. *Science*. (2013) 339:452–6. doi: 10.1126/science.1232251
- Richter KN, Revelo NH, Seitz KJ, Helm MS, Sarkar D, Saleeb RS, et al. Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. *EMBO J*. (2018) 37:139–59. doi: 10.15252/emboj.201695709
- Bacallao R, Kiai K, Jesaitis L. Guiding principles of specimen preservation for confocal fluorescence microscopy. In: *Handbook of Biological Confocal Microscopy*. Boston, MA: Springer (1995). p. 311–25.
- Almada P, Pereira PM, Culley S, Caillol G, Boroni-Rueda F, Dix CL, et al. Automating multimodal microscopy with NanoJ-Fluidics. *Nat. Commun*. (2019) 10:1223. doi: 10.1038/s41467-019-09231-9
- Gustafsson MG. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J Microsc*. (2000) 198:82–7. doi: 10.1046/j.1365-2818.2000.00710.x
- Burkel BM, Von Dassow G, Bement WM. Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin. *Cell Motil Cytoskeleton*. (2007) 64:822–32. doi: 10.1002/cm.20226

34. Giannone G, Hosy E, Levet F, Constals A, Schulze K, Sobolevsky AI, et al. Dynamic superresolution imaging of endogenous proteins on living cells at ultra-high density. *Biophys J.* (2010) 99:1303–10. doi: 10.1016/j.bpj.2010.06.005
35. Arumugam S, Petrov EP, Schuille P. Cytoskeletal pinning controls phase separation in multicomponent lipid membranes. *Biophys J.* (2015) 108:1104–13. doi: 10.1016/j.bpj.2014.12.050
36. Vogel SK, Greiss F, Khmelinskaia A, Schuille P. Control of lipid domain organization by a biomimetic contractile actomyosin cortex. *Elife.* (2017) 6:e24350. doi: 10.7554/eLife.24350
37. Wu H, Kwong PD, Hendrickson WA. Dimeric association and segmental variability in the structure of human CD4. *Nature.* (1997) 387:527. doi: 10.1038/387527a0
38. Pelchen-Matthews A, Boulet I, Littman DR, Fagard R, Marsh M. The protein tyrosine kinase p56lck inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. *J Cell Biol.* (1992) 117:279–90. doi: 10.1083/jcb.117.2.279
39. Yi J, Wu XS, Crites T, Hammer JA. Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells. *Mol Biol Cell.* (2012) 23:834–52. doi: 10.1091/mbc.E11-08-0731
40. Ashdown GW, Burn GL, Williamson DJ, Pandžić E, Peters R, Holden M, et al. Live-cell super-resolution reveals F-Actin and plasma membrane dynamics at the T cell synapse. *Biophys J.* (2017) 112:1703–13. doi: 10.1016/j.bpj.2017.01.038
41. Goñi FM. “Rafts”: a nickname for putative transient nanodomains. *Chem Phys Lipids.* (2018) 218:34–9. doi: 10.1016/j.chemphyslip.2018.11.006
42. Giulio S, Santo AL. Lipids in the cell: organisation regulates function. *Cell Mol Life Sci.* (2018) 75:1909–27. doi: 10.1007/s00018-018-2765-4
43. Nyholm TK. Lipid-protein interplay and lateral organization in biomembranes. *Chem Phys Lipids.* (2015) 189:48–55. doi: 10.1016/j.chemphyslip.2015.05.008
44. Bezaniilla M, Gladfelter AS, Kovar DR, Lee WL. Cytoskeletal dynamics: a view from the membrane. *J Cell Biol.* (2015) 209:329–37. doi: 10.1083/jcb.201502062
45. Saarikangas J, Zhao H, Lappalainen P. Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. *Physiol Rev.* (2010) 90:259–89. doi: 10.1152/physrev.00036.2009
46. Senju Y, Kalimeri M, Koskela EV, Somerharju P, Zhao H, Vattulainen I, et al. Mechanistic principles underlying regulation of the actin cytoskeleton by phosphoinositides. *Proc Natl Acad Sci USA.* (2017) 114:E8977–86. doi: 10.1073/pnas.1705032114
47. Sun M, Northup N, Marga F, Huber T, Byfield FJ, Levitan I, et al. The effect of cellular cholesterol on membrane-cytoskeleton adhesion. *J Cell Sci.* (2007) 120:2223–31. doi: 10.1242/jcs.001370
48. Raghupathy R, Anilkumar AA, Polley A, Singh PP, Yadav M, Johnson C, et al. Transbilayer lipid interactions mediate nanoclustering of lipid-anchored proteins. *Cell.* (2015) 161:581–94. doi: 10.1016/j.cell.2015.03.048
49. Stanly TA, Fritzsche M, Banerji S, García E, Bernardino de la Serna J, Jackson DG, et al. Critical importance of appropriate fixation conditions for faithful imaging of receptor microclusters. *Biol Open.* (2016) 5:1343–50. doi: 10.1242/bio.019943
50. Gudheti MV, Curthoys NM, Gould TJ, Kim D, Gunewardene MS, Gabor KA, et al. Actin mediates the nanoscale membrane organization of the clustered membrane protein influenza hemagglutinin. *Biophys J.* (2013) 104:2182–92. doi: 10.1016/j.bpj.2013.03.054
51. De Keersmaecker H, Camacho R, Rantasa DM, Fron E, Uji H, Mizuno H, et al. Mapping transient protein interactions at the nanoscale in living mammalian cells. *ACS Nano.* (2018) 12:9842–54. doi: 10.1021/acsnano.8b01227
52. Sadegh S, Higgins JL, Mannion PC, Tamkun MM, Krapf D. Plasma membrane is compartmentalized by a self-similar cortical actin meshwork. *Phys Rev X.* (2017) 7:011031. doi: 10.1103/PhysRevX.7.011031
53. Mahammad S, Parmryd I. Cholesterol homeostasis in T cells. Methyl- β -cyclodextrin treatment results in equal loss of cholesterol from Triton X-100 soluble and insoluble fractions. *Biochim Biophys Acta.* (2008) 1778:1251–8. doi: 10.1016/j.bbame.2008.02.010
54. Magee AI, Adler J, Parmryd I. Cold-induced coalescence of T-cell plasma membrane microdomains activates signalling pathways. *J Cell Sci.* (2005) 118:3141–51. doi: 10.1242/jcs.02442
55. Laine RF, Tosheva KL, Gustafsson N, Gray RDM, Almada P, Albrecht D, et al. NanoJ: a high-performance open-source super-resolution microscopy toolbox. *J. Phys. D. Appl. Phys.* (2018) 52:163001. doi: 10.1088/1361-6463/ab0261
56. Pitcher C, Höning S, Fingerhut A, Bowers K, Marsh M. Cluster of differentiation antigen 4 (cd4) endocytosis and adaptor complex binding require activation of the cd4 endocytosis signal by serine phosphorylation. *Mol Biol Cell.* (1999) 10:677–91. doi: 10.1091/mbc.10.3.677
57. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol.* (2004) 22:1567. doi: 10.1038/nbt1037
58. Shaner NC, Lin MZ, McKeown MR, Steinbach PA, Hazelwood KL, Davidson MW, et al. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat Methods.* (2008) 5:545. doi: 10.1038/nmeth.1209
59. Keller P, Toomre D, Díaz E, White J, Simons K. Multicolour imaging of post-golgi sorting and trafficking in live cells. *Nat Cell Biol.* (2001) 3:140–9. doi: 10.1038/35055042
60. Albrecht D, Winterflood CM, Ewers H. Dual color single particle tracking via nanobodies. *Methods Appl Fluoresc.* (2015) 3:024001. doi: 10.1088/2050-6120/3/2/024001
61. Dix CL, Matthews HK, Uroz M, McLaren S, Wolf L, Heatley N, et al. The role of mitotic cell-substrate adhesion re-modeling in animal cell division. *Dev Cell.* (2018) 45:132–45. doi: 10.1016/j.devcel.2018.03.009
62. Pereira PM, Almada P, Henriques R. High-content 3d multicolor super-resolution localization microscopy. *Methods Cell Biol.* (2015) 125:95–117. doi: 10.1016/bs.mcb.2014.10.004
63. Ovesný M, Křížek P, Borkovec J, Svindrych Z, Hagen GM. Thunderstorm: a comprehensive imagej plug-in for palm and storm data analysis and super-resolution imaging. *Bioinformatics.* (2014) 30:2389–90. doi: 10.1093/bioinformatics/btu202
64. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* (2012) 9:676. doi: 10.1038/nmeth.2019
65. Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. Trackmate: an open and extensible platform for single-particle tracking. *Methods.* (2017) 115:80–90. doi: 10.1016/j.jmeth.2016.09.016
66. Tarantino N, Tinevez JY, Crowell EF, Boisson B, Henriques R, Mhlanga M, et al. Tnf and il-1 exhibit distinct ubiquitin requirements for inducing nemo-ikk supramolecular structures. *J Cell Biol.* (2014) 204:231–45. doi: 10.1083/jcb.201307172

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phospholipids: Pulling Back the Actin Curtain for Granule Delivery to the Immune Synapse

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Phosphoinositides, together with the phospholipids phosphatidylserine and phosphatidic acid, are important components of the plasma membrane acting as second messengers that, with diacylglycerol, regulate a diverse range of signaling events converting extracellular changes into cellular responses. Local changes in their distribution and membrane charge on the inner leaflet of the plasma membrane play important roles in immune cell function. Here we discuss their distribution and regulators highlighting the importance of membrane changes across the immune synapse on the cytoskeleton and the impact on the function of cytotoxic T lymphocytes.

OPEN ACCESS

Edited by:

Jorge Bernardino De La Serna,
Imperial College London,
United Kingdom

Reviewed by:

Marek Cebecauer,
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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 31 October 2018

Accepted: 14 March 2019

Published: 11 April 2019

Citation:

Gawden-Bone CM and Griffiths GM
(2019) Phospholipids: Pulling Back the
Actin Curtain for Granule Delivery to
the Immune Synapse.
Front. Immunol. 10:700.
doi: 10.3389/fimmu.2019.00700

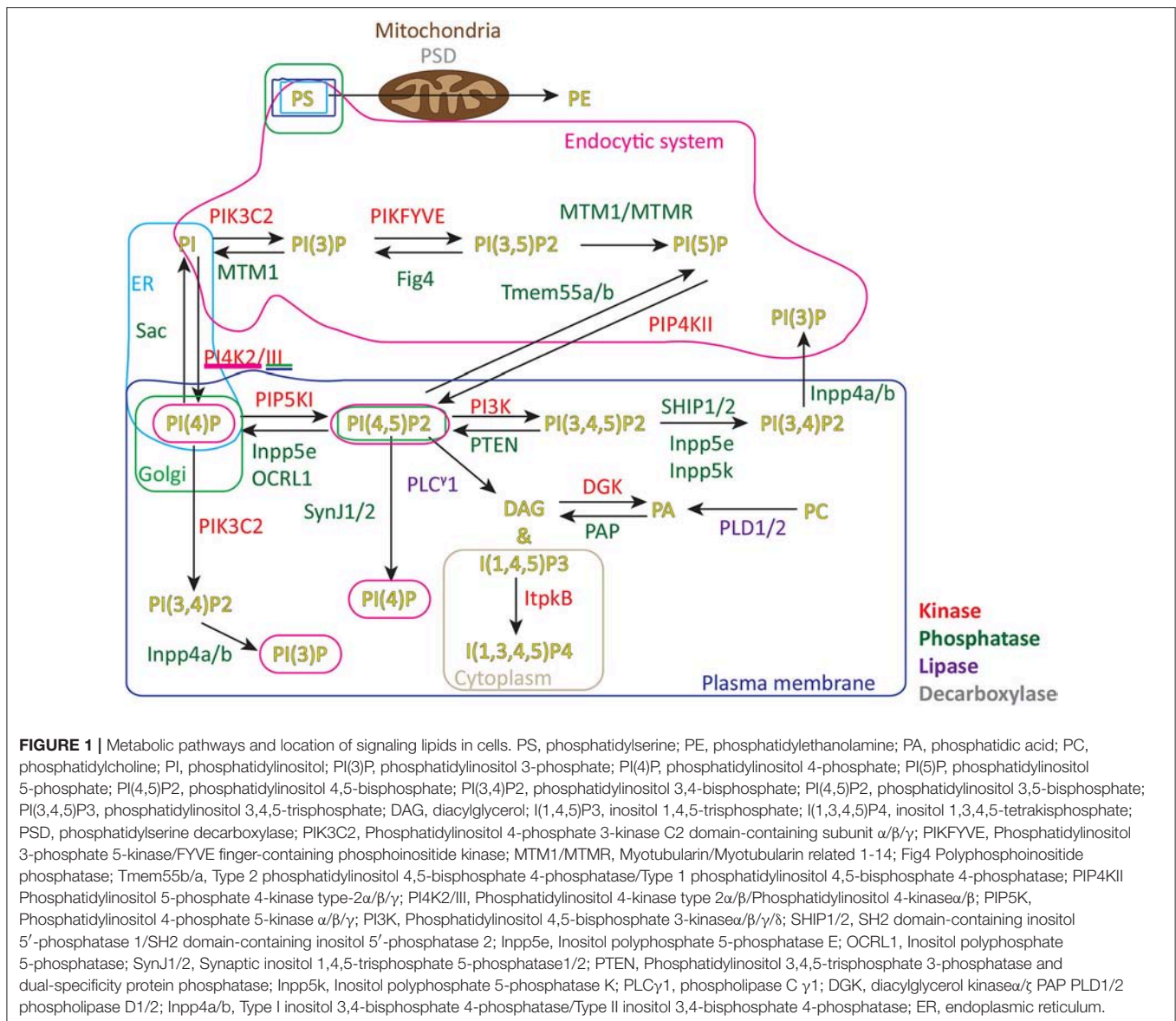
Keywords: T cells, cytotoxic T lymphocytes (CTLs), phospholipids, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), diacylglycerol (DAG), phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃)

CYTOTOXIC T CELLS, THE IMMUNE SYNAPSE AND PHOSPHOLIPIDS

Cytotoxic T lymphocytes (CTLs) are important for the clearance of cancerous and virally infected cells. Their task is more difficult as cancers and virally infected organs present as a mosaic of diseased and healthy cells within the tissue. CTLs specifically target infected or cancerous cells using the T cell receptor (TCR) recognizing peptide-loaded, major histocompatibility complex class one (pMHC I) on the surface of an antigen presenting cell [APC; reviewed in (1)]. TCR activation triggers a cascade of signaling events that results in the formation of a characteristic cell-to-cell contact between APCs and the CTLs referred to as the immune synapse [reviewed in (2)]. In CTL TCR clusters at the center of the synapse and lytic granule secretion occurs at a specialized secretory domain, next to the site of signaling (3, 4).

Actin depletion is an essential event that regulates lytic granule secretion at the synapse of both CTLs and natural killer cells (5–8). Although phospholipids and phosphoinositides have been well-characterized as regulators of actin reorganization during phagocytosis and macropinocytosis (9), their role in actin reorganization at the synapse has only recently emerged with more accurate lipid probes allowing visualization of their distribution and regulation across the synapse (10).

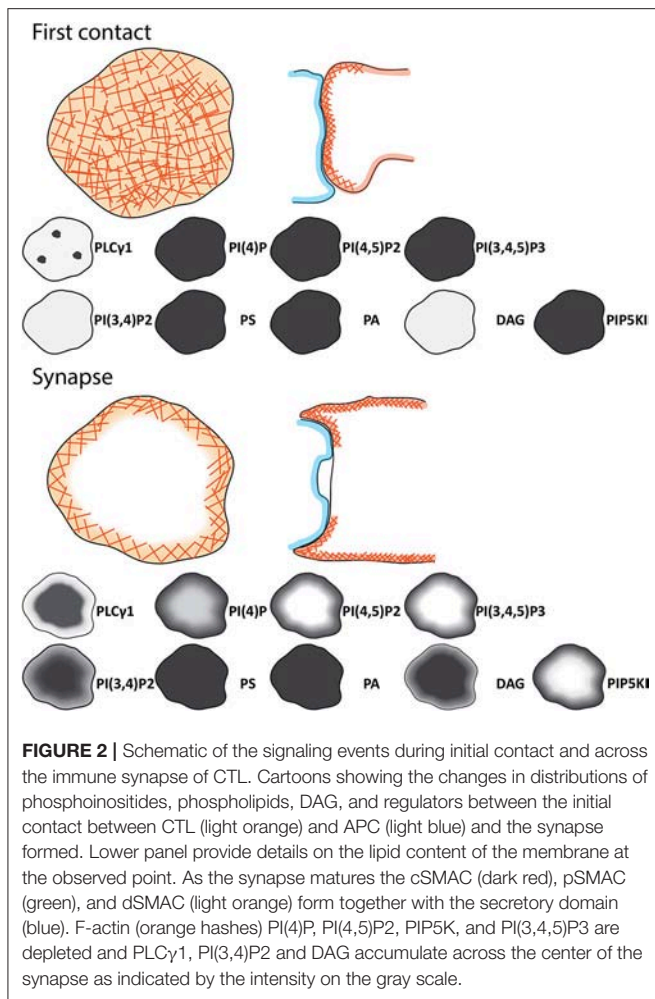
Figure 1 shows the metabolic pathways involved in lipid signaling events within the plasma membrane and endocytic system. Upon initial contact between the CTLs and APCs, F-actin initially accumulates across the forming interface before rapidly depleting as TCR activation triggers changes in the lipid composition across the immune synapse (7, 11). These changes are initiated by TCR activation of the LAT signalosome with Slp76, Gads and phospholipase C gamma 1 (PLCγ1) which rapidly accumulates in clusters as the synapse forms, recruiting other effector proteins that together potentiate signaling (12, 13). DAG, the product of PLCγ1 mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), accumulates as PI(4,5)P₂ diminishes across the synapse. Together with other changes an area of membrane specialization is formed (Figure 2).



Abbreviations: MHC I, Major histocompatibility complex I; TCR, T cell receptor; CTL, Cytotoxic T lymphocyte; PI(3)P, Phosphatidylinositol 3-phosphate; PI(4)P, Phosphatidylinositol 4-phosphate; PI(5)P, Phosphatidylinositol 5-phosphate; PI(4,5)P₂, Phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P₃, Phosphatidylinositol 3,4,5-trisphosphate; PI(3,4)P₂, Phosphatidylinositol 3,4-bisphosphate; PI(3,5)P₂, Phosphatidylinositol 3,5-bisphosphate; DAG, Diacylglycerol; PS, Phosphatidylserine; PA, Phosphatidic acid; IP₃, Inositol 1,4,5 trisphosphate; IP₄, Inositol 1,3,4,5 tetrakisphosphate; APC, Antigen presenting cells; LAT, Linker for activation of T cells; PLC γ 1, Phospholipase C gamma 1; ItpkB, Inositol 1,4,5-trisphosphate 3-kinase B; PI3K, Phosphatidylinositol 4,5-bisphosphate 3-kinase; PIP4KII, Phosphatidylinositol 5-phosphate 4-kinase; PIP5K, Phosphatidylinositol 4-phosphate 5-kinase; DGK, Diacylglycerol kinase; PTEN, Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; SHIP1, SH2 domain-containing inositol 5'-phosphatase 1; FERMT, 4.1 protein/Ezrin/Radaxin/Moesin; ERM, Ezrin/Moesin/Radaxin; WASp, Wiskott-Aldrich syndrome protein; TIRFM, Total internal reflection microscopy; PKC, Protein kinase C; PKD, Protein kinase D; IFT20, Intraflagella transport 20; ICAM-I, Intercellular adhesion molecule 1; TAPP1, Tandem PH domain-containing protein 1; PH domain, Pleckstrin homology domain; Grp1, General receptor of phosphoinositides 1; FYVE, Fab 1/YOTB/Vac 1/EEA1; F-actin, Filamentous actin; Zap70, 70 kDa zeta-chain associated protein; LFA-1, Leukocyte function-associated molecule 1.

LIPID REGULATION GENERATES MEMBRANE SPECIALIZATION

Low levels of phosphoinositides in membranes allow for exquisite regulation of signaling, with small changes modulating recruitment of signaling proteins. In CTLs the key event in response to changes initiated by TCR activation of PLC γ 1 is the loss of PI(4,5)P₂ across the immune synapse, which results in a loss of cortical actin across the membrane, allowing granule secretion to occur (10). Equally important to events driving the loss of actin across the synapse are the mechanisms that prevent PI(4,5)P₂ being rapidly replenished by the PIP5 kinases (PIP5K). Although PIP5K family members are recruited to the immune synapse in Jurkat cells, these studies did not examine dynamic temporal events (14, 15). When PIP5K dynamics were examined it was found that these kinases are also depleted across the synapse upon TCR activation, and the ability to replenish PI(4,5)P₂ via this pathway is also lost



(10). Exactly how this coordinated loss of PI(4,5)P2 and the kinase that usually maintains PI(4,5)P2 levels in the plasma membrane occurs in response to TCR activation also emerged from these studies. Several different mechanisms have been shown to control PIP5K membrane association in different cell types, including recruitment via Rac1, ARF6, AP-2, beta-arrestin, talin, and PI(4)P (16–20). However, in macrophages the mechanism for PIP5K membrane association occurs via an electrostatic switch, by virtue of a series of polybasic amino acids that project from a conserved alpha-helix and interact with negatively charged PI(4,5)P2 in the membrane (21, 22). This electrostatic mechanism also functions in T cells and provides a very elegant way of coupling TCR activation with membrane changes that control secretion. Upon TCR activation the loss of PI(4,5)P2 by PLCγ1 also causes a loss of negative charge as DAG replaces PI(4,5)P2. This rapid loss of negative charge causes PIP5K to dissociate from the plasma membrane across the synapse so that PI(4,5)P2 can no longer be replenished via this route. This rapid loss of PI(4,5)P2 drives a rapid loss of actin favoring granule secretion (10). In this way, a small shift in the balance of phosphoinositides is able to control CTL killing.

One challenge to maintaining lipid specialization is that most lipids are highly diffusible within the plasma membrane. DAG

has been reported to be a highly diffusible lipid when tracked with the PKC-theta C1 domain probe in B cells (23); nevertheless a DAG gradient is maintained at the synapse membrane by the interplay of lipases and kinases (24). Using diacylglycerol kinase (DGK) α or ζ null mice and TIRFM on activating membranes this study shows that CD8 and CD4 T cells deficient in DGKα have a centrosome polarization defect. This was due to the diffuse distribution of DAG across the synaptic membrane suggesting that the restriction of DAG in the cSMAC is an important feature for centrosome recruitment to the membrane and that DGKα acts to ensure the focus of DAG in the membrane. Importantly, DGKα localized to the dSMAC suggesting it fences in the DAG to the central synapse (24). Any DAG that moves toward the periphery at the concentration of active DGKα is metabolized to PA, which may also constrain RasGrp signaling to the central synapse (25, 26). This limits DAG to a relatively tight area of the membrane and this in turn maintains the polarizing signal (24). More importantly, DAG would create a spatially regulated docking site for centrosome delivery to the synapse. DGKζ deletion had no effect on the spatial regulation of DAG at the synapse and did not prevent centrosome polarization (24). Although, the role of DGKζ is less clear at the synapse where it is implicated in the regulation of signal strength at the synapse through its control of the Ras and Erk pathways (27).

