REGULATORY MECHANISMS OF EARLY INTRACELLULAR SIGNALING IN T LYMPHOCYTES

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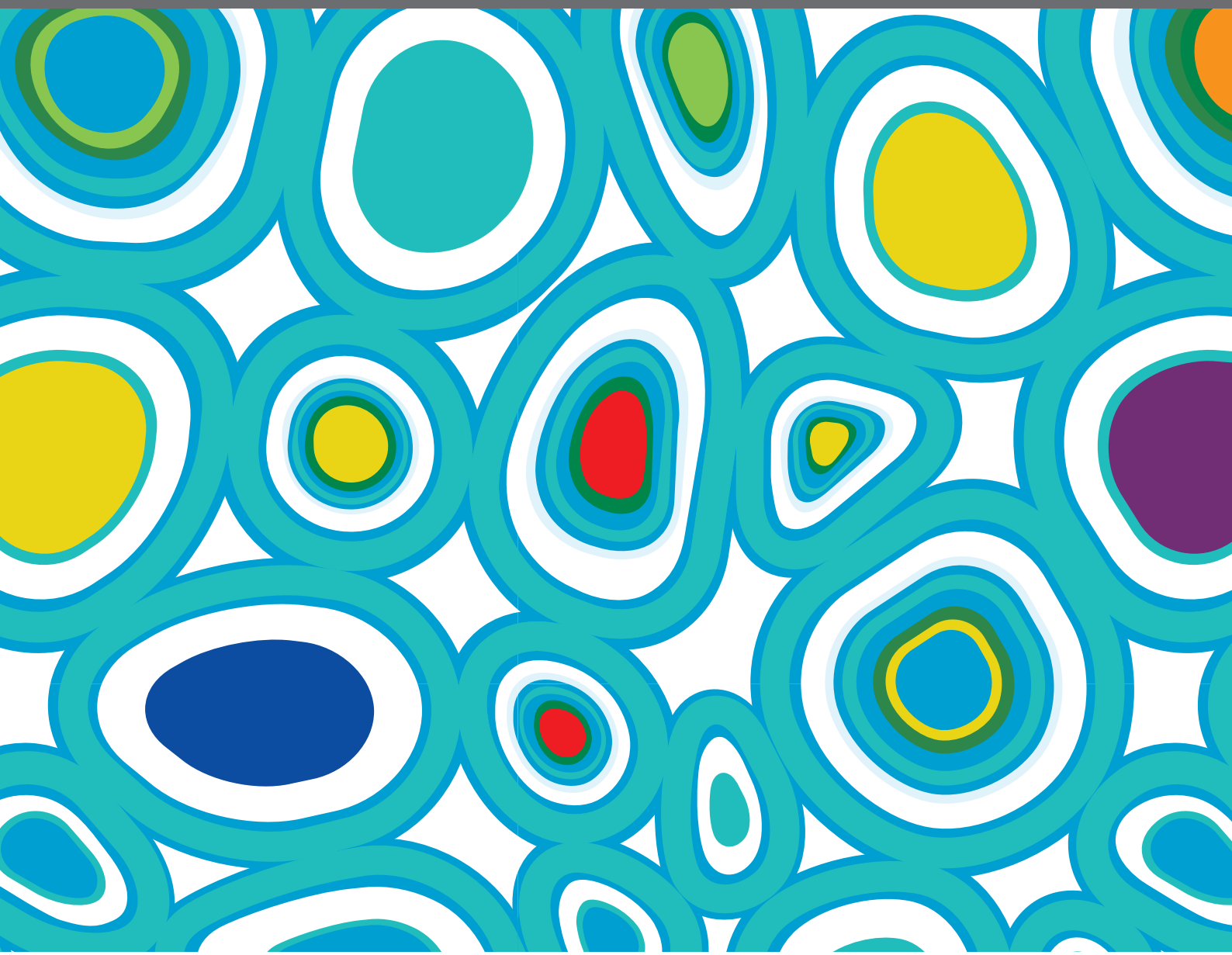

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REGULATORY MECHANISMS OF EARLY INTRACELLULAR SIGNALING IN T LYMPHOCYTES

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EDITORIAL
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Editorial: Regulatory Mechanisms of Early Intracellular Signaling in T Lymphocytes

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Editorial on the Research Topic