Intriguingly many other proteins have specific domains or positively charged outer surfaces that allow sensing of charge changes at the plasma membrane [reviewed in (21),(28–32)]. A number of synthetic probes exist to detect charge in the membrane, based on arginine and lysine rich amino acid chains, with a lipidation motif at their C-terminus. These include the Kras+8 probe, R-pre probe, and the MCS+ probe based on R-pre [described in detail in (32–34)]. The drop in charge in both the CTL synapse and in Jurkat cells has been detected with these probes (10, 33), suggesting this is a general mechanism for modulating the immune synapse membrane.

PI(4,5)P2, PIP5K, AND PLCγ1 REGULATE THE ACTIN CYTOSKELETON IN THE SYNAPSE

F-actin dynamics at the synapse are important for TCR signaling and delivery of granules to the synapse membrane (7, 35, 36). Several lines of evidence support a link between the loss of PI(4,5)P2 and the loss of actin across the synapse. First, the loss of PI(4,5)P2 correlates both spatially and temporally with the loss of actin [(7); Figure 2]. Second, the phospholipase C inhibitor, U73122, which inhibits PLCγ1, blocks actin loss from the central synapse region. Thirdly, blocking the depletion of PIP5K proteins from the synapse by tagging PIP5Kβ with the palmitoylation domain of Lyn protein, blocks actin depletion across the synapse.

The role of PI(4,5)P2 in recruiting actin to the cortical membrane is mediated by a number of actin-recruiting proteins that bind directly to PI(4,5)P2. These include the Ezrin/Radixin/Moesin (ERM) proteins which interact with PI(4,5)P2 via their FERM domains (37). Phosphorylation of ERM proteins stabilizes their open conformation and allows for bridging of the actin cytoskeleton and PI(4,5)P2 (38, 39).

PIP5Ks have also been shown to recruit ERM to the membrane of primary 5C.C7 TCR T cells by production of PI(4,5)P₂ (40). Interestingly, ERM proteins deplete across the CD4 T cell synapse after initial contact between T cell and APC (41). Furthermore, cleavage of PI(4,5)P₂ by PLC γ 1 triggers depletion of Ezrin from the membrane in CTLs (39).

Wiskott-Aldrich syndrome protein (WASp) also plays a role in actin recruitment, interacting directly with PI(4,5)P₂ via a polybasic region. Interestingly, WASp has a direct role in TCR activation and regulation of PLC γ 1 activity in the CTL membrane. WASp recruitment to TCR complexes is thought to be required for efficient activation of TCR and the subsequent recruitment of PLC γ 1, PI(4,5)P₂ metabolism and calcium signaling; all of which were defective in WASp null T cells (36). This suggests that TCR activation via WASp occurs early in signaling when the membrane has abundant levels of PI(4,5)P₂, or occurs later at the periphery of the maturing synapse where PI(4,5)P₂ is also abundant. Alternatively, low levels of PI(4,5)P₂ present in the central synapse might be sufficient to recruit WASp, or perhaps WASp might be recruited via PI(3,4)P₂, which is a mirror image of the PI(4,5)P₂ and is abundant at the central synapse membrane.

PI(3,4,5)P₃ DISTRIBUTIONS LEAD TO FORCES ACROSS THE SYNAPSE

Most research to date has focused on understanding the interplay of signaling proteins on the CTL side of the immune synapse. However, this is only half the story in the interaction between T cell and target. The membrane tension generated on the target side of the synapse is an important regulator of perforin-mediated killing. Phosphoinositides, actin and integrins are all thought to mediate forces sensed by the target. The phosphoinositide PI(3,4,5)P₃ controls actin movement in the periphery through Rac1 activity, a potent regulator of actin cytoskeletal dynamics. These actin mediated forces are focused in the dSMAC where engulfment and cell motility protein 1 (ELMO) is recruited to the plasma membrane via PI(3,4,5)P₃. Here it interacts with dedicator of cytokinesis protein 2 (DOCK2) activating Rac1 to initiate WAVE driven actin dynamics (42).

More recently a role for PI(3,4,5)P₃ in force generation at the synapse was demonstrated using deflection of micropillars to measure forces. Consistent with the earlier findings, loss of DOCK2 resulted in a loss of force across the synapse (43). This elegant system was also able to reveal force direction and showed that early forces pushed in to the periphery in a process termed mechanopotential. These results suggested that these forces, which were myosin II dependent and were increased when PTEN was silenced, increased membrane tension across the APC membrane, potentiating perforin-mediated killing (35, 43).

Integrin interactions, supported by the local lipid microenvironment, also play an important role in generating actin-driven forces across the synapse. Integrin activators kindlin-3 and talin, which stabilize the extended conformation of the integrin in to the extracellular milieu, depend on the presence of PI(3,4,5)P₃ and PI(4,5)P₂ (44, 45). Kindlin-3 may

be dispensable for synapse formation and integrin activation in T cells (46–48), whereas talin and vinculin's interaction with integrins drives TCR responses in CD4 T cells (49). PI(3,4,5)P₃ is thought to drive actin flow at the periphery of the synapse which in turn regulates integrin and WAVE activation (43). Integrins are drawn in the direction of the actin flow in CTLs, with continued ruffling between CTLs and APC generating tension (50). The actin cytoskeleton in the dendritic cell also supports T cell interaction through the stabilization of ICAM-1 contributing to the forces generated (51). Although the direction of actin flow in the synapse is controversial, with TIRF imaging interpreted as showing a centripetal inward flow of actin to the dSMAC (52), while lattice light sheet showed a rearward flow of peripheral lamellipodial actin toward the uropod of CTL (7), the direction of actin flow in the synapse will have an important role in integrin activation and forces generated at the synapse.

TCR activation in the periphery also drives inside out integrin activation and tension creation, as CasL, a phosphoprotein with multiple kinase docking sites is recruited to the peripheral TCR complexes and support larger TCR clusters when present. CasL recruitment led to integrin activation at the immune synapse (53), further building on the hypothesis that continued actin dynamics, TCR activation and integrin activation support each other in synapse formation. Activation of integrin leukocyte function-associated molecule 1 (LFA-1; CD11a/CD18 heterodimer) in the CD4 T cell immune synapse results in a significant increase in TCR activation (49, 54, 55). TCR activation, actin dynamics and integrin adhesion in the periphery clearly increase further TCR:MHC interactions, potentiating signaling and synapse lifetime. This may suggest why PI(3,4,5)P₃ supported actin dynamics in the distal synapse region is important for CTL killing.

DIACYLGLYCEROL AT THE IMMUNE SYNAPSE

As already mentioned phosphoinositide changes play a role in directing granules to the synapse. DAG, generated by the initial cleavage of PI(4,5)P₂, controls the movement of the centrosome to the synapse membrane through its activation of kinases and motor proteins (56–58). Inositol 1,4,5-trisphosphate (IP₃) either binds receptors in the endoplasmic reticulum activating calcium release from intracellular stores or is converted into IP₄ and involved in ITK signaling (59). The importance of DAG is clear from studies in which the conversion of PI(4,5)P₂ to DAG or un-caging of DAG (independent of calcium signaling) by fluorescence activation are sufficient to polarize the centrosome to the plasma membrane (58). Computer modeling of DAG in membranes suggests that DAG is a mediator of gross biophysical changes within the membrane due to changes in head group spacing and hydrophobic fatty acid packing. These changes in local membrane spacing would allow for improved localization of the DAG dependent protein kinase C (PKC) family members and C1 domain containing proteins (60). DAG plays many other roles at the synapse, supporting the activation of PKC family members, protein kinase D (PKD) and RAS guanyl-releasing protein/RasGRP (57, 61, 62).

Production of DAG at the T cell synapse membrane is important for recruiting PKD. PKC β , which is a transient resident of the synapse, activates PKD at the synapse after DAG binding (57, 62). There are multiple targets for PKD after activation including transcription factors, the actin cytoskeleton and multiple serine/threonine and tyrosine kinases. This suggests that formation of DAG at the synapse has a significant effect on the post-translational and transcriptional profile of the CTL (63).

Recruitment of the Tec kinase, ITK relies on PI(3,4,5)P3 for its localization and this is enhanced by pleckstrin homology (PH) domain binding of IP4. Inositol 1,4,5-trisphosphate (IP3) 3-kinase B (ItpkB) generates IP4 after PLC γ 1 converts PI(4,5)P2 to DAG and IP3. T cells from ItpkB deficient mice were unable to recruit ITK to the immune synapse. T cells recruited less DAG at the synapse and displayed reduced Erk signaling, which may explain why ItpkB null mice have a T cell deficiency (64). This suggests that ItpkB and ITK may act as a signal multiplier, supporting large-scale activation of PLC γ 1 at the synapse. This in turn generates more substrate for ItpkB that supports further ITK activation. ITK null CTLs show reduced killing capacity suggesting it does not only affect differentiation of T cell or T cell selection but also regulates effector cell function (65). Mathematical modeling of how IP4 levels play a role in enhancing ITK activation through increased PI(3,4,5)P3/PH domain binding, indicated there is also a negative feedback loop regulating the pathway. As IP4 increases due to continued activation of PLC γ 1 and activity of ItpkB, IP4 outcompetes the PI(3,4,5)P3 binding site in the PH domain. This results in dissociation of ITK from the membrane and loss of PLC γ 1 activation through ITK activity (66). ITK/ItpkB/IP4 may therefore regulate the total available DAG created at the membrane during synapse formation independently of other signaling events and ITK through this signaling negative feedback loop may regulate T cell activation and lipid dynamics. Pleckstrin2 is also recruited to the plasma membrane including the synapse of Jurkat cells via the PH domain, co-localizing with actin (67) although the mechanism is not as thoroughly studied as for Itk yet.

PHOSPHATIDYL SERINE AND PHOSPHATIDIC ACID AT THE SYNAPSE

Several other species of phospholipids contribute to membrane specialization in cells including PI(5)P, PI(3)P, PS, PA, and PI(3,5)P2 (Figure 1). Many have specific functions in pathways such as endosome sorting, membrane charge, endocytosis and autophagy. PS is a negatively charged phospholipid; however, it is not significantly depleted from the central synapse region as negative charge decreases (10, 33). It is possible that the influx of positive ions (such as Ca²⁺ or Mg²⁺) during TCR signaling may neutralize the charge of these lipid head groups during the charge depletion event, as seen in macrophages where calcium influx limited plasma membrane charge (32, 33, 68). In some CTL, PS appeared to concentrate at the actin-rich distal region of the synapse; F-actin is reported to slow down PS dynamic in the

membrane as PS transiently binds with actin-membrane linker proteins (69).

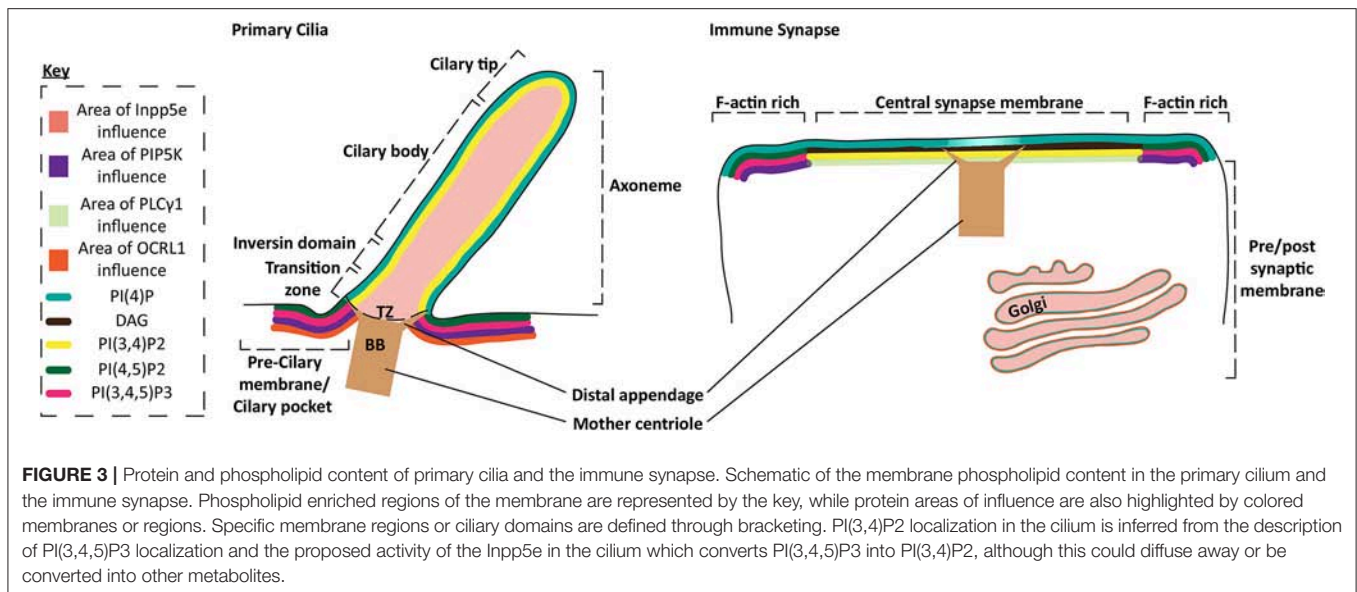
PA, which is the product of DAG phosphorylation by DGK, does not appear to change across the synapse as it forms, suggesting that DAG is not significantly modified during synapse formation (10). This is important, as PA in the plasma membrane has been shown to regulate PIP5K activity (70). As PA does not increase while actin recovers this implies that it is unlikely to be responsible for the recovery of PIP5K, PI(4,5)P2, and actin observed after granule secretion (8).

Several lipids are yet to be visualized in the synapse in T cells as the probes used to investigate their distribution are not specific (71) or express poorly in CTLs. However, it has been shown that phosphatidylinositol 5-phosphate (PI(5)P) can be produced via other routes including the 4'-phosphatase activity of Tmem55a/b on PI(4,5)P2 (Figure 1); myotubularin 3'-phosphatases acting on PI(3,5)P2 and inhibition of PIP4K activity or the expression of IpgD (72–74). Interestingly, PI(5)P increases have been shown to lead to increases in cytokine production and Src family kinase signaling in activated T cells (72, 75, 76). Until specific protein sensors become available, information on these lipids is limited.

MEMBRANE SPECIALIZATION ACROSS THE PRIMARY CILIUM AND THE IMMUNE SYNAPSE

One of the most remarkable findings about the phosphoinositide specialization observed across the synapse is its striking similarity to the phosphoinositide signature across the primary cilium with PI(4,5)P2 and PI(3,4,5)P3 depleted relative to other areas of the plasma membrane in both cilia and the synapse. Primary cilia are specialized sensory organelles that extend from the plasma membrane into the extracellular space that are important for hedgehog and platelet derived growth factor signaling in cells. Primary cilia accumulate receptors in to a specialized region of the membrane concentrating many signaling molecules important for development (77–80). While cells readily produce primary cilia in response to stress or starvation, cells of the haemopoietic lineage do not. This is somewhat surprising as studies in immortalized B and T-cells have demonstrated that these hematopoietic cells do contain the machinery required to form cilia (81).

Cilia formation is dependent on the activities of Inpp5e and Inositol polyphosphate 5-phosphatase/OCRL1/Inpp5f (5' lipid phosphatases), which convert PI(4,5)P2 and PI(3,4,5)P3 into PI(4)P and PI(3,4)P2, respectively (82–86). This acts to exclude concentrations of PI(4,5)P2 and PI(3,4,5)P3 to the transition zone at the basal body, in favor of PI(4)P and possibly PI(3,4)P2. The localization of PI(3,4)P2 in the cilium is yet to be shown, but must be present if PI(3,4,5)P3 is also converted (Figure 3). Furthermore, Bardet-Biedl Syndrome protein 5, part of a complex thought to transition G-protein coupled receptors into the cilium across the transition zone (87), have PI(3,4)P2 binding PH domains implicating the presence of PI(3,4)P2 in the cilium or as a regulator of protein transport to the cilium (88). Initiation of ciliary signaling and dissolution of cilia results in a shutdown of the lipid phosphatase pathway and production or



entry of PI(4,5)P2 into the ciliary membrane. Actin is recruited to cilia after PI(4,5)P2 accumulation (89, 90). After lytic granule delivery, actin is also recovered over the synaptic membrane of CTLs. This suggests that actin is an effective barrier to signaling at the cilia and synapse (7). Interestingly the centrosome uses the interplay between PIP5K and Inpp5e to control the balance of PI(4)P and PI(4,5)P2 in either Golgi associated vesicles or at the plasma membrane. These lipids differentially regulate the recruitment of the distal appendage protein Cep164 or Tau tubulin kinase 2 to mediate ciliation after appropriate signaling occurs (91). In T cells PIP5K and levels of PI(4,5)P2 play a similar role in synapse formation (10). These findings highlight the similarities in membrane specialization in the immune synapse and cilia, supporting the idea that there may be a functional link between the structures.

After granule delivery to the CTL membrane both PI(4,5)P2 and actin are restored across the synapse. Likewise, it appears that actin is only present before formation or after dissolution of cilia (90, 92, 93). This suggests actin may also act as a significant barrier in cilia, also regulated by changes in PI(4,5)P2 and PI(3,4,5)P3 (94) (Figure 3). Interestingly, actin dynamics slowed the flow of receptors transitioning between the cilium membrane and the plasma membrane peripheral to the cilia (93), while actin flow is also thought to play an important role in the coalescence of TCR clusters in T cells (95, 96).

THE FUTURE OF LIPIDS AT THE SYNAPSE

Phospholipids and their associated signaling molecules transmit information and support the accumulation of protein signaling

platforms. They also shape the actin cytoskeleton and thereby sculpt higher order structures in cells. The lipases, kinases, and phosphatases involved in phospholipid metabolism shape the inner leaflet of the membrane without the need for structural proteins that mediate diffusion barriers. Many questions remain, and there are gaps in our knowledge that are not understood from the current literature, or via the use of exogenously expressed, available bio-probes. For example how do TCR complexes that depend on PI(4,5)P2 transition from the periphery, into the cSMAC, where PI(4,5)P2 is depleted? Does depletion of phospholipid head groups result in significant biophysical changes in the membrane? Understanding these mechanisms will provide unique insights as to how regulators of granule delivery could be targeted, perhaps even therapeutically, and may also hold information that transfers to a diverse portfolio of cellular organelles in a diverse range of cells.

AUTHOR CONTRIBUTIONS

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

FUNDING

Research was funded by Wellcome Trust grants [103930] and [100140], to GG. We would like to thank Katharina Stregge, Phillipa Barton, Jane Stinchcombe, and Lyra Randzavola for critical reading of the manuscript.

REFERENCES

1. La Gruta NL, Gras S, Daley SR, Thomas PG, Rossjohn J. Understanding the drivers of MHC restriction of T cell receptors. *Nat Rev Immunol.* (2018) 18:467–78. doi: 10.1038/s41577-018-0007-5
2. Dieckmann NM, Frazer GL, Asano Y, Stinchcombe JC, Griffiths GM. The cytotoxic T lymphocyte immune synapse at a glance. *J Cell Sci.* (2016) 129:2881–6. doi: 10.1242/jcs.186205
3. Stinchcombe JC, Bossi G, Booth S, Griffiths GM. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity.* (2001) 15:751–61. doi: 10.1016/S1074-7613(01)00234-5