Regulatory Mechanisms of Early Intracellular Signaling in T Lymphocytes

Activation of T lymphocytes constitutes a central event in adaptive immune responses. Antigen recognition by T cells through the T cell receptor (TCR) triggers a cascade of intracellular signals leading to T cell activation and development of effector functions. Proper TCR signaling requires sequential activities of Lck and ZAP-70 tyrosine kinases, resulting in phosphorylation of tyrosine residues located in the CD3 ITAMs and adaptor protein LAT, respectively (Courtney et al., 2018). Although there has been a huge increase in knowledge on activatory intracellular signaling in recent years, much less is known about the molecular mechanisms negatively regulating early TCR intracellular signals.

This Research Topic contains 9 articles covering different aspects in the field of intracellular signaling coupled to the TCR/CD3 complex. T lymphocyte activation is initiated by interaction of TCRs with agonistic peptide-major histocompatibility complexes (pMHC) on the surface of antigen presenting cells (APCs). This triggers dramatic remodeling and reorganization of the T cell's 3D endocytic network to efficiently deliver TCR and TCR signaling proteins into a highly-organized 2D interface where signaling is integrated called the immunological synapse (IS) (Dustin and Choudhuri, 2016). Mastrogiovanni et al. comprehensively reviews the crucial role T cells' cytoskeleton plays in T cell activation by aiding the formation of the immunological synapse, regulating antigen recognition, and delivery of crucial signaling proteins into the IS.

One example of such crucial family of proteins are the co-receptors, which strengthen T-cell responses by many orders of magnitude. Morch et al. reviews three possible mechanisms explaining how co-receptors so profoundly amplify TCR signaling: (i) the Lck recruitment model, (ii) the pseudodimer model, and (iii) the two-step coreceptor recruitment to partially triggered TCRs model.

Felce et al. provides new data illustrating that other molecules recruited to the immunological synapse can contribute to T cell activation as well. More specifically, using a knock-out screen they identify various G-protein coupled receptors that contribute to T cell activation. Mutating G-protein coupled receptor CXCR4, for example, perturbs its recruitment into the immunological synapse and abolishes its contribution to activation of primary human CD4 T cells.

In parallel, Rudd summarizes the history of events leading to the development of the tyrosine kinase-mediated "TCR signaling paradigm" in T cells and discusses its importance for the design of new therapeutic approaches.

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Building on this knowledge Castro-Sanchez et al. reviews the role of tyrosine phosphatases -that remove phosphates from tyrosines- as crucial regulators of T cells activation, and their potential as therapeutic agents in autoimmune disorders and cancer. More specifically, this collection includes new data about the specific regulatory role of phosphorylation of LAT tyrosine residue 132 Arbulo-Echevarria et al.. It had previously been shown that the phosphorylation of tyrosine 132 of human LAT form has slower phosphorylation kinetics than the other functionally relevant tyrosines, due to the presence of a glycine residue preceding the tyrosine (Gly131) (Houtman et al., 2005; Lo et al., 2019). Now, we confirm that a LAT mutant in which glycine 131 has been substituted by an aspartate (LATG131D) increases TCR signaling, and also that T cells expressing the LATG131D mutant are more sensitive to inhibition of IL-2 production by pre-treatment with anti-CD3, which points to a possible role of this residue in the generation of anergy.

Two other reviews in this collection discuss how the TCR organization at the IS affects signaling. Balagopalan et al. discusses organization of microclusters, as well as the kinetics of recruitment and disassociation of molecules from microclusters in T cells. In their article, authors explain in detail the kinetics of recruitment and segregation of molecules from microclusters, and the role of post-translational modifications in the downregulation of the microcluster-associated signaling molecules. In parallel (Bunnell et al., 2002; Lee et al., 2002). Pathan-Chhatbar et al. focuses on the role of the interaction between plasma membrane cholesterol and the TCR clustering. The authors describe the opposing roles that this interaction may have in the context of T cell activation, discussing their own recently published data as well as those of other groups. In their review, Schamel and collaborators propose an interesting model by which cholesterol keeps in check TCRs that have not bound an antigenic pMHC, but at the same time is able to favor the formation of nanoclusters that increase TCR avidity, and thus their "activatability."

Finally, this collection includes a review by Castellano and Molinier-Frenkel on the role of amino acid catabolizing enzymes (IDO1/2, TDO, Arg1/2 and IL4I1) as regulators of T cell activation and differentiation Castellano and Molinier-Frenkel.

In summary, this Research Topic brings together a number of interesting contributions, focused on the mechanisms by which the TCR/CD3 complex transduces intracellular signals, and some of the mechanisms that regulate them. Technical advances are allowing to deepen into the role of tyrosine kinases and phosphatases as regulators of TCR signaling, the function of costimulatory and coinhibitory receptors, the relevance of bioenergetics for T cell activation, and the importance of membrane dynamics and nanoscale organization of TCR associated molecules in the regulation of TCR signaling.

AUTHOR CONTRIBUTIONS

EA wrote the first draft of the manuscript and updated the last version. EBC and AM corrected and completed the initial draft. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Increased Protein Stability and Interleukin-2 Production of a LAT^{G131D} Variant With Possible Implications for T Cell Anergy

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The adaptor LAT plays a crucial role in the transduction of signals coming from the TCR/CD3 complex. Phosphorylation of some of its tyrosines generates recruitment sites for other cytosolic signaling molecules. Tyrosine 132 in human LAT is essential for PLC-γ activation and calcium influx generation. It has been recently reported that a conserved glycine residue preceding tyrosine 132 decreases its phosphorylation kinetics, which constitutes a mechanism for ligand discrimination. Here we confirm that a LAT mutant in which glycine 131 has been substituted by an aspartate (LAT^{G131D}) increases phosphorylation of Tyr132, PLC-γ activation and calcium influx generation. Interestingly, the LAT^{G131D} mutant has a slower protein turnover while being equally sensitive to Fas-mediated protein cleavage by caspases. Moreover, J.CaM2 cells expressing LAT^{G131D} secrete greater amounts of interleukin-2 (IL-2) in response to CD3/CD28 engagement. However, despite this increased IL-2 secretion, J.CaM2 cells expressing the LAT^{G131D} mutant are more sensitive to inhibition of IL-2 production by pre-treatment with anti-CD3, which points to a possible role of this residue in the generation of anergy. Our results suggest that the increased kinetics of LAT Tyr132 phosphorylation could contribute to the establishment of T cell anergy, and thus constitutes an earliest known intracellular event responsible for the induction of peripheral tolerance.

Keywords: LAT, TCR, phosphorylation, anergy, IL-2 (interleukin-2)

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INTRODUCTION

After the specific recognition of a peptide antigen bound to an MHC molecule on the surface of an Antigen Presenting Cell, a cascade of intracellular signaling events are triggered in T lymphocytes (Malissen and Bongrand, 2015; Alcover et al., 2018; Courtney et al., 2018). The transmembrane adaptor LAT (Linker for the Activation of T cells) constitutes a major ZAP-70 substrate, and initiates most of the intracellular events that characterize the T cell receptor (TCR) signaling pathway (Weber et al., 1998; Zhang et al., 1998). Early on after initial LAT cloning and characterization, experiments performed with the J.CaM2 LAT-deficient T cells analyzed the

contribution of some of the nine conserved LAT tyrosines to the TCR/CD3 signaling cascade (Finco et al., 1998; Zhang et al., 2000; Lin and Weiss, 2001; Paz et al., 2001). Those early works showed that the three most distal tyrosines (171, 191 and 226 in the human form of LAT) are necessary for Grb2-SOS and Gads-SLP76 binding and Erk activation and that the sixth tyrosine residue (132 in human LAT) is essential for PLC-γ1 binding and activation and calcium influx generation. Moreover, the phenotype of LAT-knockout (KO) mice revealed the essential role of this molecule for the transduction of intracellular signals emanating from the pre-TCR, since thymic development was completely blocked at the CD4-CD8- Double Negative (DN) stage (Zhang et al., 1999).