4. Ritter AT, Angus KL, Griffiths GM. The role of the cytoskeleton at the immunological synapse. *Immunol Rev.* (2013) 256:107–17. doi: 10.1111/immr.12117
5. Brown AC, Oddos S, Dobbie IM, Alakoskela JM, Parton RM, Eissmann P, et al. Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol.* (2011) 9:e1001152. doi: 10.1371/journal.pbio.1001152
6. Rak GD, Mace EM, Banerjee PP, Svitkina T, Orange JS. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol.* (2011) 9:e1001151. doi: 10.1371/journal.pbio.1001151
7. Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen BC, Gawden-Bone C, et al. Actin depletion initiates events leading to granule secretion at the immunological synapse. *Immunity.* (2015) 42:864–76. doi: 10.1016/j.immuni.2015.04.013
8. Ritter AT, Kapnick SM, Murugesan S, Schwartzberg PL, Griffiths GM, Lippincott-Schwartz J. Cortical actin recovery at the immunological synapse leads to termination of lytic granule secretion in cytotoxic T lymphocytes. *Proc Natl Acad Sci USA.* (2017) 114:E6585–94. doi: 10.1073/pnas.1710751114
9. Levin R, Grinstein S, Schlam D. Phosphoinositides in phagocytosis and macropinocytosis. *Biochim Biophys Acta.* (2015) 1851:805–23. doi: 10.1016/j.bbalip.2014.09.005
10. Gawden-Bone CM, Frazer GL, Richard AC, Ma CY, Strega K, Griffiths GM. PIP5 kinases regulate membrane phosphoinositide and actin composition for targeted granule secretion by cytotoxic lymphocytes. *Immunity.* (2018) 49:427–37.e4. doi: 10.1016/j.immuni.2018.08.017
11. Jenkins MR, Stinchcombe JC, Au-Yeung BB, Asano Y, Ritter AT, Weiss A, et al. Distinct structural and catalytic roles for Zap70 in formation of the immunological synapse in CTL. *Elife.* (2014) 3:e01310. doi: 10.7554/eLife.01310
12. Balagopalan L, Coussens NP, Sherman E, Samelson LE, Sommers CL. The LAT story: a tale of cooperativity, coordination, and choreography. *Cold Spring Harb Perspect Biol.* (2010) 2:a005512. doi: 10.1101/cshperspect.a005512
13. Balagopalan L, Kortum RL, Coussens NP, Barr VA, Samelson LE. The linker for activation of T cells (LAT) signaling hub: from signaling complexes to microclusters. *J Biol Chem.* (2015) 290:26422–9. doi: 10.1074/jbc.R115.665869
14. Kallikourdis M, Trovato AE, Roselli G, Muscolini M, Porciello N, Tuosto L, et al. Phosphatidylinositol 4-phosphate 5-kinase beta controls recruitment of lipid rafts into the immunological synapse. *J Immunol.* (2016) 196:1955–63. doi: 10.4049/jimmunol.1501788
15. Porciello N, Kunkl M, Viola A, Tuosto L. Phosphatidylinositol 4-phosphate 5-kinases in the regulation of T cell activation. *Front Immunol.* (2016) 7:186. doi: 10.3389/fimmu.2016.00186
16. Aikawa Y, Martin TF. ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. *J Cell Biol.* (2003) 162:647–59. doi: 10.1083/jcb.200212142
17. de Pereda JM, Wegener KL, Santelli E, Bate N, Ginsberg MH, Critchley DR, et al. Structural basis for phosphatidylinositol phosphate kinase type Igamma binding to talin at focal adhesions. *J Biol Chem.* (2005) 280:8381–6. doi: 10.1074/jbc.M413180200
18. Halstead JR, Savaskan NE, van den Bout I, Van Horck F, Hajdo-Milasovic A, Snell M, et al. Rac controls PIP5K localisation and PtdIns(4,5)P(2) synthesis, which modulates vinculin localisation and neurite dynamics. *J Cell Sci.* (2010) 123:3535–46. doi: 10.1242/jcs.062679
19. Nelson CD, Kovacs JJ, Nobles KN, Whalen EJ, Lefkowitz RJ. Beta-arrestin scaffolding of phosphatidylinositol 4-phosphate 5-kinase Ialpha promotes agonist-stimulated sequestration of the beta2-adrenergic receptor. *J Biol Chem.* (2008) 283:21093–101. doi: 10.1074/jbc.M800431200
20. van den Bout I, Divecha N. PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions. *J Cell Sci.* (2009) 122:3837–50. doi: 10.1242/jcs.056127
21. Fairn GD, Ogata K, Botelho RJ, Stahl PD, Anderson RA, De Camilli P, et al. An electrostatic switch displaces phosphatidylinositol phosphate kinases from the membrane during phagocytosis. *J Cell Biol.* (2009) 187:701–14. doi: 10.1083/jcb.200909025
22. Liu A, Sui D, Wu D, Hu J. The activation loop of PIP5K functions as a membrane sensor essential for lipid substrate processing. *Sci Adv.* (2016) 2:e1600925. doi: 10.1126/sciadv.1600925
23. Xu C, Xie H, Guo X, Gong H, Liu L, Qi H, et al. A PIP2-derived amplification loop fuels the sustained initiation of B cell activation. *Sci Immunol.* (2017) 2:eaan0787. doi: 10.1126/sciimmunol.aan0787
24. Chauveau A, Le Floch A, Bantilan NS, Koretzky GA, Huse M. Diacylglycerol kinase alpha establishes T cell polarity by shaping diacylglycerol accumulation at the immunological synapse. *Sci Signal.* (2014) 7:ra82. doi: 10.1126/scisignal.2005287
25. Sanjuan MA, Jones DR, Izquierdo M, Merida I. Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. *J Cell Biol.* (2001) 153:207–20. doi: 10.1083/jcb.153.1.207
26. Sanjuan MA, Pradet-Balade B, Jones DR, Martinez AC, Stone JC, Garcia-Sanz JA, et al. T cell activation *in vivo* targets diacylglycerol kinase alpha to the membrane: a novel mechanism for Ras attenuation. *J Immunol.* (2003) 170:2877–83. doi: 10.4049/jimmunol.170.6.2877
27. Merida I, Andrada E, Gharbi SI, Avila-Flores A. Redundant and specialized roles for diacylglycerol kinases alpha and zeta in the control of T cell functions. *Sci Signal.* (2015) 8:re6. doi: 10.1126/scisignal.aaa0974
28. Corbalan-Garcia S, Gomez-Fernandez JC. Protein kinase C regulatory domains: the art of decoding many different signals in membranes. *Biochim Biophys Acta.* (2006) 1761:633–54. doi: 10.1016/j.bbalip.2006.04.015
29. Corbalan-Garcia S, Gomez-Fernandez JC. Signaling through C2 domains: more than one lipid target. *Biochim Biophys Acta.* (2014) 1838:1536–47. doi: 10.1016/j.bbamem.2014.01.008
30. El Amri M, Fitzgerald U, Schlosser G. MARCKS and MARCKS-like proteins in development and regeneration. *J Biomed Sci.* (2018) 25:43. doi: 10.1186/s12929-018-0445-1
31. Lemmon MA. Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol.* (2008) 9:99–111. doi: 10.1038/nrm2328
32. Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S. Membrane phosphatidylserine regulates surface charge and protein localization. *Science.* (2008) 319:210–3. doi: 10.1126/science.1152066
33. Ma Y, Yamamoto Y, Nicovich PR, Goyette J, Rossy J, Gooding JJ, et al. A FRET sensor enables quantitative measurements of membrane charges in live cells. *Nat Biotechnol.* (2017) 35:363–70. doi: 10.1038/nbt.3828
34. Roy MO, Leventis R, Silvius JR. Mutational and biochemical analysis of plasma membrane targeting mediated by the farnesylated, polybasic carboxy terminus of K-ras4B. *Biochemistry.* (2000) 39:8298–307. doi: 10.1021/bi000512q
35. Basu R, Huse M. Mechanical communication at the immunological synapse. *Trends Cell Biol.* (2017) 27:241–54. doi: 10.1016/j.tcb.2016.10.005
36. Kumari S, Depoil D, Martinelli R, Judokusumo E, Carmona G, Gertler FB, et al. Actin foci facilitate activation of the phospholipase C-gamma in primary T lymphocytes via the WASP pathway. *Elife.* (2015) 4. doi: 10.7554/eLife.04953
37. Janmey PA, Bucki R, Radhakrishnan R. Regulation of actin assembly by PI(4,5)P2 and other inositol phospholipids: an update on possible mechanisms. *Biochem Biophys Res Commun.* (2018) 506:307–14. doi: 10.1016/j.bbrc.2018.07.155
38. Faure S, Salazar-Fontana LI, Semichon M, Tybulewicz VL, Bismuth G, Trautmann A, et al. ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. *Nat Immunol.* (2004) 5:272–9. doi: 10.1038/ni1039
39. Hao JJ, Liu Y, Kruhlak M, Debell KE, Rellahan BL, Shaw S. Phospholipase C-mediated hydrolysis of PIP2 releases ERM proteins from lymphocyte membrane. *J Cell Biol.* (2009) 184:451–62. doi: 10.1083/jcb.200807047
40. Sun Y, Dandekar RD, Mao YS, Yin HL, Wulfe C. Phosphatidylinositol (4,5) bisphosphate controls T cell activation by regulating T cell rigidity and organization. *PLoS ONE.* (2011) 6:e27227. doi: 10.1371/journal.pone.0027227
41. Shaffer MH, Dupree RS, Zhu P, Saotome I, Schmidt RF, McClatchey AI, et al. Ezrin and moesin function together to promote T cell activation. *J Immunol.* (2009) 182:1021–32. doi: 10.4049/jimmunol.182.2.1021
42. Le Floch A, Tanaka Y, Bantilan NS, Voisinne G, Altan-Bonnet G, Fukui Y, et al. Annular PIP3 accumulation controls actin architecture and modulates cytotoxicity at the immunological synapse. *J Exp Med.* (2013) 210:2721–37. doi: 10.1084/jem.20131324
43. Basu R, Whitlock BM, Husson J, Le Floch A, Jin W, Oyler-Yaniv A, et al. Cytotoxic T cells use mechanical force to potentiate target cell killing. *Cell.* (2016) 165:100–10. doi: 10.1016/j.cell.2016.01.021

44. Moretti FA, Moser M, Lyck R, Abadier M, Ruppert R, Engelhardt B, et al. Kindlin-3 regulates integrin activation and adhesion reinforcement of effector T cells. *Proc Natl Acad Sci USA*. (2013) 110:17005–10. doi: 10.1073/pnas.1316032110
45. Ni T, Kalli AC, Naughton FB, Yates LA, Nanah O, Kozorog M, et al. Structure and lipid-binding properties of the kindlin-3 pleckstrin homology domain. *Biochem J*. (2017) 474:539–56. doi: 10.1042/BCJ20160791
46. Fagerholm SC, Lek HS, Morrison VL. Kindlin-3 in the immune system. *Am J Clin Exp Immunol*. (2014) 3:37–42.
47. Moretti FA, Klapproth S, Ruppert R, Margraf A, Weber J, Pick R, et al. Differential requirement of kindlin-3 for T cell progenitor homing to the non-vascularized and vascularized thymus. *Elife*. (2018) 7:e35816. doi: 10.7554/eLife.35816
48. Morrison VL, MacPherson M, Savinko T, Lek HS, Prescott A, Fagerholm SC. The beta2 integrin-kindlin-3 interaction is essential for T-cell homing but dispensable for T-cell activation *in vivo*. *Blood*. (2013) 122:1428–36. doi: 10.1182/blood-2013-02-484998
49. Jankowska KI, Williamson EK, Roy NH, Blumenthal D, Chandra V, Baumgart T, et al. Integrins modulate T cell receptor signaling by constraining actin flow at the immunological synapse. *Front Immunol*. (2018) 9:25. doi: 10.3389/fimmu.2018.00025
50. Comrie WA, Babich A, Burkhardt JK. F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *J Cell Biol*. (2015) 208:475–91. doi: 10.1083/jcb.201406121
51. Comrie WA, Li S, Boyle S, Burkhardt JK. The dendritic cell cytoskeleton promotes T cell adhesion and activation by constraining ICAM-1 mobility. *J Cell Biol*. (2015) 208:457–73. doi: 10.1083/jcb.201406120
52. Yu CH, Wu HJ, Kaizuka Y, Vale RD, Groves JT. Altered actin centripetal retrograde flow in physically restricted immunological synapses. *PLoS ONE*. (2010) 5:e11878. doi: 10.1371/journal.pone.0011878
53. Santos LC, Blair DA, Kumari S, Cammer M, Iskratsch T, Herbin O, et al. Actin polymerization-dependent activation of Cas-L promotes immunological synapse stability. *Immunol Cell Biol*. (2016) 94:981–93. doi: 10.1038/icb.2016.61
54. Bachmann MF, McCall-Faienza K, Schmits R, Bouchard D, Beach J, Speiser DE, et al. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity*. (1997) 7:549–57. doi: 10.1016/S1074-7613(00)80376-3
55. Roy NH, Burkhardt JK. Embracing the enemy: cell-to-cell force transmission enhances cytotoxicity. *Dev Cell*. (2016) 36:592–4. doi: 10.1016/j.devcel.2016.03.007
56. Liu X, Kapoor TM, Chen JK, Huse M. Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin II. *Proc Natl Acad Sci USA*. (2013) 110:11976–81. doi: 10.1073/pnas.1306180110
57. Quann EJ, Liu X, Altan-Bonnet G, Huse M. A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat Immunol*. (2011) 12:647–54. doi: 10.1038/ni.2033
58. Quann EJ, Merino E, Furuta T, Huse M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol*. (2009) 10:627–35. doi: 10.1038/ni.1734
59. Joseph N, Reicher B, Barda-Saad M. The calcium feedback loop and T cell activation: how cytoskeleton networks control intracellular calcium flux. *Biochim Biophys Acta*. (2014) 1838:557–68. doi: 10.1016/j.bbame.2013.07.009
60. Alwarawrah M, Dai J, Huang J. Modification of lipid bilayer structure by diacylglycerol: a comparative study of diacylglycerol and cholesterol. *J Chem Theory Comput*. (2012) 8:749–58. doi: 10.1021/ct200790q
61. Ebinu JO, Stang SL, Teixeira C, Bottorff DA, Hooton J, Blumberg PM, et al. RasGRP links T-cell receptor signaling to Ras. *Blood*. (2000) 95:3199–203.
62. Spitaler M, Emslie E, Wood CD, Cantrell D. Diacylglycerol and protein kinase D localization during T lymphocyte activation. *Immunity*. (2006) 24:535–46. doi: 10.1016/j.immuni.2006.02.013
63. Navarro MN, Goebel J, Hukelmann JL, Cantrell DA. Quantitative phosphoproteomics of cytotoxic T cells to reveal protein kinase D 2 regulated networks. *Mol Cell Proteomics*. (2014) 13:3544–57. doi: 10.1074/mcp.M113.037242
64. Huang YH, Grasis JA, Miller AT, Xu R, Soonthornvacharin S, Andreotti AH, et al. Positive regulation of Itk PH domain function by soluble IP4. *Science*. (2007) 316:886–9. doi: 10.1126/science.1138684
65. Kapnick SM, Stinchcombe JC, Griffiths GM, Schwartzberg PL. Inducible T cell kinase regulates the acquisition of cytolytic capacity and degranulation in CD8(+) CTLs. *J Immunol*. (2017) 198:2699–711. doi: 10.4049/jimmunol.1601202
66. Mukherjee S, Rigaud S, Seok SC, Fu G, Prochenka A, Dworkin M, et al. *In silico* modeling of Itk activation kinetics in thymocytes suggests competing positive and negative IP4 mediated feedbacks increase robustness. *PLoS ONE*. (2013) 8:e73937. doi: 10.1371/journal.pone.0073937
67. Bach TL, Kerr WT, Wang Y, Bauman EM, Kine P, Whiteman EL, et al. PI3K regulates pleckstrin-2 in T-cell cytoskeletal reorganization. *Blood*. (2007) 109:1147–55. doi: 10.1182/blood-2006-02-001339
68. Ma Y, Poole K, Goyette J, Gaus K. Introducing membrane charge and membrane potential to T cell signaling. *Front Immunol*. (2017) 8:1513. doi: 10.3389/fimmu.2017.01513
69. Kay JG, Koivusalo M, Ma X, Wohland T, Grinstein S. Phosphatidylserine dynamics in cellular membranes. *Mol Biol Cell*. (2012) 23:2198–212. doi: 10.1091/mbc.e11-11-0936
70. Jenkins GH, Fisette PL, Anderson RA. Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J Biol Chem*. (1994) 269:11547–54.
71. Varnai P, Gulyas G, Toth DJ, Sohn M, Sengupta N, Balla T. Quantifying lipid changes in various membrane compartments using lipid binding protein domains. *Cell Calcium*. (2017) 64:72–82. doi: 10.1016/j.ceca.2016.12.008
72. Shim H, Wu C, Ramsamooj S, Bosch KN, Chen Z, Emerling BM, et al. Deletion of the gene Pip4k2c, a novel phosphatidylinositol kinase, results in hyperactivation of the immune system. *Proc Natl Acad Sci USA*. (2016) 113:7596–601. doi: 10.1073/pnas.1600934113
73. Tronchere H, Laporte J, Pendaries C, Chaussade C, Liaubet L, Pirola L, et al. Production of phosphatidylinositol 5-phosphate by the phosphoinositide 3-phosphatase myotubularin in mammalian cells. *J Biol Chem*. (2004) 279:7304–12. doi: 10.1074/jbc.M311071200
74. Ungewickell A, Hugge C, Kisseleva M, Chang SC, Zou J, Feng Y, et al. The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases. *Proc Natl Acad Sci USA*. (2005) 102:18854–9. doi: 10.1073/pnas.0509740102
75. Guittard G, Mortier E, Tronchere H, Firaguay G, Gerard A, Zimmermann P, et al. Evidence for a positive role of PtdIns5P in T-cell signal transduction pathways. *FEBS Lett*. (2010) 584:2455–60. doi: 10.1016/j.febslet.2010.04.051
76. Nunes JA, Guittard G. An emerging role for PI5P in T cell biology. *Front Immunol*. (2013) 4:80. doi: 10.3389/fimmu.2013.00080
77. Briscoe J, Therond PP. The mechanisms of Hedgehog signalling and its roles in development and disease. *Nat Rev Mol Cell Biol*. (2013) 14:416–29. doi: 10.1038/nrm3598
78. Christensen ST, Clement CA, Satir P, Pedersen LB. Primary cilia and coordination of receptor tyrosine kinase (RTK) signalling. *J Pathol*. (2012) 226:172–84. doi: 10.1002/path.3004
79. Satir P, Pedersen LB, Christensen ST. The primary cilium at a glance. *J Cell Sci*. (2010) 123:499–503. doi: 10.1242/jcs.050377
80. Wang L, Dynlacht BD. The regulation of cilium assembly and disassembly in development and disease. *Development*. (2018) 145:dev151407. doi: 10.1242/dev.151407
81. Prosser SL, Morrison CG. Centrin2 regulates CP110 removal in primary cilium formation. *J Cell Biol*. (2015) 208:693–701. doi: 10.1083/jcb.201411070
82. Chavez M, Ena S, Van Sande J, de Kerchove d'Exaerde A, Schurmans S, Schiffmann S. N. Modulation of ciliary phosphoinositide content regulates trafficking and sonic hedgehog signaling output. *Dev Cell*. (2015) 34:338–50. doi: 10.1016/j.devcel.2015.06.016
83. Dyson JM, Conduit SE, Feeney SJ, Hakim S, DiTommaso T, Fulcher AJ, et al. INPP5E regulates phosphoinositide-dependent cilia transition zone function. *J Cell Biol*. (2017) 216:247–63. doi: 10.1083/jcb.2015.11055

84. Garcia-Gonzalo FR, Phua SC, Roberson EC, Garcia G III, Abedin M, Schurmans S, Inoue T, et al. Phosphoinositides regulate ciliary protein trafficking to modulate hedgehog signaling. *Dev Cell*. (2015) 34:400–9. doi: 10.1016/j.devcel.2015.08.001
85. Hakim S, Dyson JM, Feeney SJ, Davies EM, Sriratana A, Koenig MN, et al. Inpp5e suppresses polycystic kidney disease via inhibition of PI3K/Akt-dependent mTORC1 signaling. *Hum Mol Genet*. (2016) 25:2295–313. doi: 10.1093/hmg/ddw097
86. Jensen VL, Li C, Bowie RV, Clarke L, Mohan S, Blacque OE, et al. Formation of the transition zone by Mks5/Rpgrip1L establishes a ciliary zone of exclusion (CIZE) that compartmentalises ciliary signalling proteins and controls PIP2 ciliary abundance. *EMBO J*. (2015) 34:2537–56. doi: 10.15252/embj.201488044
87. Klink BU, Zent E, Juneja P, Kuhlee A, Raunser S, Wittinghofer A. A recombinant BBSome core complex and how it interacts with ciliary cargo. *Elife*. (2017) 6:e27434. doi: 10.7554/eLife.27434
88. Jin H, White SR, Shida T, Schulz S, Aguiar M, Gygi SP, et al. The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia. *Cell*. (2010) 141:1208–19. doi: 10.1016/j.cell.2010.05.015
89. Nager AR, Goldstein JS, Herranz-Perez V, Portran D, Ye F, Garcia-Verdugo JM, et al. An actin network dispatches ciliary GPCRs into extracellular vesicles to modulate signaling. *Cell*. (2017) 168:252–63 e214. doi: 10.1016/j.cell.2016.11.036
90. Phua SC, Chiba S, Suzuki M, Su E, Roberson EC, Pusapati GV, et al. Dynamic remodeling of membrane composition drives cell cycle through primary cilia excision. *Cell*. (2017) 168:264–79 e215. doi: 10.1016/j.cell.2016.12.032
91. Xu Q, Zhang Y, Wei Q, Huang Y, Hu J, Ling K. Phosphatidylinositol phosphate kinase PIPKγ and phosphatase INPP5E coordinate initiation of ciliogenesis. *Nat Commun*. (2016) 7:10777. doi: 10.1038/ncomms10777
92. Avasthi P, Marshall WF. Stages of ciliogenesis and regulation of ciliary length. *Differentiation*. (2012) 83:S30–42. doi: 10.1016/j.diff.2011.11.015
93. Lee S, Tan HY, Geneva II, Kruglov A, Calvert PD. Actin filaments partition primary cilia membranes into distinct fluid corrals. *J Cell Biol*. (2018) 217:2831–49. doi: 10.1083/jcb.201711104
94. Phua SC, Nihongaki Y, Inoue T. Autonomy declared by primary cilia through compartmentalization of membrane phosphoinositides. *Curr Opin Cell Biol*. (2018) 50:72–8. doi: 10.1016/j.ceb.2018.01.008
95. Hashimoto-Tane A, Saito T. Dynamic regulation of TCR-microclusters and the microsynapse for T cell activation. *Front Immunol*. (2016) 7:255. doi: 10.3389/fimmu.2016.00255
96. Kaizuka Y, Douglass AD, Varma R, Dustin ML, Vale RD. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc Natl Acad Sci USA*. (2007) 104:20296–301. doi: 10.1073/pnas.0710258105

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Protein Kinase C δ Regulates the Depletion of Actin at the Immunological Synapse Required for Polarized Exosome Secretion by T Cells

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 11 October 2018

Accepted: 02 April 2019

Published: 26 April 2019

Citation:

Herranz G, Aguilera P, Dávila S,
Sánchez A, Stancu B, Gómez J,
Fernández-Moreno D, de Martín R,
Quintanilla M, Fernández T,
Rodríguez-Silvestre P,
Márquez-Expósito L, Bello-Gamboa A,
Fraile-Ramos A, Calvo V and
Izquierdo M (2019) Protein Kinase C δ
Regulates the Depletion of Actin at the
Immunological Synapse Required for
Polarized Exosome Secretion by T
Cells. *Front. Immunol.* 10:851.
doi: 10.3389/fimmu.2019.00851

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Multivesicular bodies (MVB) are endocytic compartments that enclose intraluminal vesicles (ILVs) formed by inward budding from the limiting membrane of endosomes. In T lymphocytes, ILVs are secreted as Fas ligand-bearing, pro-apoptotic exosomes following T cell receptor (TCR)-induced fusion of MVB with the plasma membrane at the immune synapse (IS). In this study we show that protein kinase C δ (PKC δ), a novel PKC isotype activated by diacylglycerol (DAG), regulates TCR-controlled MVB polarization toward the IS and exosome secretion. Concomitantly, we demonstrate that PKC δ -interfered T lymphocytes are defective in activation-induced cell death. Using a DAG sensor based on the C1 DAG-binding domain of PKC δ and a GFP-PKC δ chimera, we reveal that T lymphocyte activation enhances DAG levels at the MVB endomembranes which mediates the association of PKC δ to MVB. Spatiotemporal reorganization of F-actin at the IS is inhibited in PKC δ -interfered T lymphocytes. Therefore, we propose PKC δ as a DAG effector that regulates the actin reorganization necessary for MVB traffic and exosome secretion.