Therefore, TCR engagement triggers the assembly of a LAT signalosome linking the TCR to activatory signaling pathways that govern T-cell development and activation (Malissen et al., 2005). However, two LAT-knockin (KI) strains of mice harboring point mutations in the four most distal tyrosines developed lymphoproliferative disorders involving helper *T* (T_H) cells (Aguado et al., 2002; Sommers et al., 2002; Nunez-Cruz et al., 2003). The analysis of those mice strains revealed for the first time that the LAT adaptor acts not only as a transducer of activation signals, but also constitutes a negative regulator of TCR signaling and T-cell homeostasis. One of these strains of mice had a Tyr to Phe mutation in tyrosine 136 of LAT (mouse ortholog of human tyrosine 132, LAT^{Y136F} KI), and presented a paradoxical phenotype with a lymphoproliferative disorder of polyclonal CD4 T cells along with high Th2 cytokine production, despite a reduction in thymic development (Aguado et al., 2002; Sommers et al., 2002). The phenotype of these mice constituted the first evidence of a special inhibitory function for LAT, mainly played by the sixth tyrosine residue.

The order of signaling events taking place after TCR engagement is critical for productive immune responses. Lck initiates intracellular signaling in T cells by phosphorylating CD3 and ζ-chain ITAMs, leading to ZAP-70 recruitment and activation (Chan et al., 1991; Wange et al., 1993; Iwashima et al., 1994). Once activated ZAP-70, and not Lck, phosphorylates LAT and SLP-76 tyrosines by utilizing an electrostatic mechanism that favors phosphorylation of tyrosines surrounded by negatively charged residues, and excludes phosphorylation of tyrosines close to positively charged amino acids (Shah et al., 2016, 2018). Tyrosine residues 127, 171, 191, and 226 of human LAT are all preceded by aspartic or glutamic acid residues, and, as shown by means of an approach using bacterial surface-display, cell sorting, and deep sequencing, these tyrosines are efficiently phosphorylated by ZAP-70. However, tyrosine 132 of LAT is preceded by a well-conserved glycine residue, which should preclude its efficient phosphorylation, and so PLC-γ activation, calcium influx generation, and, ultimately, the activation of T lymphocytes. Indeed, in agreement with such a hindrance, in both Jurkat cells and primary human T cells, the kinetics of tyrosine 132 phosphorylation of LAT is much slower than the one of tyrosine 191 (Houtman et al., 2005). This is bewildering, given the essential role of tyrosine 132 phosphorylation for complete T cells activation (Zhang et al., 2000; Lin and Weiss, 2001; Paz et al., 2001; Aguado et al., 2002; Sommers et al., 2002). Recent work has shown that glycine 131 mutation by an aspartate residue in LAT increases LAT-Y132 phosphorylation, but not the one of tyrosines 171, 191 or 226 (Lo et al., 2019). Moreover, more distal signaling events are also increased in cells expressing the LAT^{G131D} mutant, for example, PLC-γ phosphorylation, Ca^{2+} influx, Erk phosphorylation or CD69 expression. Interestingly, Lo et al. demonstrate in this work that cells expressing the LAT^{G131D} mutant responded with greater intensity to lower anti-CD3 concentrations than did wild-type cells, and the same behavior was observed for low-affinity ligands in Jurkat cells expressing the OTI TCR, or primary T cells from a mouse strain expressing a floxed *Lat* allele, which allowed authors to delete endogenous LAT expression and express wild-type LAT or a LAT^{G131D} mutant. Lentiviral expression in mouse primary cells of a LAT^{G131D} mutant also increased the production of IFN-γ, which constitutes a piece of evidence that the brake imposed by Gly 131 has effects in the final activation of T lymphocytes. However, Weiss and collaborators did not analyze the production of IL-2 in either Jurkat cells or primary cells. This is of relevance since the increase in calcium responses shown by cells expressing LAT^{G131D} may induce a greater production of this cytokine.

In the present report, we analyze the effects of expressing a LAT^{G131D} mutant in the J.CaM2 LAT deficient cell line. We verify the findings of Lo et al., showing that this LAT mutant induces increased tyrosine phosphorylation of LAT specifically at residue 132, increased phosphorylation of PLC-γ and Ca^{2+} responses after CD3 stimulation. Moreover, we observe an increase in LAT protein stability, despite normal Fas-mediated cleavage, and augmentation of IL-2 production after CD3/CD28 cross-linking. Interestingly, J.CaM2 cells expressing the LAT^{G131D} mutant are more sensitive to inhibition of IL-2 production by pre-treatment with anti-CD3, which points to a possible role of this residue in the generation of anergy.