Keywords: T lymphocytes, immune synapse, protein kinase C δ , multivesicular bodies, exosomes, cytotoxic activity, cell death

INTRODUCTION

T cell receptor (TCR) stimulation by antigen presented by major histocompatibility complex (MHC) molecules on an antigen-presenting cell (APC) induces the formation of the immunological synapse (IS), the convergence of the secretory granules of T lymphocytes toward the microtubule-organizing center (MTOC) and, almost simultaneously, the polarization of the MTOC to the IS (1, 2). This ensures the specificity of T cell effector responses by enabling polarized secretory traffic toward the APC (1, 2), spatially and temporally focusing secretion at the synaptic cleft (3). The polarization of the MTOC toward the IS is conducted by a transient increase in cortical actin at the IS, followed by a decrease in cortical actin density at the central region of the immune

synapse (cIS) that contains the secretory domain. The central supramolecular activation cluster (cSMAC) next to this secretory domain is also located within the F-actin-low region at cIS (Fact-low cIS) (2, 4, 5). In parallel, F-actin accumulation occurs at the edge of the T lymphocyte/APC interface, which constitutes the distal SMAC (dSMAC) and delimits the synaptic contact region (6, 7). The secretory granules from cytotoxic T lymphocytes (CTL) (also called “lytic” or “cytotoxic” granules) contain diverse apoptosis-inducing molecules (8), including Fas ligand (FasL). Among several pro-apoptotic mechanisms, CTL kill Fas⁺ target cells by rapidly exposing intact, pre-formed FasL on the plasma membrane at the IS (9). FasL induces cross-linking of the Fas death receptor on the target cell and subsequent apoptosis (10). In resting CTL, FasL is located at the limiting membrane of secretory multivesicular bodies (MVB) (9). In addition, FasL can be sorted from the limiting membrane of the MVB to the intraluminal vesicles (ILV) via inward budding, which occurs during maturation of MVB in CTL, CD4⁺ T lymphoblasts and Jurkat, a CD4⁺ T helper (Th) type cell line (11–13). Upon TCR activation of CTL and MTOC reorientation, lytic granules undergo fusion with the plasma membrane at the IS (5). As a consequence, two mechanisms for the transport of pro-apoptotic FasL to the extracellular milieu may coexist: relocation of FasL to the cell surface (9), and secretion of FasL-containing ILV as lethal extracellular nanovesicles (50–100 nm size) called exosomes (13–15). While exosomes are constitutively secreted by a variety of cell lineages and tumor cells, in T and B lymphocytes exosome secretion is triggered upon activation of cell surface receptors, which in turn regulates antigen-specific immune responses (16). Exosomes are involved in important processes related to TCR-triggered immune responses, including T lymphocyte-mediated cytotoxicity, activation-induced cell death (AICD) of CD4⁺ lymphocytes, antigen presentation, intercellular miRNA exchange (11–13, 17, 18) and thymic development (19). However, the mechanisms underlying MVB traffic and exosome secretion are poorly understood. In this context, it is known that MTOC reorientation in CTL is initially guided by a diacylglycerol (DAG) gradient centered at the IS (20),

which is *de novo* produced by TCR-stimulated phospholipase C (PLC) activation. DAG activates, among others, several members of the protein kinase C (PKC) and the protein kinase D (PKD) families (21). Phosphorylation of DAG by diacylglycerol kinase α (DGK α) to produce phosphatidic acid (PA) (22) is one of the mechanisms involved in the spatiotemporal control of the DAG gradient (23) and MTOC reorientation to the IS (20). Furthermore, several authors have described DGK α as a crucial factor in the polarization of late endosomes/MVB (24). We have shown that DGK α controls the polarized secretion of exosomes containing FasL in Th lymphocytes (13, 25) and that the kinase activity of DGK α inhibits ILV formation during MVB maturation (25). In addition, we have identified a DAG-activated enzyme, PKD1/2, as a key component of this DGK α -controlled pathway involved in MVB maturation and exosome secretion (26). Besides this early regulation, DGK α also controls MTOC and MVB polarization toward the IS both in CTL and CD4⁺ T lymphocytes (20, 25, 27), although the molecular basis underlying this second checkpoint remains unclear. The fact that the novel PKC family member PKC δ , a DAG-activated PKC isotype, is necessary for the polarization of lytic granules and cytotoxicity in mouse CTL (28, 29) prompted us to study the function of PKC δ in MVB polarized trafficking and exosome secretion in human T lymphocytes.

MATERIALS AND METHODS

Cells

J-HM1-2.2 Jurkat cells expressing human muscarinic type 1 receptor (HM1R) and high levels of PKC δ have been used as a model system to trigger phosphatidylinositol turnover and DAG production at the plasma membrane upon carbachol (CCH) stimulation (30). Raji B and Jurkat T (clone JE6.1) cell lines were obtained from the ATCC. Cell lines were cultured in RPMI 1640 medium containing L-glutamine (Invitrogen) with 10% heat-inactivated FCS (Gibco) and penicillin/streptomycin (Gibco). Jurkat cells (clone JE6.1) transfected with control and PKC δ shRNA-encoding plasmids were selected with puromycin (1 μ g/ml) and clones isolated by limiting dilution. Human primary T lymphoblasts from healthy volunteers were obtained and cultured as described previously (31).

ShRNA Plasmids, Expression Vectors, Transfection Assays, and Isolation of Clones

Plasmids used in this study were as follows: pEFbos-GFP was described previously (13, 23); pEFGFP-C1bosCD63 and pEGFP-C1CD63 were provided by G. Griffiths; mouse pEGFP-PKC δ wt (GFP-PKC δ WT), pEGFP-PKC δ DR144/145A constitutively active mutant (GFP-PKC δ CA) (32) and pEGFP-PKC δ K376A kinase-dead mutant (GFP-PKC δ KD) (33, 34) were obtained from A. Zweifach and D. M. Reyland. GFP-C1bPKC θ expression plasmid was kindly provided by I. Mérida; UpwardDAG2 (U.DAG2) (35) was generously provided by A.M. Quinn (Montana Molecular Inc.). In some experiments, human DGK α was silenced using the pSUPER RNAi System (pSR-GFP bicistronic or pSuperplasmids; Oligoengine, Seattle, WA, USA)

Abbreviations: Ab, antibody; AICD, activation-induced cell death; APC, antigen-presenting cell; C, center of mass; CCH, carbachol; cIS, central region of the immune synapse; Fact-low cIS, F-actin-low region at the center of the immune synapse; CMAC, cell tracker blue-CellTrackerTM (7-amino-4-chloromethylcoumarin); cSMAC, central supramolecular activation cluster; CTL, cytotoxic T lymphocytes; DAG, diacylglycerol; DGK α , diacylglycerol kinase α ; dSMAC, distal supramolecular activation cluster; ECL, enhanced chemiluminescence; ESCRT, endosomal sorting complex required for traffic; F-actin, filamentous actin; FasL, Fas ligand; FI, fluorescence intensity; fps, frames *per second*; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; ILV, intraluminal vesicles; IS, immune synapse; MHC, major histocompatibility complex; MIP, maximal intensity projection; MVB, multivesicular bodies; MTOC, microtubule-organizing center; NS, not significant; NTA, nanoparticle tracking analysis; PA, phosphatidic acid; PBL, peripheral blood lymphocytes; PKC, protein kinase C; PKC δ , protein kinase C δ isotype; PKD, protein kinase D; PLC, phospholipase C; PMA, phorbol myristate acetate; Pol. Index, polarization index; pSMAC, peripheral supramolecular activation cluster; PSF, point spread function; ROI, region of interest; SD, standard deviation; shRNA, short hairpin RNA; SEE, Staphylococcus enterotoxin E; SMAC, supramolecular activation cluster; TCR, T-cell receptor for antigen; Th, helper T lymphocyte; TRANS, Transmittance; U.DAG2, Upward DAG2; WB, western blot.

with the appropriate hairpin as described (25). pDsRed2-PKD1wt plasmid was previously described (26). U.DAG2 is a genetically encoded, fluorescent protein-containing DAG sensor based on the insertion of the circularly permuted (cp) EGFP into a PKC δ coding sequence that was modified by deleting only the N-terminal region containing the C2 domain (35). The U.DAG2 sensor maintains the C1, DAG-binding domain and the catalytic domain of PKC δ and, upon DAG production, is recruited to cellular membranes following DAG binding and undergoes conformational changes, leading to a rapid fluorescence increase (35, 36). This sensor was demonstrated to produce rapid, robust and reversible changes in green fluorescence in a live-cell assay (35).

Control short-hairpin RNA (Cont shRNA) plasmid-A (Santa Cruz Biotechnology), PKC δ shRNA plasmid (h) (Santa Cruz Biotechnology) or a mixture of three pSIREN-RetroQ retroviral vectors (Clontech) encoding shRNAs against human PKC δ (37) were used to generate stable JE6.1 Jurkat clones. All these plasmids expressed a puromycin resistance gene for the selection of stably transfected clones. The plasmids were verified by sequencing. For characterization of control and PKC δ -interfered Jurkat stable clones, PKC δ levels were analyzed by WB and cell surface levels of CD3/TCR, CD2, CD4, LFA-1, CD28, CD45, and CD95 (Fas) were analyzed by flow cytometry after expansion of the cell clones obtained by limiting dilution. For transient transfection experiments, J-HM1-2.2 and Jurkat clones were transiently transfected with 20–30 μ g of the plasmids as described (13). For exosome secretion experiments, mouse PKC δ expression constructs were transiently co-transfected with exosome reporter GFP-CD63 expression plasmid in a 3:1 molecular ratio (26). Human primary T lymphoblasts were cultured in the presence of IL-2 as previously described (31) and were transfected, between 3 and 7 days after the addition of IL-2, with 2 μ g of the indicated expression and interference plasmids, by using an appropriate nucleofector kit (Amaxa[®] Human T Cell Nucleofector[®] Kit, Program T-20 or T-23 for Nucleofector[®] I).

Antibodies and Reagents

Antibodies used in this study were obtained from the indicated sources: rabbit monoclonal anti-human PKC δ EP1486Y (Abcam) for WB (this antibody does not recognize mouse PKC δ); rabbit polyclonal anti-rat PKC δ C-17 (Santa Cruz Biotechnology) for WB (recognizes both human and mouse PKC δ); anti-human CD3 UCHT1 (BD Biosciences and Santa Cruz Biotechnology) for cell stimulation and immunofluorescence; rabbit polyclonal anti-phospho-PKC δ Thr505 (Cell Signaling Technology) for WB; mouse monoclonal anti-CD63 clone NKI-C-3 (Oncogene) for WB; mouse monoclonal anti-CD63 clone TA3/18 (Immunostep) for immunofluorescence; and mouse monoclonal anti- γ -tubulin (SIGMA) for immunofluorescence. Fluorochrome-coupled secondary antibodies (goat-anti-mouse IgG AF488 A-11029, goat-anti-rabbit IgG AF488 A-11034, goat-anti-mouse IgG AF546 A-11030, goat-anti-mouse IgG AF647 A-21236) for immunofluorescence were from ThermoFisher. All horseradish peroxidase (HRP)-coupled secondary Abs (goat anti-mouse IgG-HRP, sc-2005 and goat anti-rabbit IgG-HRP, sc-2004) were obtained from Santa Cruz Biotechnology. Cell tracker

blue (CMAC) and phalloidin were from ThermoFisher. Annexin V-PE was from Immunostep. Carbachol (CCH) and staphylococcal enterotoxin E (SEE) were from SIGMA and Toxin Technology, Inc (USA), respectively. Blocking antibody directed against CD95 (Fas), clone DX2, was from BDBiosciences.

Isolation and Quantitation of Exosomes

Exosomes produced by equal numbers of cells for each experimental condition were isolated from cell culture supernatants as previously described (14, 15, 26). No significant differences in β -actin levels (i.e., **Figure 3**) were observed in the lysates of cells, stimulated or not, at the end of the cell culture period for exosome secretion, showing that the exosomes were produced by equal numbers of viable cells. Using these standard protocols, culture supernatants of 20×10^6 Jurkat cells were centrifuged in sequential steps to eliminate cells and cell debris/apoptotic bodies (38), and the exosomes were recovered by ultracentrifugation (100,000xg for 12 h) as described (14). In some experiments, to quantify exosomes and to analyze their size distribution, the cell culture supernatant collected just before the ultracentrifugation step was diluted (1/5) in Hank's balanced salt solution (HBSS) and analyzed by Nanoparticle Tracking Analysis (NTA) with the use of NANOSIGHT equipment (LM10, Malvern) that was calibrated with 50 nm, 100 nm and 400 nm fluorescent calibration beads (Malvern). The hydrodynamic diameter measured by NTA, although apparently higher than that originally described for exosomes using electron microscopy (50–100 nm), certainly corresponds to the real size of canonic, unfixed exosomes in solution, as described (39). The NTA measurements of exosome concentration (particles/ml) were normalized by the exosome-producing cell number, by referring exosome concentration to β -actin or endogenous CD63 signals in the WB of the cell lysates. CD63 is characteristically present in MVB, ILVs and hence in exosomes, but also in secretory lysosomes and the plasma membrane. Plasma membrane CD63 localization is produced by degranulation of MVB and diffusion of CD63 from the limiting membrane of MVB to the plasma membrane upon MVB fusion (25, 26). This protein and its chimeras (GFP-CD63) have been used as appropriate reporters for MVB/exosomes (13, 40, 41), and allow the quantitation of exosome secretion in Jurkat cells and primary human T lymphoblasts (13, 25, 38). To analyze the exosomes from cells expressing the exosome reporter GFP-CD63, a similar protocol was performed, although 1×10^6 Jurkat cells or human T lymphoblasts were used and the WB signals in exosome lysates were normalized by the expression levels of GFP-CD63 among different transfections and stimuli in the WB corresponding to the cell lysates (25, 41). It has been established that the exosomal CD63 WB signal correlates well with the results of exosome number obtained by flow cytometry (42), by electron microscopy (43) and by nanoparticle concentration analysis (nanoparticles/ml), using NTA (26). Thus, WB analysis of endogenous or GFP-tagged CD63 in isolated exosomes constitutes a *bona fide* method to measure exosome production (25, 26).

Western Blot Analysis of Cell and Exosome Lysates

Cells and isolated exosomes were lysed in RIPA lysis buffer containing protease inhibitors. Approximately 50 μ g of exosomal proteins was recovered in the 100,000 $\times$ g pellet from 20×10^6 cells. Exosomes were resuspended in 60 μ l of RIPA lysis buffer and 20 μ l of exosomal or cell lysates were separated on SDS-PAGE under reducing conditions and transferred to HybondTM ECLTM membranes (GE Healthcare). For CD63 detection, proteins were separated under non-reducing conditions as described (13). For WB analysis of exosomes, each lane contained the total exosomal protein that was recovered in the culture medium from the same number of cells, untreated or treated with stimuli. Blots were incubated with mouse anti-CD63 (clone NKI-C-3, Oncogene) and developed with the appropriate HRP-conjugated secondary antibody using enhanced chemiluminescence (ECL). Autoradiography films were scanned and the bands were quantified with the use of Quantity One 4.4.0 (Bio-Rad) and ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2004) software.

Time-Lapse Microscopy, Immunofluorescence Experiments, and Image Analysis

Jurkat clones transfected with the different expression plasmids were attached to glass-bottom IBIDI microwell culture dishes using fibronectin (0.1 mg/ml) at 24–48 h post-transfection and stimulated in culture medium at 37°C. In some experiments, Raji cells attached to glass-bottom IBIDI microwell culture dishes using fibronectin were labeled with CMAC and pulsed with 1 μ g/ml SEE, and then mixed with transfected Jurkat clones and the immune synapses were analyzed as described (25). In other experiments, transfected Jurkat clones were stimulated with plastic-bound anti-TCR UCHT1 Ab (10 μ g/ml) or directly in suspension with CCH (500 μ M) or phorbol myristate acetate (PMA, 100 ng/ml). Immunofluorescence of fixed synapses was performed as previously described (44), and additional fixation was made between each fluorochrome-coupled secondary antibody and subsequent fluorochrome-coupled primary antibody staining, to exclude any potential cross-reaction of secondary antibodies. Time-lapse experiments were performed using an OKO-lab stage incubator (OKO) on a Nikon Eclipse TiE microscope equipped with a DS-Qi1MC digital camera and a PlanApo VC 60 $\times$ NA 1.4 objective (NIKON). Time-lapse acquisition and analysis were performed by using NIS-AR software (NIKON). Subsequently, epi-fluorescence images were improved by Huygens Deconvolution Software from Scientific Volume Image (SVI) using the “widefield” optical option. Deconvolution is a computational image processing technique that can improve image resolution and contrast up to two times, down to 150–100 nm in XY and 500 nm in Z-axis (<https://svi.nl/Deconvolution>). Deconvolution requires the knowledge of the idealized or measured point spread function (PSF) of the microscope and the imaging technique used (45). One example of the power of deconvolution applied to epifluorescence

videos on the polarized traffic of MVB at the IS is provided (25), (video before deconvolution, <https://www.youtube.com/watch?v=mID0m3usQOQ>; video after deconvolution, <https://www.youtube.com/watch?v=Aj0vPj6WAI>). For quantification, digital images were analyzed using NIS-AR (Nikon) or ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2004). For quantification of relative fluorescence intensity (FI) in time-lapse experiments, analysis of average FI in floating regions of interest (ROI) (i.e., ROI changing over time) was performed using NIS-AR software. These measurements were performed in deconvoluted time-lapse series because of the enhanced signal-to-noise ratio of the images, although raw time-lapse series yielded comparable results. In several experiments, to define the central immune synapse region (cIS), subcellular relocation of DsRed2-PKD1 to the synapse was evaluated in parallel (i.e., **Supplementary Video 6**), since PKD1 relocates to central synapse and cSMAC via DAG binding (46). Confocal microscopy imaging was performed by using a SP8 Leica confocal microscope, with sequential acquisition, bidirectional scanning and the following laser lines: UV (405 nm, intensity: 33.4%), supercontinuum visible (633 nm, intensity: 15.2%), supercontinuum visible (550 nm, intensity: 20.8%), supercontinuum visible (488 nm, intensity: 31.2%). Deconvolution of confocal images was performed by using Huygens Deconvolution Software from Scientific Volume Image (SVI) with the “confocal” optical option. Colocalization analyses were accomplished by using the Jacop plugin from ImageJ. The velocity of movement of MVB was measured by analyzing the trajectories of CFP-CD63⁺ vesicles in videos with the use of NIS-AR software and the ImageJ MJTrack plugin. In polarization experiments, to establish the relative ability of the MTOC and MVB to polarize toward the IS, MTOC and MVB polarization indexes (Pol. Indexes) were calculated by measuring the distance of the cell’s center of mass (cell^C) to the IS (“B” distance), and the distances between the projections of the MTOC or MVB centers of mass (MTOC^C and MVB^C, respectively) to the cell^C (“A” distance) (**Supplementary Figure 1C**). Cell^C position was taken as the origin to measure distances, and those “A” values in the opposite direction to the synapse were taken as negative. Pol. Indexes were calculated, as described in **Supplementary Figure 1C**, as the ratio of distances A and B (Pol. Index = A/B), ranging from +1 to –1. Therefore, Pol. Index values were normalized by cell size and shape (**Supplementary Figure 1C**). The cut-off value for Pol. Indexes was arbitrarily set up at 0.25 (synapses displaying Pol. Index >0.25 were scored as “polarized” and those with Pol. Index <0.25 as “not polarized”) (**Supplementary Figure 1D**). The percentage of synapses with polarized MVB/MTOC that measures polarization efficiency was calculated considering this cut-off value (**Supplementary Figure 1D, Figures 1C, 2C**).

The relative area of the F-actin-low region at the cIS (Fact-low cIS) was measured on confocal microscopy images using the 3D Viewer ImageJ plugin. Briefly, a 2D face on view of the synapse observing from the T lymphocyte (IS interface) (i.e., **Supplementary Video 10** and **Figure 9A**) was generated, and the boundary of the T lymphocyte/APC synaptic contact

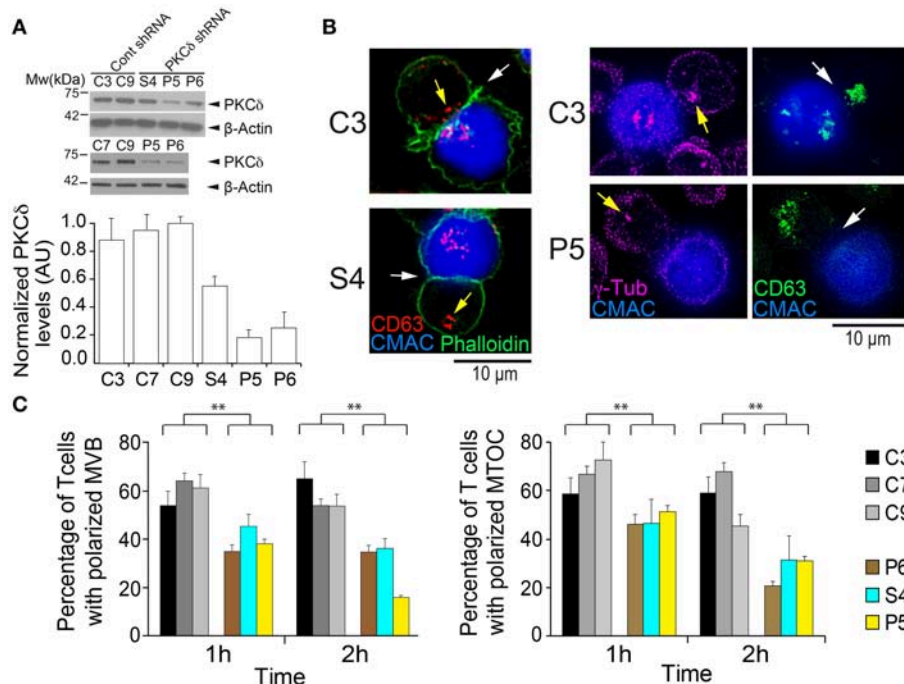


FIGURE 1 | PKC δ regulates the polarization of the MTOC and MVB. **(A)** PKC δ expression levels in different control (C3, C7, and C9) and PKC δ -interfered (S4, P5, and P6) Jurkat clones. Cell lysates were analyzed by WB with anti-PKC δ antibody, and β -actin was used as a loading control. In the lower panel, normalized levels of PKC δ expressed as arbitrary units (AU), means plus standard deviations (SD) ($n = 3$), is represented. **(B)** C3, C7, C9 (control) and S4, P5, P6 (PKC δ -interfered) Jurkat clones were challenged with CMAC-labeled SEE-pulsed Raji cells to induce synaptic conjugate formation. After 1 h cells were fixed, permeabilized, stained with phalloidin and anti-CD63 or anti- γ -tubulin Abs, and imaged by fluorescence microscopy to analyze MVB and MTOC polarization toward the IS. Representative examples of the polarized C3 control clone and non-polarized (S4 and P5) PKC δ -interfered clones are shown. White arrows indicate the IS areas and yellow arrows CD63 $^{+}$ MVB or MTOC. CMAC labeling of Raji cells in blue, phalloidin in green, CD63 in red in left panels and green in right panels and anti- γ -tubulin in magenta. Scale bars, 10 μ m.) **(C)** The percentage of the control and PKC δ -interfered Jurkat clones forming synapses with polarized MVB (left) and MTOC (right) after 1 and 2 h challenge with SEE-pulsed Raji cells was determined as indicated in the Materials and Methods section by measuring the respective MVB and MTOC polarization indexes in control and PKC δ -interfered Jurkat clones (see also **Supplementary Figure 1**). Data are means plus SD ($n = 3$, analyzing at least 50 synapses from 15 different microscopy fields per experiment). Single-factor analysis of variance (ANOVA) was performed between the indicated groups. ** $p \leq 0.05$.

is defined by the distal SMAC (dSMAC), which consists of a circular array with F-actin accumulating at the edge of the T cell/APC interface (6, 47). Thus, the IS area was delimited by the edge of the F-actin signal. When necessary, the definition of the regions of interest (ROI) to measure the areas of the F-actin-low region at the center of the IS (Fact-low cIS area) and the F-actin accumulation region at the IS (IS area) was performed by using the automated algorithm “auto-detect ROI/segmentation” from NIS-AR or by ImageJ segmentation software. Next, these defined ROI areas (Fact-low cIS area and IS area) were measured, and the relative area of the F-actin depleted region at cIS (Fact-low cIS area/IS area) was determined (**Supplementary Figures 7B,C**). This allowed normalization by cell size and IS contact area. The cut-off value for synapses substantiating a F-actin depleted region at cIS was arbitrarily set up at 0.1 (those synapses displaying area ratio >0.1 were scored as “depleted”; **Supplementary Figure 7C**). The percentage of synapses with F-actin depleted at cIS was calculated considering this cut-off value (**Supplementary Figure 7C**, **Figure 9C**). The plot profile analyses of FI corresponding to phalloidin along the indicated ROIs at the IS interfaces were performed by using ImageJ. Image analysis data correspond to at least three different

experiments, analyzing a minimum of 30 synapses from 15 different, randomly selected microscopy fields per experiment. ANOVA analysis was performed for statistical significance of the results using Excel and IBM's SPSS Statistics software.

Apoptosis Experiments

GFP-PKC δ transfected and untransfected Jurkat clones were challenged with CMAC-labeled, SEE-pulsed Raji cells or Raji cells as a control. After the indicated culture periods, the percentage of apoptotic cells (Annexin-V $^{+}$ cells) was analyzed by end point, flow cytometry analysis. Since both size and complexity of Jurkat clones and Raji cells are similar it is not possible, using Forward Scatter (FS) and Side Scatter (SS), to gate cell types using these parameters. Therefore, we have only used CMAC fluorescence gating to exclude the death of Raji cells of the analyses. In some experiments, for the optimal correlation between the formation of synaptic conjugates with apoptosis measurements, these co-cultured cells were continuously imaged by time-lapse microscopy to visualize some early signs of AICD (i.e., plasma membrane blebbing and subsequent cell shrinkage) in the Jurkat clones (CMAC negative) forming stable synapses (**Supplementary Video 3**). In other experiments, Jurkat clones

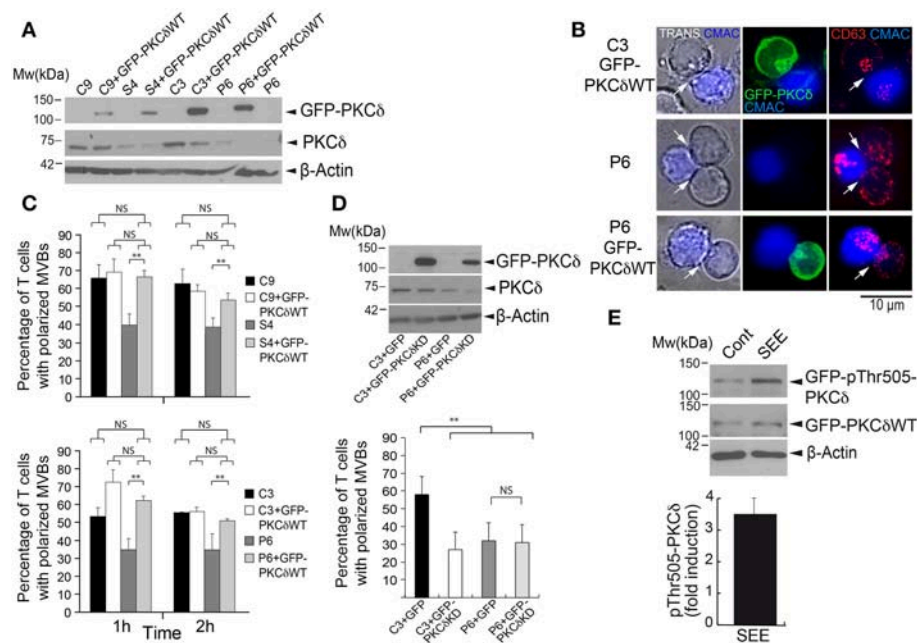


FIGURE 2 | GFP-PKC δ expression restores the polarization of MVB. C3 and C9 control and S4 and P6 PKC δ -interfered Jurkat clones were transfected or not with GFP, GFP-PKC δ WT, or GFP-PKC δ KD mutant. **(A)** WB of lysates from C3, C9, S4, and P6 clones, transfected or not with GFP-PKC δ WT, was carried out with an anti-ratPKC δ , anti-humanPKC δ and anti- β -actin Abs. **(B)** Cells were challenged with CMAC-labeled SEE-pulsed Raji cells to induce synaptic conjugate formation. After 1 h cells were fixed, permeabilized, stained with anti-CD63 Abs and imaged by fluorescence microscopy. Transmittance (TRANS) plus CMAC is shown in the left panel, and the white arrows indicate the IS areas. CMAC labeling of Raji cells in blue, GFP-PKC δ in green and CD63 in red. Representative examples of polarized C3 GFP-PKC δ WT⁺ clone, non-polarized P6 clone and polarized P6 GFP-PKC δ WT⁺ clone are shown. Scale bar, 10 μ m. **(C)** Quantification of MVB polarization in C3 (control) and S4, P6 (PKC δ -interfered) Jurkat clones transfected or not with GFP-PKC δ WT and challenged with CMAC-labeled, SEE-pulsed Raji cells for 1 and 2 h. The percentage of synapse-forming clones with polarized MVB, expressing or not expressing GFP-PKC δ WT, was determined as indicated in Materials and Methods section (see also **Supplementary Figure 1**). Data are means plus SD ($n = 3$, analyzing at least 50 synapses from 15 different microscopy fields per experiment). Single-factor ANOVA was performed between the indicated groups. NS, not significant, $**p \leq 0.05$. **(D)** Upper panel: Lysates of C3 and P6 clones transfected with either GFP or GFP-PKC δ KD mutant were analyzed by WB with an anti-ratPKC δ , anti-humanPKC δ and anti- β -actin Abs. Lower panel: quantification of MVB polarization efficiency in C3 control and P6 PKC δ -interfered Jurkat clones transfected with either GFP or GFP-PKC δ KD and challenged with CMAC-labeled, SEE-pulsed Raji cells for 1 h. The percentage of synapse-forming clones with polarized MVB, expressing or not expressing GFP-PKC δ KD, was determined as in **(C)** Data are means plus SD ($n = 3$, analyzing at least 40 synapses from 15 different microscopy fields per experiment) and single-factor ANOVA was performed between the indicated groups. NS, not significant, $**p \leq 0.05$. **(E)** C3 control Jurkat clone was transfected with GFP-PKC δ WT and challenged with non-pulsed (Cont) or SEE-pulsed (SEE) Raji cells for 1 h. Cells were lysed and lysates analyzed by WB with anti-phosphoThr505-PKC δ , anti-rat PKC δ Ab (C-17) and anti- β -actin to normalize. In the lower panel, mean fold induction plus SD ($n = 3$) of normalized phosphoThr505-PKC δ signal is represented.

were pre-incubated for 30 min with a blocking antibody directed against human Fas (clone DX2, 1 μ g/ml), before the challenge with the SEE-pulsed Raji cells.

RESULTS

PKC δ Regulates the Polarized Traffic of MTOC and MVB Toward the IS

To study the role of PKC δ in the polarized traffic of MVB in human T lymphocytes, we generated several PKC δ -interfered clones. PKC δ -interference was analyzed by WB, and 3 Jurkat clones expressing PKC δ -shRNA-encoding plasmids showed a reduction in PKC δ levels (P5 and P6, and in lower extension S4, **Figure 1A**), when compared to Jurkat clones transfected with control shRNA plasmid (C3, C7, and C9), and were used in further studies. The PKC δ -interfered clones P5, P6, and S4 had similar levels of the cell surface molecules relevant for T lymphocyte interaction with APC when compared to control

clones (**Supplementary Figure 1A**). We then analyzed the formation of synaptic conjugates in a human IS model with the use of CMAC-labeled Raji cells presenting superantigen (SEE). The formation of synaptic conjugates was not affected by PKC δ interference (i.e., **Supplementary Video 1**), and the percentage of cells undergoing conjugate formation after 1 h of challenge with SEE-pulsed APC was similar in the different clones (i.e., $67 \pm 4\%$ of C3 control clone cells underwent conjugate formation vs. $70 \pm 7\%$ of P5 PKC δ -interfered clone cells, mean $\pm$ SD in 3 different experiments, not significant, **Supplementary Figure 1B**). Next, to analyze the traffic of MVB and to study synaptic architecture, we used an approach based on time-lapse studies combined with end point immunofluorescence analysis in fixed cells. This double approach allowed us, on one hand, to study the traffic of CD63⁺ MVB shortly after IS formation in living cells and, on the other hand, to label endogenous MVB and MTOC with anti-CD63 or anti- γ -tubulin, respectively, in fixed cells to perform end point 3D analysis of F-actin and synaptic architecture. Both

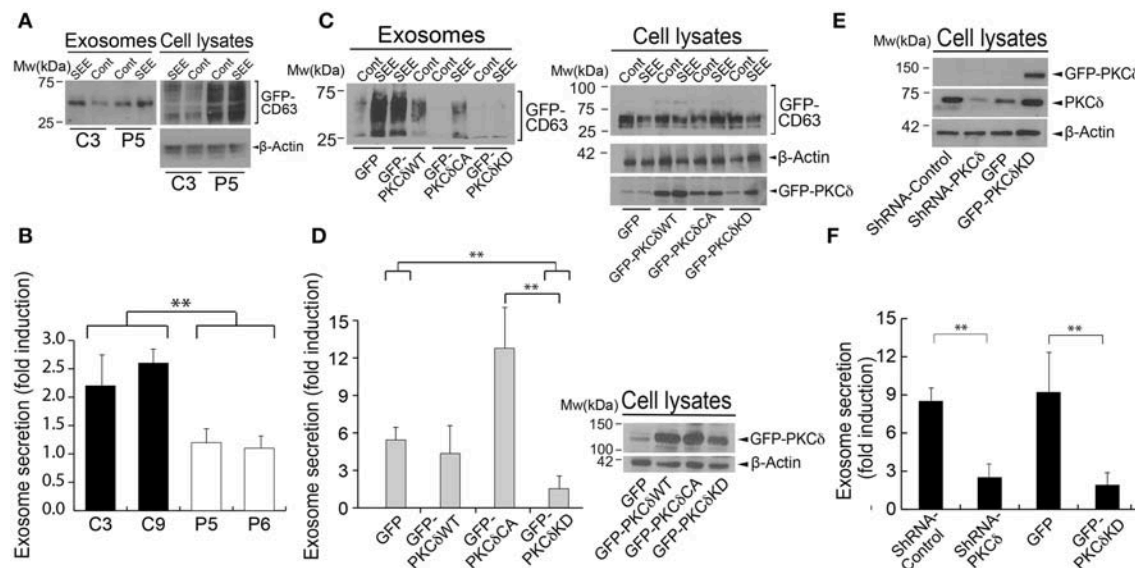


FIGURE 3 | PKC δ regulates polarized exosome secretion. **(A)** GFP-CD63-expressing C3 control and P5 PKC δ -interfered Jurkat clones were challenged with either untreated (Cont) or SEE-pulsed (SEE) Raji cells for 6 h to induce synaptic conjugate formation and subsequent secretion of GFP-CD63 $^{+}$ exosomes. Exosomes were isolated, quantified by WB analysis of the GFP-CD63 reporter (using anti-CD63 Ab) (left panel) and normalized for cell number and cellular GFP-CD63 content in the corresponding cell lysates (right panel). **(B)** GFP-CD63-expressing C3 and C9 control, P5 and P6 PKC δ -interfered Jurkat clones were challenged with either non-pulsed (Cont) or SEE-pulsed (SEE) Raji cells for 6 h to induce synaptic conjugate formation and subsequent secretion of GFP-CD63 $^{+}$ exosomes. Exosomes were isolated and quantified by WB as in **(A)**. Data are means of normalized exosome secretion expressed as x-fold induction with respect to the non-stimulated control plus SD obtained from several experiments as described in **(A)** ($n = 3$), and single-factor ANOVA was performed between the indicated groups. $^{**}p \leq 0.05$. **(C)** C3 control clone that co-expressed GFP-CD63 together with GFP, GFP-PKC δ WT, GFP-PKC δ CA, or GFP-PKC δ KD was challenged as described above and exosomes were isolated. WB analysis of the GFP-CD63 reporter in exosomes (left panel) and cellular GFP-CD63 and β -actin (right panel) for normalization was performed as in **(A)**. In addition, WB of cell lysates was developed with anti-ratPKC δ to analyze the expression of the different PKC δ mutants in all conditions after the synaptic challenge ($\pm$ SEE). **(D)** In the left panel, data are means of normalized exosome secretion expressed as x-fold induction plus SD from several experiments as described in **(C)** ($n = 3$). In the right panel, cell lysates were immunoblotted with anti-ratPKC δ to compare the levels of the different GFP-PKC δ constructs before the synaptic challenge. A non-specific band with an apparent mobility near the GFP-PKC δ chimeras was observed in lysates from cells co-transfected with GFP. **(E)** Human primary T-lymphoblasts were transduced by nucleofection with GFP-CD63 and the indicated interference or expression plasmids, and subsequently analyzed for endogenous PKC δ expression or ectopic GFP-mouse-PKC δ expression. **(F)** Subsequently, cells were stimulated with plastic-bound anti-TCR and exosome secretion was analyzed as in **(A)**. Data are means of normalized exosome secretion expressed as x-fold induction with respect to the non-stimulated control plus SD obtained from several experiments ($n = 3$); and single-factor ANOVA was performed between the indicated groups. $^{**}p \leq 0.05$.

approaches were complementarily required, since when the formation of synaptic conjugates was analyzed at the single cell level by time-lapse microscopy, we observed that IS formation constitutes an asynchronous (Figure 8A), stochastic process (26, 45), as previously shown (48). We have studied the kinetics of conjugate formation by time-lapse microscopy in control and PKC δ -interfered clones, and we observed that after 30 min of challenge only 30% of cells formed conjugates, whereas after 1 h of challenge the efficiency rose to 65% and after 2 h reached 75% (Supplementary Figure 1B). In addition to this fact, and since preliminary results with the different clones showed both the maximal conjugate formation (measured as the percentage of Jurkat cells forming synaptic conjugates) and the maximal polarization (the percentage of Jurkat cells forming synapses with polarized MVB/MTOC, see below) occurred between 1 and 2 h after challenge with APCs, we decided to use these time points in our end point experiments with fixed synapses (Figure 1). However, it should be pointed out that these time points indicate only the period elapsed after Jurkat clone addition to the SEE-pulsed APCs, but not the beginning of the conjugate

formation period (Figure 8A). For all these reasons, end point analysis in fixed cells provided neither temporal information regarding the onset of these synapses nor a dynamic, realistic view of the progression of MVB convergence and MVB/MTOC polarization (48), however this was indeed achieved by time-lapse studies (45) (i.e., Supplementary Video 1). For both approaches, CD63 staining was analyzed since this molecule is the canonical marker of MVB and exosomes across a multitude of cell types (http://exocarta.org/exosome_markers_new). Thus, we analyzed the polarization of MVB in a human IS model with the use of CMAC-labeled Raji cells presenting superantigen (SEE) to challenge the different Jurkat clones, either untransfected or expressing the MVB reporter CFP-CD63 (25). Time-lapse microscopy showed that upon IS formation, CFP-CD63 vesicles in the C3 control clone progressively accumulate in the vicinity of the IS, as previously reported (25, 26). This feature was not observed in the P5 PKC δ -interfered clone (Supplementary Video 1, Supplementary Figure 2A). End point analysis of the polarization of endogenous CD63 $^{+}$ vesicles and the MTOC were performed in synapses established

by the different clones during the two different times (1 and 2 h) after challenge with SEE-presenting Raji cells (**Figures 1B,C**). In **Figure 1B**, images of polarized (C3) and non-polarized (S4 and P5) clones forming synapses are shown, representing the data obtained when all control (C3, C7 and C9) and PKC δ -interfered clones (P6, P5, and S4) were compared (**Figure 1C**, **Supplementary Figure 1D**). The MVB and MTOC Pol. Indexes were determined and the percentage of synapses with polarized MVB/MTOC (i.e. synapses substantiating Pol. Indexes >0.25) was calculated (**Supplementary Figure 1**). Despite remarkable dispersion in Pol. Index values for each clone, significant differences among all the control and all the PKC δ -interfered clones existed (**Supplementary Figures 1C,D**). PKC δ -interfered clones forming IS exhibited lower polarization percentages (15–50%) for both MVB and the MTOC when compared with control clones (50–75%) (**Figure 1C**). Remarkably, we found a strong linear correlation between MVB and MTOC Pol. Indexes for each clone (i.e. Pearson's lineal correlation coefficient of 0.962 and 0.949 for C3 and P5 clones, respectively). Furthermore, in all the analyzed synapses the MTOC^C was coincident or very proximal to the MVB^C (i.e., white crosses in **Supplementary Figure 1C**), regardless of polarization. In addition, when we continuously analyzed the Pol. Index during 4 and 15 h of synapse formation in time-lapse experiments, the lower efficiency in MVB polarization in PKC δ -interfered clones was maintained (**Supplementary Figure 2B**). No significant differences were found between the average velocity of MVB movement in the PKC δ -interfered clones when compared with that of control clones (mean $\pm$ SD = 3.2 ± 0.4 μ m/s and 3.1μ m/s ± 0.3 for C3 and P5 clones, respectively, analyzing 200 CFP-CD63⁺ vesicles per clone, not significant). Therefore, PKC δ -interfered clones exhibited a continuously reduced ability to polarize the secretory machinery toward the IS, although the velocity of MVB remained unaffected. To determine the specificity of PKC δ silencing in MVB polarization, we carried out rescue experiments with a mouse PKC δ GFP-tagged construct resistant to shRNA inhibition (**Figure 2A**). Untransfected or GFP-PKC δ -transfected control and PKC δ -interfered Jurkat clones were challenged with SEE-pulsed Raji cells to induce IS formation, and the MVB polarization was analyzed by time-lapse microscopy and immunofluorescence. CFP-CD63⁺ vesicles polarized toward the IS in P6 PKC δ -interfered cells expressing high levels of GFP-PKC δ (i.e., **Supplementary Video 2**, lower right panel), while very low expression levels of GFP-PKC δ did not restore MVB polarization when analyzed at the single cell level (i.e., **Supplementary Video 2**, lower left panel). More extensive end point analyses in fixed synapses were performed in C3 control and P6 PKC δ -interfered clones expressing high levels of GFP-PKC δ , and C9 control and S4 PKC δ -interfered clones expressing low levels GFP-PKC δ (**Figure 2A**). When we determined the percentage of synapses with polarized MVB we found that MVB polarization was restored in P6 PKC δ -interfered, GFP-PKC δ WT-transfected clone to the levels observed for the C3 control clone (**Figures 2B,C**, lower panel). Similar results were obtained when low levels of GFP-PKC δ WT were expressed in S4 PKC δ -interfered clone (**Figure 2C**, upper panel). In contrast, the expression of a kinase-dead, a dominant-negative PKC δ mutant

(GFP-PKC δ KD) (**Figure 2D**, upper panel), inhibited MVB polarization in the C3 control clone, and did not reestablish the polarization of the P6 PKC δ -interfered clone to the levels obtained in the C3 control clone (**Figure 2D**, lower panel). Thus, PKC δ kinase activity appears to be necessary for the positive effect of PKC δ on MVB polarization. In addition, we observed that GFP-PKC δ underwent activation upon IS formation, as assessed by WB analysis of T505 phosphorylation (3–4 fold induction) at the activation loop of GFP-PKC δ (**Figure 2E**) (34), in agreement with PKC δ activation described in mouse CTL (28). Altogether, these data indicate that PKC δ is required for the polarization of both MVB and the MTOC toward the IS in T lymphocytes.

PKC δ Regulates Exosome Secretion

Since the polarization and degranulation of MVB at the IS are necessary for exosome secretion and apoptosis induction in T lymphocytes (13, 14, 25), we next studied the consequences of deficient MVB polarization on exosome secretion by several approaches. For the first approach, control and PKC δ -interfered Jurkat clones expressing the MVB/exosome reporter GFP-CD63 were challenged with untreated (control) or SEE-pulsed Raji cells (SEE). After 6 h of stimulation, exosomes were purified from the cell culture supernatants and quantified by WB analysis of GFP-CD63 (26). This allowed us, on one hand, to enhance the sensitivity of the assay (25) and, on the other hand, to exclusively quantitate and normalize the exosomes secreted by the Jurkat clones, avoiding the exosomes secreted by the Raji cells. We found that normalized inducible exosome secretion was decreased in P5 and P6 PKC δ -interfered clones when compared with C3 and C9 control clones (**Figures 3A,B**). Second, we transiently co-expressed in the C3 control clone the exosome reporter plasmid GFP-CD63 together with different GFP-PKC δ versions or GFP, as a control, and challenged them with SEE-pulsed Raji cells. WB analysis showed similar levels of expression of the GFP-PKC δ chimeras before the synaptic challenge (**Figure 3D**, right panel) and somewhat variable after the challenge (**Figure 3C**, right panel). The expression of a dominant-negative, kinase-dead PKC δ mutant (GFP-PKC δ KD) strongly reduced the normalized secretion of exosomes induced upon IS formation (**Figures 3C,D**), in agreement with the data obtained in PKC δ -interfered clones (**Figure 3B**). By contrast, expression of a constitutively active PKC δ mutant (GFP-PKC δ CA) strongly enhanced exosome secretion induced after IS formation (**Figures 3C,D**). Third, we stimulated control and PKC δ -interfered Jurkat clones with anti-TCR and, subsequently, we measured the number of secreted exosomes by nanoparticle tracking analysis (NTA). We observed a significant reduction in the concentration of exosomes (particles/ml) secreted upon TCR stimulation in the P5 PKC δ -interfered clone when compared with the C3 control clone (**Supplementary Figure 3**). However, the size distribution of the exosomes was not affected by PKC δ interference (**Supplementary Figure 3**). Next, we extended the results obtained in the Jurkat cells to human primary T lymphoblasts. As shown in **Figure 3**, both the interference in PKC δ expression and the transient expression of GFP-PKC δ KD mutant (**Figure 3E**) inhibited TCR-stimulated exosome secretion

(**Figure 3F**). Together, these results demonstrate that PKC δ is necessary for inducible exosome secretion in T lymphocytes.

PKC δ Regulates AICD

FasL-containing exosomes secreted upon IS formation are involved in autocrine T lymphocyte AICD (13, 14). Thus, it is conceivable that the decreased exosome secretion observed in PKC δ -interfered clones may affect AICD. To test this hypothesis, control and PKC δ -interfered Jurkat clones were challenged with non-pulsed or SEE-pulsed Raji cells for 6 h and end point apoptosis was measured by Annexin-V binding and flow cytometry analysis. We observed a 3.4-fold increase in apoptosis in the C3 control clone (9% apoptotic cells without SEE vs. 32% apoptotic cells with SEE). However, the apoptosis induction in P6 PKC δ -interfered clone was lower, with a 1.8-fold increase (8% apoptotic cells without SEE vs. 15% apoptotic cells with SEE) (**Figure 4A**, left panel). Comparable results were obtained when other control and PKC δ -interfered clones were challenged in parallel (C9 and P5, **Figure 4B**). The AICD produced upon synaptic challenge was dependent of Fas ligand/Fas interaction, since apoptosis was inhibited when C3 and P6 clones were pre-incubated with a blocking antibody directed against human Fas (clone DX2) (13) before the challenge with SEE-pulsed Raji cells (**Figure 4A**, right panel). In addition, time-lapse imaging of GFP-PKC δ -expressing clones showed evidence for early apoptosis (i.e. plasma membrane blebs and cell shrinkage) between 2 and 5 h after IS formation (**Supplementary Video 3**, red arrows), in agreement with our previous results (25). Furthermore, expression of both high and low levels of GFP-PKC δ in the C3 control and P6 PKC δ -interfered clones (**Figure 4C**, right panel) showed a similar percentage of apoptotic cells upon stable IS formation (**Supplementary Video 3**), as assessed by flow cytometry (**Figure 4C**). Thus, deficient apoptosis was rescued when GFP-PKC δ WT was expressed in P6 PKC δ -interfered Jurkat clone, in accordance with the observed increase in MVB polarization toward the IS (**Figure 2C**). However, the expression of a kinase-dead PKC δ mutant (GFP-PKC δ KD) in the P6 PKC δ -interfered clone did not restore the AICD to the levels obtained in the C3 control clone (**Figure 4D**). Thus, PKC δ kinase activity seems to be required for the positive effect of PKC δ on AICD as it was for MVB polarization and exosome secretion. Together these data indicate that PKC δ is required for the secretion of FasL-containing exosomes and subsequently AICD.

DAG Recruits PKC δ to Polarized MVB but Not to the IS

To improve our understanding of the molecular basis underlying the PKC δ effect on MVB polarization, we first analyzed the dynamics of PKC δ subcellular localization upon IS formation. Imaging of control Jurkat clones expressing GFP-PKC δ showed that IS formation induced a partial redistribution of GFP-PKC δ from cytosol to a ring-shaped area nearby the MVB and proximal to the IS that was concomitant to its activation (**Supplementary Video 2** -top right panel-, **Figures 2B**, **5**). It has been described that DAG recruits PKC δ to membranes through its C1 DAG-binding domains, and this leads to PKC δ activation (49). Thus, in order to optimally detect subcellular

changes in the PKC δ activator DAG, we used U.DAG2, which upon DAG binding increases its fluorescence (35, 36). We first validated the sensor transfecting J-HM1-2.2 cells with U.DAG2 and stimulated them with either CCH or PMA. We used GFP-C1bPKC θ , another DAG sensor based on the C1b domain of PKC θ , as a control since it does not enhance its fluorescence when it binds DAG (50). The FI ratio along time for each construction was calculated as the average FI corresponding to the cell ROI at different time points relative to the average FI of the same cell ROI at the initial time point. CCH and PMA induced recruitment of both DAG sensors to the plasma membrane (although the effect with PMA was stronger than CCH), and a concomitant increase in relative U.DAG2 FI, as opposed to relative GFP-C1bPKC θ FI (**Supplementary Figure 4** and **Supplementary Video 4**, first concatenated video). We then co-expressed CFP-CD63 and U.DAG2 in J-HM1-2.2 cells and stimulated them with either plastic-bound anti-TCR (51) or with CMAC-labeled SEE-pulsed Raji cells to test whether DAG was generated during MVB polarization. Time-lapse imaging showed an increase in the U.DAG2 FI ratio (**Figure 5B**) upon TCR stimulation, concomitant with the centripetal convergence of MVB toward the center of the cell in contact with the coverslip surface, that corresponded to the secretory domain next to cSMAC (52) (**Supplementary Video 4**, 2nd concatenated video, **Figure 5B**). Furthermore, during IS formation, accumulations of U.DAG2 co-migrating with MVB and a simultaneous enhancement of U.DAG2 FI, were also observed (yellow arrows in **Figure 5C**, **Supplementary Video 5** and **Supplementary Figure 6**). Interestingly, when we co-expressed CFP-CD63 and GFP-PKC δ , we observed GFP-PKC δ accumulations that may co-migrate with MVB toward the IS (yellow arrows in J-HM1-2.2 and P6 panels, **Figure 5C** and **Supplementary Video 2**), although temporal segregation between these subcellular structures was also evident (yellow arrows in C3 panel, **Figure 5C** and **Supplementary Video 2**). To further study this potential association and to analyze the subcellular localization of U.DAG2 and GFP-PKC δ with respect to the MVB, we carried out confocal microscopy. We found that the accumulation of U.DAG2 observed in living cells upon IS formation colocalized with the MVB marker CD63 (**Figure 6A**). Furthermore, GFP-PKC δ also partially colocalized with CD63+ structures (**Figure 6B**). These partial colocalizations of U.DAG2 and GFP-PKC δ with CD63 were indeed specific, since Pearson's correlation coefficients of scatter plot fluorograms were significantly different from those corresponding to the analysis of U.DAG2 or GFP-PKC δ vs. cytosolic CMAC, (**Figure 6C**). Remarkably, we could not detect any recruitment of GFP-PKC δ to the synaptic membrane (**Supplementary Video 6**) whereas, in parallel, relocalization of PKD1 or C1bPKC θ domain to this membrane was evident (**Supplementary Videos 6**, **7**). The relocalization of both PKD1 and C1bPKC θ was dependent on transient DAG production at the IS, since interference in DGK α that enhances both DAG levels (24) and PKD1 activation (26), increased PKD1 and C1bPKC θ residence half-life at the synapse as assessed by time-lapse video analysis (**Supplementary Video 7** and **Supplementary Figure 5**). DGK α attenuation significantly enhanced mean DsRed2-PKD1 residence half-life at the synaptic

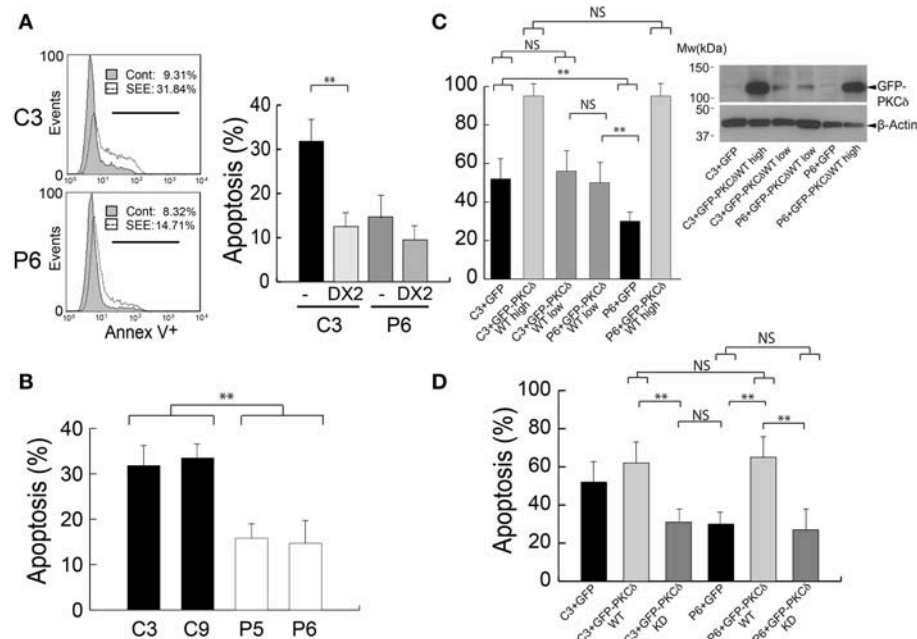


FIGURE 4 | PKC δ regulates AICD. **(A)** Left panel, C3 control and P6 PKC δ -interfered Jurkat clones were challenged either with CMAC-labeled non-pulsed (Cont) or SEE-pulsed (SEE) Raji cells for 6 h, and the percentage of Annexin-V⁺ apoptotic cells (line marker) was determined by flow cytometry analysis after gating to exclude CMAC⁺ Raji cells. Right panel, same as left panel, but the Jurkat clones were preincubated or not (-) with blocking anti-Fas antibody (DX2) before the challenge with SEE-pulsed Raji cells. Data are means of the percentage of apoptosis plus SD obtained from several experiments ($n = 3$). **(B)** C3, C9 (control) and P5, P6 (PKC δ -interfered) Jurkat clones were challenged as in **(A)**, and the percentage of Annexin-V⁺ apoptotic cells was determined by flow cytometry analysis after gating to exclude CMAC⁺ Raji cells. Data are means of the percentage of apoptosis plus SD obtained from several experiments ($n = 3$). **(C)** Left panel, C3 control and PKC δ -interfered P6 Jurkat clones transfected with GFP or different amounts of GFP-PKC δ WT expression plasmids (high = 30 μ g, low = 10 μ g) were challenged with CMAC-labeled, SEE-pulsed Raji cells for 12 h, and apoptosis induction was assessed in the transfected cells as shown in **(B)**. Right panel, WB analysis of GFP-PKC δ expression in cells used in the experiment from the left panel. **(D)** C3 control and P6 PKC δ -interfered Jurkat clones were transfected with the indicated expression plasmids and were challenged with CMAC-labeled, SEE-pulsed Raji cells for 12 h and apoptosis induction in the transfected cells was assessed as shown in **(B,C)**. Data are means plus SD ($n = 3$). Single-factor ANOVA was performed between the indicated groups. NS, not significant; $^{**}p \leq 0.05$.

membrane from 15–20 up to 40 min and GFP-C1bPKC θ half-life from 40 up to 75 min (**Supplementary Figure 5**). Together, these results suggest that upon synaptic activation DAG species, capable of recruiting a portion of PKC δ to endomembranes including MVB but not the IS, are produced.

PKC δ Regulates the Spatiotemporal Reorganization of Cortical Actin at the IS

It has been reported that the polarizations of the MTOC and cytotoxic granules toward the IS are conducted by a transient increase in cortical actin reorganization at the IS (4). This burst in cortical actin at the IS is followed by the clearance, and subsequent recovery, of cortical actin density at cIS. These events appear to be required for lytic granule and cytokine secretion in CTL and CD4⁺ lymphocytes, respectively (4, 5, 53). Thus, we studied whether actin reorganization at the IS occurs during traffic of MVB in T lymphocytes and whether PKC δ regulates this reorganization. To perform this analysis and to correlate actin reorganization at the IS with MVB polarization, we challenged control and PKC δ -interfered Jurkat clones, co-expressing GFP-actin and CFP-CD63, with SEE-pulsed Raji cells, and analyzed the changes of relative actin fluorescence intensity (FI) at the IS and at the cIS by time-lapse microscopy (**Figures 7,**

8; Supplementary Videos 8, 9). In actin reorganization kinetic experiments (**Figure 8A**), the actin FI ratio was calculated as the average actin FI corresponding to each subcellular ROI (synapse or central synapse regions) relative to the average FI of the indicated ROI (cell or synapse) at the same time point. In the C3 control clone, we observed a transient accumulation of cortical actin at the IS (closed white arrow, **Figure 7**), followed by the temporary depletion of actin at the cIS (open white arrows, **Figure 7**) and, almost simultaneously, the convergence and polarization of the initially scattered MVB toward the F-actin-low region at the cIS (yellow arrows in **Figure 7** and **Supplementary Video 8**). Next, we analyzed the kinetics of the accumulation of cortical actin at the IS, defined as the time period showing a relative actin FI at the synapse (IS FI/cell FI) > 1 (**Supplementary Video 9** and **Figure 8**, first and third rows). In the left side of **Figure 8A**, some representative frames from **Supplementary Video 9** at the indicated times after the addition of clones to the SEE-pulsed Raji cells and, below, the superimposed ROIs (cell, IS and cIS) used in these analyses are depicted. The C3 and C9 control clones showed significantly longer actin accumulation at the IS than the P5 and P6 PKC δ -interfered clones (**Figure 8B, Supplementary Video 9**). In addition, a difference in the magnitude of the F-actin

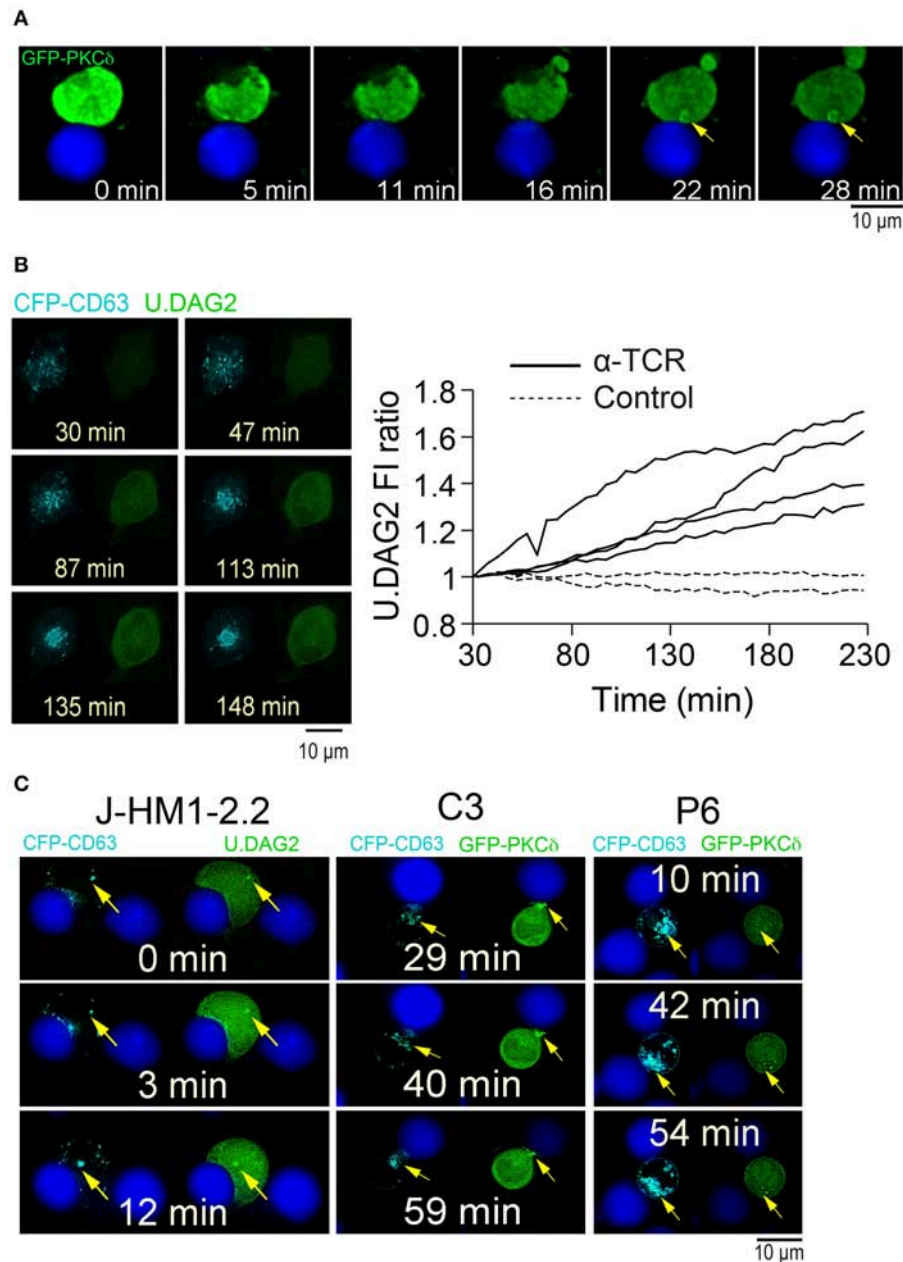
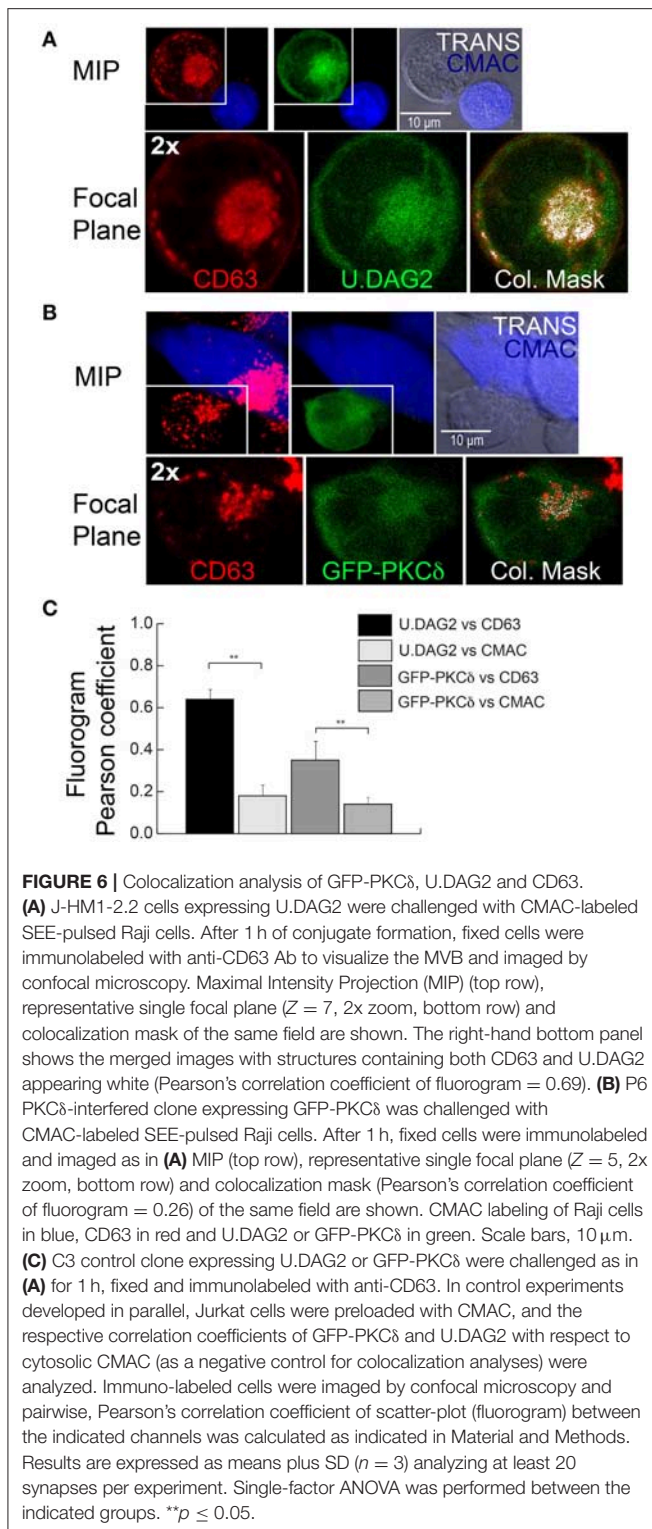


FIGURE 5 | Subcellular localization of GFP-PKC δ and U.DAG2 upon activation. **(A)** C3 control Jurkat clone expressing GFP-PKC δ WT was challenged with CMAC-labeled, SEE-pulsed Raji cells and the synaptic conjugates imaged by time-lapse fluorescence microscopy. Relevant frames corresponding to **Supplementary Video 2** (top right panel) are shown. The yellow arrow indicates accumulation of GFP-PKC δ . **(B)** J-HM1-2.2 cells co-expressing CFP-CD63 and U.DAG2 were untreated (Control) or challenged with plate-bound anti-TCR (α -TCR), imaged by time-lapse fluorescence microscopy, and the U.DAG2 FI ratio was calculated. Left panels show relevant frames of two fluorescence channels corresponding to the 2nd time-lapse video from concatenated **Supplementary Video 4** (TCR stimulation). Right panel, U.DAG2 FI ratio was calculated as the average FI corresponding to each cell ROI at different times relative to the average FI at $t = 30$ min (FI at the indicated time point/FI at $t = 30$ min) and corresponded to untreated cells ($n = 2$, discontinuous lines) and cells challenged with plate-bound anti-TCR ($n = 4$, continuous lines). **(C)** J-HM1-2.2 cells co-expressing CFP-CD63 and U.DAG2 (left panels), and C3 control and P6 PKC δ -interfered Jurkat clones co-expressing CFP-CD63 and GFP-PKC δ (right panels) were challenged with CMAC-labeled, SEE-pulsed Raji cells and imaged by time-lapse microscopy. Relevant frames corresponding to J-HM1-2.2 cells (**Supplementary Video 5**), C3 control clone (**Supplementary Video 2**, top left panel) and P6 PKC δ -interfered clone (**Supplementary Video 2**, lower right panel) are shown. The yellow arrow indicates the accumulations of MVB, U.DAG2 and GFP-PKC δ . CMAC labeling of Raji cells in blue, CFP-CD63 in cyan and U.DAG2 or GFP-PKC δ in green. Scale bars, 10 μ m.



reorganization process (measured as the maximal relative actin FI at the synapse) between the control and the PKC δ -interfered clones was observed (Figure 8A, right graphs, and Figure 8C). Moreover, no depletion of cortical actin at the cIS was observed in the P5 PKC δ -interfered clone, since the relative value of actin

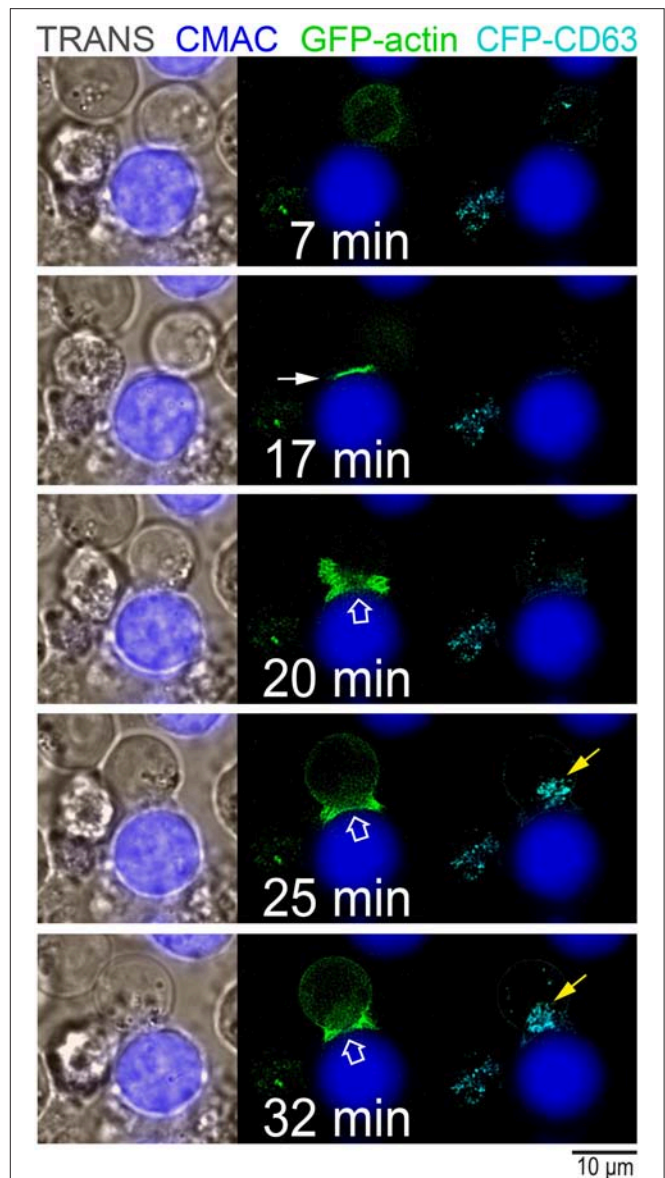


FIGURE 7 | Reorganization of cortical actin and polarization of MVB at the IS. C3 control clone co-expressing GFP-actin and CFP-CD63 was challenged with CMAC-labeled, SEE-pulsed Raji cells and imaged by time-lapse fluorescence microscopy. Some representative frames from **Supplementary Video 8** are shown. Left panel, transmittance and CMAC channels; middle panel, GFP-actin and CMAC channels; right panel, CFP-CD63 and CMAC channels. The initial burst of actin reorganization at the IS occurring upon synaptic contact (closed white arrow), the cortical actin depletion at the cIS (open white arrow) and the polarization of MVB (yellow arrow) toward the IS are indicated. CMAC labeling of Raji cells in blue, CFP-CD63 in cyan and GFP-actin in green. Scale bars, 10 μ m.

FI in the cIS (cIS FI/IS FI) remained >1 (Figure 8A, fourth row, **Supplementary Video 9**), while a transient actin depletion at cIS (cIS FI/IS FI remained <1) was observed in the C3 control clone (Figure 8A, second row).

Since 2D (X, Y) time-lapse analysis did not provide spatial information of the synaptic actin architecture, we

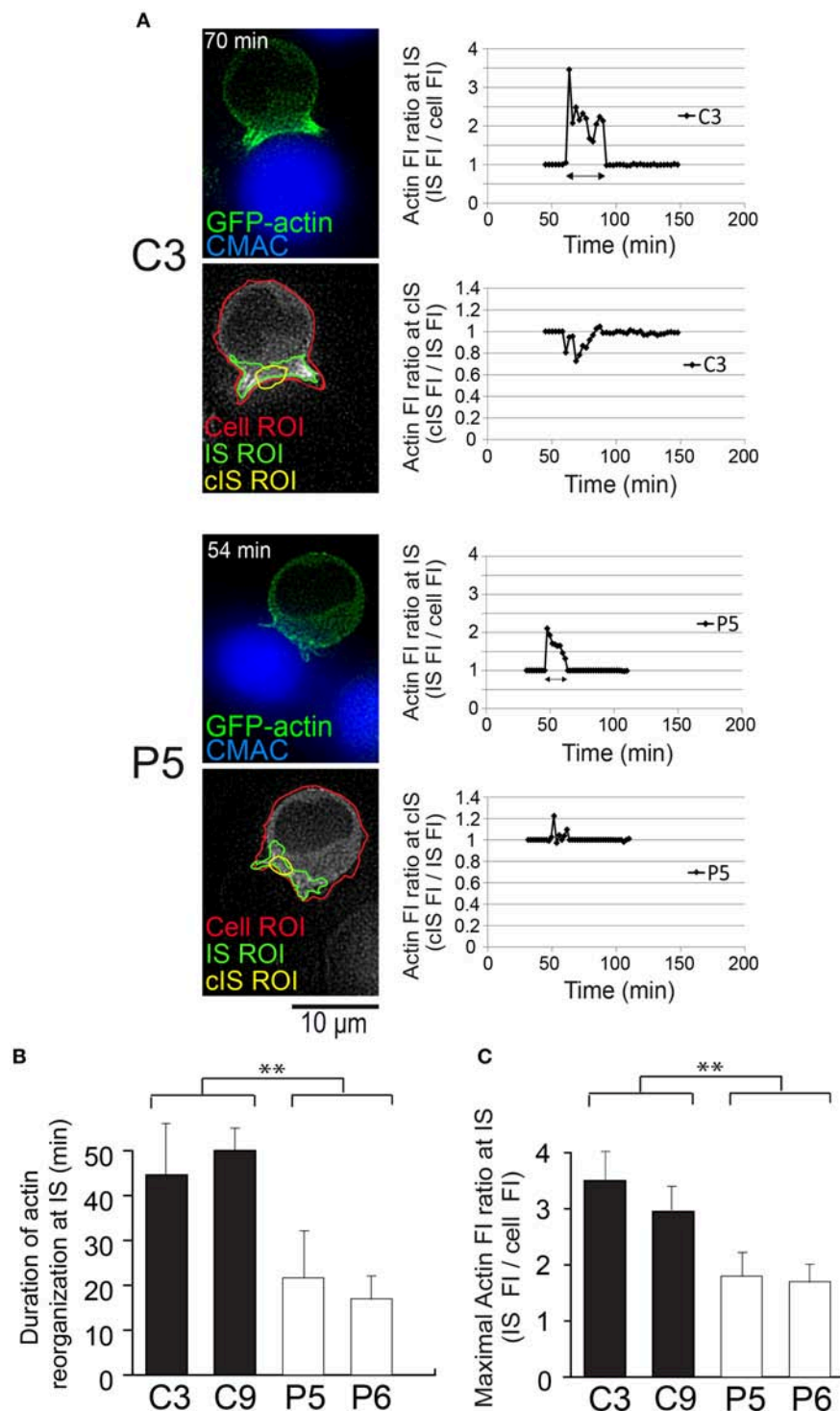


FIGURE 8 | Kinetic analysis of cortical actin reorganization at the IS in PKC δ -interfered cells. C3, C9 (control) and P5, P6 (PKC δ -interfered) clones expressing GFP-actin were challenged with CMAC-labeled SEE-pulsed Raji cells and imaged by time-lapse fluorescence microscopy. **(A)** Kinetic image analysis to evaluate actin FI ratio at the synapse (IS) and at the central synapse (cIS) using the indicated ROIs (cell ROI, red line; synapse ROI, green line; central synapse ROI, yellow line) for C3 and P5 clones asynchronously developing synapses. Representative frames from **Supplementary Video 9** at the indicated times after the addition of clones to the SEE-pulsed Raji cells and below the corresponding, superimposed ROIs, are depicted (left panels) as a reference. Kinetic analyses of the relative cortical actin FI at the IS (IS FI/cell FI) and at the cIS (cIS FI/IS FI) are shown (right graphs) for C3 (upper graphs) and P5 (lower graphs). The time $t = 0$ of the X axis scale corresponds to the addition of clones to the SEE-pulsed Raji cells, which occurred 45 min (for C3) and 30 min (for P5) before the beginning of the time-lapse capture (thick lines in the

(Continued)

FIGURE 8 | graphs of **Supplementary Video 9**. The beginning of conjugate formation, which corresponds to the peak of actin FI ratio at the IS, occurred at 62 min (17 plus 45 min) for the C3 clone and 46 min (16 plus 30 min) for the P5 clone after the addition of clones to the SEE-pulsed Raji cells. Double-headed dark arrows correspond to the time intervals during which actin ratio values were different to 1 (i.e., length of the actin reorganization period). CMAC labeling of Raji cells in blue and GFP-actin in green. Scale bars, 10 μ m. **(B)** Results are expressed as average duration of the interval of actin reorganization for C3, C9 (control) and P5, P6 (PKC δ -interfered) clones. **(C)** Same as **(B)** but results are expressed as average maximal actin FI ratio at the IS. Data are means plus SD ($n = 3$, analyzing at least 12 synapses from several different microscopy fields per experiment). Single-factor ANOVA was performed between the indicated groups. $^{**}p \leq 0.05$. This figure is related to **Supplementary Video 9**.

analyzed 3D distribution of F-actin at the IS in confocal microscopy image stacks. To perform these measurements, we generated 2D projections of the IS interface of both control and PKC δ -interfered clones, labeled with phalloidin (**Supplementary Video 10** and **Figure 9**). The boundary of the T lymphocyte/APC synaptic contact was defined by the distal SMAC (dSMAC), which consists of a circular array of F-actin accumulation at the edge of the T cell/APC interface (6) (47) (**Supplementary Figure 7B**). Thus, the IS area was delimited by the edge of the F-actin signal. When necessary, the definition of the regions of interest (ROI) to measure the F-actin-low (Fact-low) cIS area and the IS area was performed by using automated algorithms as described in the Materials and Methods section. Next, these defined ROI areas (Fact-low cIS area and IS area) were measured, and the relative area of the F-actin depleted region at cIS (Fact-low cIS area/IS area) was determined (**Supplementary Figures 7B,C**). In addition, the intensity of F-actin along the IS interface was analyzed (**Supplementary Video 10** and **Figures 9A,B**). We found a significant reduction in the percentage of synapses substantiating a F-actin-low region at cIS, with relative area > 0.1 in the P5 PKC δ -interfered cells when compared with C3 control cells ($80\% \pm 6$ F-actin depleted synapses in C3 clone vs. $48\% \pm 4$ in P5 clone; **Supplementary Figure 7C** and **Figure 9C**). Similar results were obtained when C9 control and P6 (PKC δ -interfered) clones were compared (**Figure 9C**). In addition, the expression of GFP-PKC δ WT both in the P5 and P6 PKC δ -interfered clones restored the percentage of synapses exhibiting the depletion of F-actin at cIS to the levels observed for the C3 and C9 control clones (i.e., $48\% \pm 4$ in P5 clone vs. $85\% \pm 9$ in P5 clone expressing GFP-PKC δ (**Figure 9C**). In contrast, the expression of a kinase-dead PKC δ mutant (GFP-PKC δ KD) in P5, PKC δ -interfered clone, did not recover the depletion of F-actin at cIS to the levels obtained in C3 control clone (**Figure 9C**). Together these data indicate that PKC δ participates in the spatiotemporal reorganization of actin at the IS and the subsequent polarization of MVB toward the F-actin-low region at the cIS and exosome secretion.

DISCUSSION

In this work we have established that activation of TCR at the IS induces PKC δ activation, which is necessary for the polarization of MTOC and the polarized secretory traffic of MVB toward the IS and exosome secretion. Concomitantly, we demonstrate that PKC δ -interfered T lymphocytes are defective in Fas-FasL dependent AICD. Since exosomes containing FasL have been shown to be involved in AICD (13, 14), a key process in regulating T lymphocyte homeostasis (10), our data

support a new role of PKC δ in exosome-controlled immune regulatory processes. Both FasL and Fas deficiencies are involved in the development of several autoimmune lymphoproliferative syndromes (ALPS) both in humans and mice (10, 54). In this context, human patients were identified with a homozygous loss-of-function mutation of PKC δ resulting in deficient PKC δ expression. These patients developed ALPS resembling the phenotype of PKC δ KO mice (55). Thus, PKC δ is important for the maintenance of T lymphocyte homeostasis as well as the FasL/Fas system. Regarding the functional consequences of defective exosome secretion occurring in PKC δ -interfered cells, it has been reported that exosomes contribute to thymic development (19). Negative selection in thymocytes, which is induced via TCR-controlled apoptosis (AICD), is considered an important mechanism regulating thymocyte development and immune tolerance (56). In addition, immature thymocytes from PKC δ KO mice were protected from apoptosis, indicating a clear pro-apoptotic role of PKC δ (57). It remains to be established whether the systemic autoimmune phenotype and lack of tolerance observed in PKC δ KO mice and human patients with autosomal recessive mutations in PKC δ (58) might be due to defective exosome secretion and subsequent AICD occurring during thymic development.

T and B lymphocytes are the only cells in which triggering of cell surface receptors such as TCR and BCR controls induce exosome secretion (16). Following their secretion, exosomes participate in several antigen-dependent, important immune functions (59). We have described how exosome secretion in Th lymphocytes follows TCR stimulation and IS formation (13, 25). Regarding the TCR-regulated secretory traffic of MVB, several reports have previously established the essential role of DAG and its negative regulator DGK α in the polarized traffic of MVB/late endosomes and lytic granule secretion (13, 20, 24, 25), but the molecular basis by which DAG regulates this traffic remained largely unknown. To address this important point, we have analyzed Jurkat-Raji synapses because of the closeness of this experimental system to the biological reality (T cell-APC synaptic conjugates). This analysis has used scanning confocal microscopy and time-lapse fluorescence microscopy combined with post-acquisition deconvolution, due to the enhanced signal-to-noise ratio of the images, high temporal resolution, and adaptability to the simultaneous acquisition of multiple fluorochromes in developing synapses (45). As a drawback, only those APC-T cell interfaces that were perpendicular to the plane of focus along the Z-axis could be properly analyzed (45). Since other experimental models that may facilitate image capture and analyses do not mimic the complex, irregular surface of an APC or a target cell, and may raise to non-physiological interactions in the

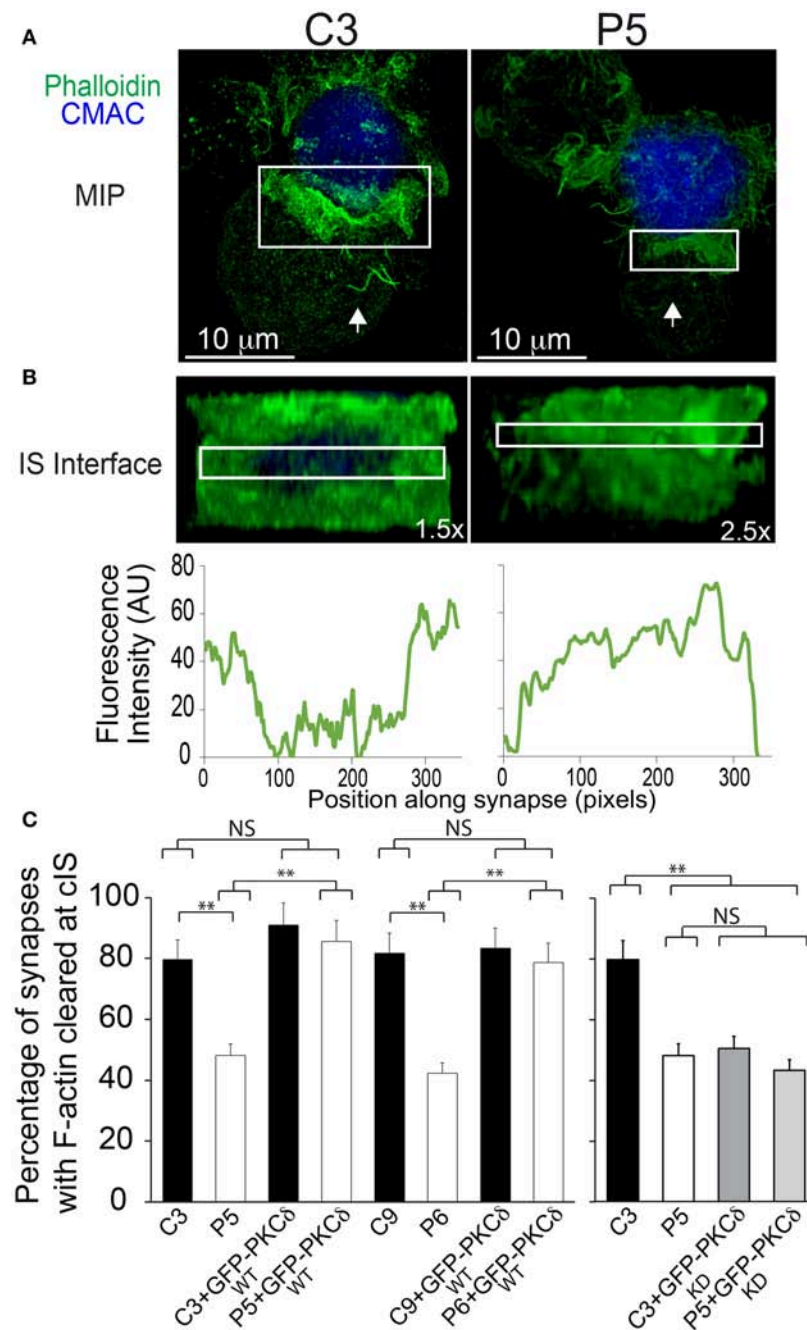


FIGURE 9 | PKC δ regulates the spatial organization of cortical actin at the IS. C3 control and P5 PKC δ -interfered clones were challenged with CMAC-labeled SEE-pulsed Raji cells for 1 h, fixed, stained with phalloidinAF488 and imaged by confocal fluorescence microscopy. **(A)** Top views correspond to the Maximal Intensity Projection (MIP) of the indicated, two merged channels, in a representative example. White arrows indicate the direction to visualize the face on views of the synapse (IS interface) enclosed by the boxed ROIs (white rectangles) as shown in **Supplementary Video 10**. **(B)** Face on views of the IS. The enlarged ROIs from **(A)** (1.5x and 2.5x zoom, respectively) were used to generate (as shown in **Supplementary Video 10**) the IS interface images shown in the upper panels. The IS interface images of the phalloidin channel correspond to frame no. 44 of **Supplementary Video 10**. The plot profile analysis of phalloidin FI for this frame along the indicated rectangular ROIs is shown in the lower diagrams. CMAC labeling of Raji cells in blue and phalloidin in green. Scale bars, 10 μ m. **(C)** Left panel, C3, C9 (control) and P5, P6 (PKC δ -interfered) clones were untransfected or transfected with GFP-PKC δ WT or GFP-PKC δ KD. Subsequently the clones were challenged with CMAC-labeled SEE-pulsed Raji cells for 1 h, fixed, immunolabeled with phalloidin AF546 and imaged by fluorescence microscopy. Subsequently, the face on views of synapses were generated as in **(B)** and the percentage of synapses with an F-actin depleted region at the central IS for the different cellular groups was calculated as described in **Supplementary Figure 7**. Data are means plus SD ($n = 3$) analyzing at least 54 synapses per experiment. Single-factor ANOVA was performed between the indicated groups. ** $p \leq 0.05$.

IS (60–63), we consider the experimental system used here appropriate to address some biological events occurring at the IS. In addition, with the use of the PKC δ C1-based U.DAG2 sensor, we have shown that upon synaptic activation, DAG species are produced that may recruit PKC δ at endomembranes including MVB. The U.DAG2 sensor was described to produce rapid and robust changes in green fluorescence in a live-cell assay, and these changes were reversible, since fluorescence returns to baseline levels 20–30 s after stimuli removal (35). In addition, there was an additional fluorescence increase of the U.DAG2 sensor due to the conformational changes (35, 36); this was an additional reason to use the sensor in our effort to reveal potential subtle DAG changes that may occur. Thus, the sensor indeed appears to be sensitive enough, although we cannot fully exclude that some very transient or very small DAG changes may not be detectable by the sensor. In addition, we cannot entirely exclude that the PKC δ C2 domain (absent in the U.DAG2 probe) may have an indirect, additional role on the selectivity of PKC δ for DAG species produced at the plasma membrane or endomembranes. In this context, we could not detect any U.DAG2 accumulation or PKC δ recruitment at the IS membrane, although DAG-mediated accumulation of C1bPKC θ or PKD1 at the IS was induced in parallel (**Supplementary Videos 6, 7**), as shown by other researchers (20, 50, 64). Thus, most probably, DAG species that were produced upon PLC activation by TCR were capable of recruiting PKC θ (65) or PKD1 (46) to the IS via their C1 domains, but not PKC δ (66). In addition, our results support that, also upon synaptic activation, distinct DAG species to those generated at the IS are produced into endomembranes (such as MVB), which are capable of recruiting and activate PKC δ . Supporting our data, PKC δ has been found in lytic granules from CTLs (28). In this context, it has been shown that differences in intrinsic affinities and selectivities among the C1 and C2 DAG-binding domains for different DAG species control the rate, magnitude, duration, and subcellular localization of the diverse PKC isotypes (67, 68). The data from other authors, based on the lack of recruitment of PKC δ to the IS (64), may apparently argue against a contribution of PKC δ to MTOC polarization. However, we propose that TCR signaling regulates the production of distinct DAG species at different subcellular locations (IS and MVB endomembranes), with each species recruiting different PKC isotypes, which may be together necessary for MTOC polarization. Indeed, this may conciliate these data with our results regarding the involvement of PKC δ in MTOC polarization toward the IS. Regarding the molecular bases that may underlie the PKC δ effect on F-actin reorganization, it has been described that phosphorylation of paxillin, an actin regulatory protein, by PKC δ regulates integrin-mediated adhesion and migration of lymphoid cells (69). In particular, PKC δ phosphorylates paxillin at T538, leading to the depolymerization of the actin cytoskeleton (69). In addition, paxillin phosphorylation is required for the degranulation of CTL (70). Thus, we are analyzing the phosphorylation of paxillin at T538 in control Jurkat clones. Our preliminary data suggest that both pharmacologic (PMA), anti-TCR stimulation and synaptic stimulation of Jurkat control clones induced a strong phosphorylation of paxillin at T538 (not shown). Further

experiments are required to analyze whether PKC δ -interfered clones substantiate comparable paxillin phosphorylation upon stimulation. The results from these experiments may provide some clue to explain the PKC δ role on F-actin reorganization leading to MTOC polarization.

Here we have studied the role of cortical actin reorganization in the polarized traffic of MVB leading to exosome secretion at the IS in Th lymphocytes. PKC δ -interfered clones forming synapses exhibited quantitative alterations (duration and magnitude of actin reorganization) but also qualitative differences (absence of depletion of F-actin at the cIS) in actin rearrangement at the IS when compared with control clones. Any of these alterations acting alone or in combination, have been described to affect T-cell activation, polarized secretion, AICD and CTL effector functions [reviewed in (3)]. In fact, the phenotype we describe here resembles the alterations found in T lymphocytes deficient in TAGLN2 (71) or HS1 (51). Thus, the altered actin stabilization at the IS we found may underlie the deficient MVB polarization occurring in PKC δ -interfered clones. The initial increase in F-actin at the IS was followed by a decrease in F-actin density at the cIS, and this event appears to be the limiting step for exosome secretion. This process is similar to the degranulation of cytotoxic granules and cytokine-containing secretory granules at the F-actin-depleted region that contains a specialized secretory domain in CTL and Th lymphocytes (4, 5, 53). Interestingly, in both cell types cortical actin reorganization at the IS, followed by the polarization of MTOC and secretory granules toward the IS, was also reported (4, 47, 53, 72). Thus, these sequential events are essentially common to IS formed by CTL or Th cells, although both the nature and cargo of the secretory granules in these cells are quite different. In addition, CTL form much more transient synapses than Th cells, lasting only a few minutes, as the target cells are killed (2, 5). This is probably due to the fact that the optimal CTL function requires rapid and transient contact in order to deliver as many lethal hits as possible to several target cells, whereas stable, lengthy synapses (>20–30 min up to several hours) formed by Th cells, as we studied here, are necessary for both directional and continuous secretion of stimulatory cytokines (2, 5). Accordingly, in CTL the directional movement of MTOC toward the synapse lasts very few minutes, whereas in long-lived synapses made by Th lymphocytes the MTOC, but also the MVB as we have shown (26), takes from several minutes up to hours to move and dock to the IS (2, 5, 73). Thus, the contribution of the actin reorganization to the polarization of secretory granules is a qualitative feature shared by CTL and Th cells, as well as some of the molecular components that control this reorganization (i.e., WASP, TAGLN2) (3, 71, 74). Nevertheless, there must be quantitative differences in signals, and probably in some molecular mechanisms that govern the actin cytoskeleton dynamics between the cytolytic and helper synapses (5, 75).

Recently, it has been shown in CTL and Jurkat cells that secretory granule convergence toward the MTOC and MTOC polarization to the IS are two mechanistically distinct processes (76). Interestingly, in our analysis of MVB and MTOC migration to the IS at the single cell level, we have found that both MVB

and MTOC do not efficiently polarize in PKC δ -interfered cells, but their center of mass converge at nearby positions in control and interfered cells (**Figure 1B** and **Supplementary Figure 1C**), correlating in their migration tendency. In addition, our data show that diminished levels of PKC δ did not affect the average centripetal velocity of MVB. Instead, PKC δ appears to specifically regulate the subsequent step of the secretory traffic (i.e., MTOC translocation to the IS). These findings are in part compatible with what was found in PKC δ -KO mouse CTL, in which the lytic granules underwent convergence toward the MTOC, but these granules did not polarize to the IS and the subsequent cytotoxicity was inhibited (29). However, although in these cells the absence of PKC δ inhibited polarization of cytolytic granules, the MTOC polarization toward the IS was not affected (29). Currently, we lack a clear explanation for the observed partial discrepancy, but it is certain that in the mentioned study no simultaneous assessment of MVB and MTOC polarization was performed at the single cell level as we have performed here. Additionally, and most likely, differences between cytotoxic and helper synapses may cause this apparent disagreement (see above).

In conclusion, aside from the well-known role of the PKC θ isotype and the redundant action of PKC ϵ and PKC η isoforms in the polarization of the secretory machinery in CD4⁺ T cells (2, 77), we have identified a positive regulatory role of the PKC δ isotype in MVB polarization and exosome secretion upon IS formation in Th lymphocytes. Regarding the biological significance of such a regulatory mechanism, it is remarkable that melanocytes, among other cell types, undergo multidirectional dispersion of secretory organelles for efficient distribution of their granule contents (78). In these cells, convergence of secretory granules toward the MTOC, which remains at the perinuclear area, prevents (but does not promote) degranulation (78). In contrast, the MTOC polarization to the IS, acting in coordination with the convergence of secretory granules toward the MTOC, is necessary for optimal polarized and focused secretion in many cell types of the immune system, including innate NK cells (79, 80), CTL (4, 76), primary CD4⁺ T cells (47) and Jurkat cells [(76) and the present report]. This mechanism that now includes the key player PKC δ appears to specifically provide the immune system with a finely-tuned strategy to increase the efficiency of crucial secretory effector functions, while minimizing nonspecific, bystander cytokine stimulation, target cell killing and AICD.

REFERENCES

- de la Roche M, Asano Y, Griffiths GM. Origins of the cytolytic synapse. *Nat Rev Immunol.* (2016) 16:421–32. doi: 10.1038/nri.2016.54
- Huse M. Microtubule-organizing center polarity and the immunological synapse: protein kinase C and beyond. *Front Immunol.* (2012) 3:235. doi: 10.3389/fimmu.2012.00235
- Billadeau DD, Nolz JC, Gomez TS. Regulation of T-cell activation by the cytoskeleton. *Nat Rev Immunol.* (2007) 7:131–43. doi: 10.1038/nri2021
- Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen BC, Gawden-Bone C, et al. Actin depletion initiates events leading to granule

AUTHOR CONTRIBUTIONS

VC and MI conceived and designed all the experiments. GH and VC did most of the experiments, analyzed data, and contributed to the writing of the manuscript. PA, SD, BS, DF-M, JG, RdM, MQ, LM-E, AB-G, and TF contributed to the MTOC/MVB polarization experiments and time-lapse studies. AS performed the WB analysis, exosome measurement, and also contributed to rescue experiments. PR-S contributed to actin reorganization experiments, image analysis, statistical analysis of the results, and helped in writing of the manuscript. AF-R contributed to design some experiments and contributed to writing the manuscript. MI conceptualized and coordinated the research, directed the study, analyzed data, and wrote the manuscript. All the authors contributed to the planning and designing of the experiments and to helpful discussions.

FUNDING

This work was supported by grants from the Spanish Ministerio de Economía y Competitividad (MINECO), Plan Nacional de Investigación Científica (SAF2016-77561-R to MI, which was in part granted with FEDER-EC- funding). This work was partially supported by grant BFU2012-35067 to AF-R.

ACKNOWLEDGMENTS

We acknowledge the excellent technical support from A. M. García, A. Martínez, A. Merchán, A. Sánchez, and H. Serrano. We acknowledge Dr. M.A. Alonso (CBM, CSIC) for suggestions, reagents and critical reading of this manuscript. Thanks to Lola Morales (SIDI-UAM) and Silvia Gutiérrez (CNB, CSIC) for their superb expertise with confocal microscopy. We are indebted to Dr. I. Mérida (CNB, CSIC Madrid) for her generous and continuous support and reagents. We acknowledge M. Ware (Nanosight Ltd., UK) and J. Puebla (MALVERN) for their support in NANOSIGHT studies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00851/full#supplementary-material>

- secretion at the immunological synapse. *Immunity.* (2015) 42:864–76. doi: 10.1016/j.immuni.2015.04.013
- Griffiths GM, Tsun A, Stinchcombe JC. The immunological synapse: a focal point for endocytosis and exocytosis. *J Cell Biol.* (2010) 189:399–406. doi: 10.1083/jcb.201002027
- Ritter AT, Angus KL, Griffiths GM. The role of the cytoskeleton at the immunological synapse. *Immunol Rev.* (2013) 256:107–17. doi: 10.1111/imr.12117
- Le Floch A, Huse M. Molecular mechanisms and functional implications of polarized actin remodeling at the T cell immunological synapse. *Cell Mol Life Sci.* (2015) 72:537–56. doi: 10.1007/s00018-014-1760-7

8. Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, et al. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med.* (1991) 173:1099–109. doi: 10.1084/jem.173.5.1099
9. Bossi G, Griffiths GM. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat Med.* (1999) 5:90–6. doi: 10.1038/4779
10. Nagata S, Golstein P. The Fas death factor. *Science.* (1995) 267:1449–56. doi: 10.1126/science.7533326
11. Monleon I, Martinez-Lorenzo MJ, Monteagudo L, Lasiera P, Taules M, Iturralde M, et al. Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. *J Immunol.* (2001) 167:6736–44. doi: 10.4049/jimmunol.167.12.6736
12. Zuccato E, Blott EJ, Holt O, Sigismund S, Shaw M, Bossi G, et al. Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. *J Cell Sci.* (2007) 120(Pt 1):191–9. doi: 10.1242/jcs.03315
13. Alonso R, Rodriguez MC, Pindado J, Merino E, Merida I, Izquierdo M. Diacylglycerol kinase α regulates the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes. *J Biol Chem.* (2005) 280:28439–50. doi: 10.1074/jbc.M501112200
14. Martinez-Lorenzo MJ, Anel A, Gamen S, Monle n I, Lasiera P, Larrad L, et al. Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. *J Immunol.* (1999) 163:1274–81.
15. Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol.* (2002) 168:3235–41. doi: 10.4049/jimmunol.168.7.3235
16. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* (2009) 9:581–93. doi: 10.1038/nri2567
17. Peters PJ, Geuze HJ, Van der Donk HA, Slot JW, Griffith JM, Stam NJ, et al. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *Eur J Immunol.* (1989) 19:1469–75. doi: 10.1002/eji.1830190819
18. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun.* (2011) 2:282. doi: 10.1038/ncomms1285
19. Lundberg V, Berglund M, Skogberg G, Lindgren S, Lundqvist C, Gudmundsdottir J, et al. Thymic exosomes promote the final maturation of thymocytes. *Sci Rep.* (2016) 6:36479. doi: 10.1038/srep36479
20. Quann EJ, Merino E, Furuta T, Huse M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol.* (2009) 10:627–35. doi: 10.1038/ni.1734
21. Carrasco S, Merida I. Diacylglycerol, when simplicity becomes complex. *Trends Biochem Sci.* (2007) 32:27–36. doi: 10.1016/j.tibs.2006.11.004
22. Topham MK, Prescott SM. Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J Biol Chem.* (1999) 274:11447–50. doi: 10.1074/jbc.274.17.11447
23. Sanjuan MA, Jones DR, Izquierdo M, Merida I. Role of diacylglycerol kinase α in the attenuation of receptor signaling. *J Cell Biol.* (2001) 153:207–20. doi: 10.1083/jcb.153.1.207
24. Rainero E, Caswell PT, Muller PA, Grindlay J, McCaffrey MW, Zhang Q, et al. Diacylglycerol kinase α controls RCP-dependent integrin trafficking to promote invasive migration. *J Cell Biol.* (2012) 196:277–95. doi: 10.1083/jcb.201109112
25. Alonso R, Mazzeo C, Rodriguez MC, Marsh M, Fraile-Ramos A, Calvo V, et al. Diacylglycerol kinase α regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of Fas ligand-containing exosomes in T lymphocytes. *Cell Death Differ.* (2011) 18:1161–73. doi: 10.1038/cdd.2010.184
26. Mazzeo C, Calvo V, Alonso R, Merida I, Izquierdo M. Protein kinase D1/2 is involved in the maturation of multivesicular bodies and secretion of exosomes in T and B lymphocytes. *Cell Death Differ.* (2016) 23:99–109. doi: 10.1038/cdd.2015.72
27. Chauveau A, Le Floch A, Bantilan NS, Koretzky GA, Huse M. Diacylglycerol kinase α establishes T cell polarity by shaping diacylglycerol accumulation at the immunological synapse. *Sci Signal.* (2014) 7:ra82. doi: 10.1126/scisignal.2005287
28. Ma JS, Haydar TF, Radoja S. Protein kinase C δ localizes to secretory lysosomes in CD8+ CTL and directly mediates TCR signals leading to granule exocytosis-mediated cytotoxicity. *J Immunol.* (2008) 181:4716–22. doi: 10.4049/jimmunol.181.7.4716
29. Ma JS, Monu N, Shen DT, Mecklenbrauker I, Radoja N, Haydar TF, et al. Protein kinase C δ regulates antigen receptor-induced lytic granule polarization in mouse CD8+ CTL. *J Immunol.* (2007) 178:7814–21. doi: 10.4049/jimmunol.178.12.7814
30. Desai DM, Newton ME, Kadlecsek T, Weiss A. Stimulation of the phosphatidylinositol pathway can induce T-cell activation. *Nature.* (1990) 348:66–9. doi: 10.1038/348066a0
31. Izquierdo M, Downward J, Graves JD, Cantrell DA. Role of protein kinase C in T-cell antigen receptor regulation of p21ras: evidence that two p21ras regulatory pathways coexist in T cells. *Mol Cell Biol.* (1992) 12:3305–12. doi: 10.1128/MCB.12.7.3305
32. Hirai S, Izumi Y, Higa K, Kaibuchi K, Mizuno K, Osada S, et al. Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKC δ . *EMBO J.* (1994) 13:2331–40. doi: 10.1002/j.1460-2075.1994.tb06517.x
33. DeVries TA, Neville MC, Reyland ME. Nuclear import of PKC δ is required for apoptosis: identification of a novel nuclear import sequence. *EMBO J.* (2002) 21:6050–60. doi: 10.1093/emboj/cdf606
34. Pores-Fernando AT, Ranaghan MY, Zweifach A. No specific subcellular localization of protein kinase C is required for cytotoxic T cell granule exocytosis. *J Biol Chem.* (2009) 284:25107–15. doi: 10.1074/jbc.M109.011866
35. Tewson PH, Quinn AM, Hughes TE. A multiplexed fluorescent assay for independent second-messenger systems: decoding GPCR activation in living cells. *J Biomol Screen.* (2013) 18:797–806. doi: 10.1177/1087057113485427
36. Tewson P, Westenberg M, Zhao Y, Campbell RE, Quinn AM, Hughes TE. Simultaneous detection of Ca²⁺ and diacylglycerol signaling in living cells. *PLoS ONE.* (2012) 7:e42791. doi: 10.1371/journal.pone.0042791
37. Singh R, Andreadis ST. EGF receptor activation decreases retroviral gene transfer through protein kinase C- δ . *Mol Therap.* (2007) 15:369–77. doi: 10.1038/sj.mt.6300050
38. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* (2006) Chapter 3:Unit 3.22. doi: 10.1002/0471143030.cb0322s30
39. Skliar M, Chernyshev VS, Belnap DM, Sergey GV, Al-Hakami SM, Bernard PS, et al. Membrane proteins significantly restrict exosome mobility. *Biochem Biophys Res Commun.* (2018) 501:1055–9. doi: 10.1016/j.bbrc.2018.05.107
40. Raiborg C, Rusten TE, Stenmark H. Protein sorting into multivesicular endosomes. *Curr Opin Cell Biol.* (2003) 15:446–55. doi: 10.1016/S0955-0674(03)00080-2
41. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science.* (2008) 319:1244–7. doi: 10.1126/science.1153124
42. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol.* (2010) 12:19–30. doi: 10.1038/ncb2000
43. Ventimiglia LN, Fernandez-Martin L, Martinez-Alonso E, Anton OM, Guerra M, Martinez-Menarguez JA, et al. Cutting Edge: Regulation of Exosome Secretion by the Integral MAL Protein in T Cells. *J Immunol.* (2015) 195:810–4. doi: 10.4049/jimmunol.1500891
44. Jambrina E, Alonso R, Alcalde M, del Carmen Rodriguez M, Serrano A, Martinez AC, et al. Calcium influx through receptor-operated channel induces mitochondria-triggered paraptotic cell death. *J Biol Chem.* (2003) 278:14134–45. doi: 10.1074/jbc.M211388200
45. Calvo V, Izquierdo M. Imaging polarized secretory traffic at the immune synapse in living T lymphocytes. *Front Immunol.* (2018) 9:684. doi: 10.3389/fimmu.2018.00684
46. Spitaler M, Emslie E, Wood CD, Cantrell D. Diacylglycerol and protein kinase D localization during T lymphocyte activation. *Immunity.* (2006) 24:535–46. doi: 10.1016/j.immuni.2006.02.013
47. Ueda H, Zhou J, Xie J, Davis MM. Distinct roles of cytoskeletal components in immunological synapse formation and directed secretion. *J Immunol.* (2015) 195:4117–25. doi: 10.4049/jimmunol.1402175

48. Yi J, Wu X, Chung AH, Chen JK, Kapoor TM, Hammer JA. Centrosome repositioning in T cells is biphasic and driven by microtubule end-on capture-shrinkage. *J Cell Biol.* (2013) 202:779–92. doi: 10.1083/jcb.201301004
49. Giorgione JR, Lin JH, McCammon JA, Newton AC. Increased membrane affinity of the C1 domain of protein kinase C δ compensates for the lack of involvement of its C2 domain in membrane recruitment. *J Biol Chem.* (2006) 281:1660–9. doi: 10.1074/jbc.M510251200
50. Carrasco S, Merida I. Diacylglycerol-dependent Binding Recruits PKC θ and RasGRP1 C1 Domains to specific subcellular localizations in living T lymphocytes. *Mol Biol Cell.* (2004) 15:2932–42. doi: 10.1091/mbc.e03-11-0844
51. Gomez TS, McCarney SD, Carrizosa E, Labno CM, Comiskey EO, Nolz JC, et al. HS1 functions as an essential actin-regulatory adaptor protein at the immune synapse. *Immunity.* (2006) 24:741–52. doi: 10.1016/j.immuni.2006.03.022
52. Comrie WA, Babich A, Burkhardt JK. F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *J Cell Biol.* (2015) 208:475–91. doi: 10.1083/jcb.201406121
53. Chemin K, Bohineust A, Dogniaux S, Turret M, Guegan S, Miro F, et al. Cytokine secretion by CD4+ T cells at the immunological synapse requires Cdc42-dependent local actin remodeling but not microtubule organizing center polarity. *J Immunol.* (2012) 189:2159–68. doi: 10.4049/jimmunol.1200156
54. Nagata S, Suda T. Fas and Fas ligand: lpr and gld mutations. *Immunol Today.* (1995) 16:39–43. doi: 10.1016/0167-5699(95)80069-7
55. Kuehn HS, Niemela JE, Rangel-Santos A, Zhang M, Pittaluga S, Stoddard JL, et al. Loss-of-function of the protein kinase C δ (PKC δ) causes a B-cell lymphoproliferative syndrome in humans. *Blood.* (2013) 121:3117–25. doi: 10.1182/blood-2012-12-469544
56. Palmer E. Negative selection: clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol.* (2003) 3:383–91. doi: 10.1038/nri1085
57. Pfeiffer-Obermair C, Thuille N, Baier G. Involvement of distinct PKC gene products in T cell functions. *Front Immunol.* (2012) 3:220. doi: 10.3389/fimmu.2012.00220
58. Salzer E, Santos-Valente E, Keller B, Warnatz K, Boztug K. Protein Kinase C δ : a Gatekeeper of immune homeostasis. *J Clin Immunol.* (2016) 36:631–40. doi: 10.1007/s10875-016-0323-0
59. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol.* (2014) 14:195–208. doi: 10.1038/nri3622
60. Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, et al. Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol.* (2010) 28:79–105. doi: 10.1146/annurev-immunol-030409-101308
61. Bertolet G, Liu D. The planar lipid bilayer system serves as a reductionist approach for studying NK cell immunological synapses and their functions. *Methods Mol Biol.* (2016) 1441:151–65. doi: 10.1007/978-1-4939-3684-7_13
62. Dustin ML. Supported bilayers at the vanguard of immune cell activation studies. *J Struct Biol.* (2009) 168:152–60. doi: 10.1016/j.jsb.2009.05.007
63. Martinelli R, Carman CV. An endothelial planar cell model for imaging immunological synapse dynamics. *J Vis Exp.* (2015) 2015:e53288. doi: 10.3791/53288
64. Quann EJ, Liu X, Altan-Bonnet G, Huse M. A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat Immunol.* (2011) 12:647–54. doi: 10.1038/ni.2033
65. Monks CRE, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature.* (1998) 395:82–6. doi: 10.1038/25764
66. Melowic HR, Stahelin RV, Blatner NR, Tian W, Hayashi K, Altman A, et al. Mechanism of Diacylglycerol-induced Membrane Targeting and Activation of Protein Kinase C θ . *J Biol Chem.* (2007) 282:21467–76. doi: 10.1074/jbc.M700119200
67. Kamiya Y, Mizuno S, Komenoi S, Sakai H, Sakane F. Activation of conventional and novel protein kinase C isozymes by different diacylglycerol molecular species. *Biochem Biophys Rep.* (2016) 7:361–6. doi: 10.1016/j.bbrep.2016.07.017
68. Gallegos LL, Newton AC. Spatiotemporal dynamics of lipid signaling: protein kinase C as a paradigm. *IUBMB Life.* (2008) 60:782–9. doi: 10.1002/iub.122
69. Romanova LY, Holmes G, Bahte SK, Kovalchuk AL, Nelson PJ, Ward Y, et al. Phosphorylation of paxillin at threonine 538 by PKC δ regulates LFA1-mediated adhesion of lymphoid cells. *J Cell Sci.* (2010) 123(Pt 9):1567–77. doi: 10.1242/jcs.060996
70. Robertson LK, Mireau LR, Ostergaard HL. A role for phosphatidylinositol 3-kinase in TCR-stimulated ERK activation leading to paxillin phosphorylation and CTL degranulation. *J Immunol.* (2005) 175:8138–45. doi: 10.4049/jimmunol.175.12.8138
71. Na B-R, Kim H-R, Piragyte I, Oh H-M, Kwon M-S, Akber U, et al. TAGLN2 regulates T cell activation by stabilizing the actin cytoskeleton at the immunological synapse. *J Cell Biol.* (2015) 209:143–62. doi: 10.1083/jcb.201407130
72. Ueda H, Morpew MK, McIntosh JR, Davis MM. CD4+ T-cell synapses involve multiple distinct stages. *Proc Natl Acad Sci USA.* (2011) 108:17099–104. doi: 10.1073/pnas.1113703108
73. Xie J, Tato CM, Davis MM. How the immune system talks to itself: the varied role of synapses. *Immunol Rev.* (2013) 251:65–79. doi: 10.1111/imr.12017
74. De Meester J, Calvez R, Valitutti S, Dupre L. The Wiskott-Aldrich syndrome protein regulates CTL cytotoxicity and is required for efficient killing of B cell lymphoma targets. *J Leukoc Biol.* (2010) 88:1031–40. doi: 10.1189/jlb.0410197
75. Huse M, Quann EJ, Davis MM. Shouts, whispers and the kiss of death: directional secretion in T cells. *Nat Immunol.* (2008) 9:1105–11. doi: 10.1038/ni.f.215
76. Nath S, Christian L, Tan SY, Ki S, Ehrlich LIR, Poenie M. Dynein separately partners with NDE1 and dynactin to orchestrate T cell focused secretion. *J Immunol.* (2016) 197:2090–101. doi: 10.4049/jimmunol.1600180
77. Kong K-F, Altman A. In and out of the bull's eye: PKCs in the immunological synapse. *Trends Immunol.* (2013) 34:234–42. doi: 10.1016/j.it.2013.01.002
78. Alexandra A, Nascimento, Joseph T, Roland a, Gelfand VI. Pigment cells: a model for the study of organelle transport. *Ann Rev Cell Dev Biol.* (2003) 19:469–91. doi: 10.1146/annurev.cellbio.19.11401.092937
79. Mentlik AN, Sanborn KB, Holzbaur EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolitic commitment. *Mol Biol Cell.* (2010) 21:2241–56. doi: 10.1091/mbc.e09-11-0930
80. Hsu H-T, Mace EM, Carisey AF, Viswanath DI, Christakou AE, Wiklund M, et al. NK cells converge lytic granules to promote cytotoxicity and prevent bystander killing. *J Cell Biol.* (2016) 215:875–89. doi: 10.1083/jcb.2016.04136

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