METHOD

Antibodies and Reagents

The anti-Fas (IgM) antibody was from Merck-Millipore; anti-LAT LAT-01 mAb was from EXBIO (Praha, Czech Republic); anti-LAT 11B.12, anti-PLC- γ, anti-PTP1B, and anti-caspase-3 monoclonal antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany); antibodies binding phospho-Erk, β-actin, phospho-PLC-γ1-Tyr783 and phospho-LAT-Tyr171 were from Cell Signaling Technology; anti-6His-HRP antibody and anti-phospho-LAT-Tyr132 were from Abcam (Cambridge, United Kingdom). The protein synthesis inhibitor cycloheximide was purchased from Merck-Millipore. Stimulations were performed with the anti-human CD3 OKT3 monoclonal antibody (eBioscience).

Enzyme−Linked Immunosorbent Assay (ELISA) and Anergy Induction

IL-2 release from lentivirally transduced J.CaM2 cells was measured by human IL−2 ELISA set (MAX Standard, Biolegend, Fell, Germany), using 96−well Nunc MaxiSorp microtiter plates. Supernatants of resting or activated cells were analyzed in

comparison to a standard curve of IL-2. Absorbance was determined using a Synergy MX Multi-Mode Reader (Biotek, Bad Friedrichshall, Germany) set to 405 and 450 nm. For anergy induction, 24 well plates were coated overnight at 4°C with 10, 2, 0.5, or 0.2 μg/ml OKT3 mAb in Tris 0.1 M buffer (pH 8.2; 200 μl/well). After washing three times with phosphate−buffered saline (PBS), cells expressing wild-type or the mutant form of LAT were incubated overnight and then stimulated in 96 well plates with anti-CD3/CD28 microbeads (at a bead-to-cell ratio of 3:1) for 48 h. Supernatants were analyzed by the human IL−2 ELISA set.

Cell Culture

The LAT-deficient J.CaM2 cell line was generously provided by Dr. Arthur Weiss, University of California, San Francisco (CA, United States). Cells were grown in complete RPMI 1640 medium (Lonza) supplemented with 10% FCS (Lonza) and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 10% CO^2.

Mutagenesis and Lentiviral Transduction

LAT cDNA cloning was performed as previously described (Garcia-Blesa et al., 2013). Site-directed mutagenesis was performed to change the sequence coding for glycine 131. Coding sequences in the plasmids were verified by sequencing and then subcloned in frame with GFP in the SIN lentiviral transfer plasmid pHR'SINcPPT-Blast through site-specific recombination (Gateway LR Clonase, Invitrogen). Lentiviral supernatants were generated as previously described (Garcia-Blesa et al., 2013) and used to induce expression of WT-LAT or the LAT-NIL mutant in J.CaM2 cells. Blasticidin selection (20 μg/ml) was applied to transduced cells after 72 h of culture, and the expression of GFP was analyzed using FACS analysis (CytoFLEX, Beckman Coulter).

Preparation of Cell Lysates and Western Blotting

Lentivirally transduced J.CaM2 cells were starved in RPMI 1640 without FCS for 18 h before being stimulated with anti-CD3 mAb at 37°C. Cells were then lysed at 2.0×10^7 cells/ml in 2X Laemmli buffer, followed by incubation at 99°C for 5 min and sonication. For anti-Fas stimulation, cells were incubated with 100 ng/ml of anti-Fas mAb at 1×10^6 cells/ml in RPMI 1640, supplemented with 10% FCS, and then pelleted and lysed as described above. For Western blotting, whole-cell lysates were separated by SDS-PAGE and transferred to PVDF membranes, which were incubated with the indicated primary antibodies, followed by the appropriate secondary antibody conjugated to IRDye 800CW (Li-Cor, Lincoln, NE, United States) or horseradish peroxidase (HRP). Reactive proteins were visualized using the Odyssey CLx Infrared Imaging System (Li-Cor) or by enhanced chemiluminescence (ECL) acquired in a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). For reprobing, PVDF membranes were incubated for 10 min at room temperature with WB Stripping Solution (Nacalai Tesque, Kyoto, Japan), followed by a TTBS wash. For the cycloheximide chase assay, cells were treated with 0.1 mM cycloheximide for up to 10 h. At the indicated time points, cell samples were obtained and lysed in 2X Laemmli buffer, and LAT protein levels were determined by immunoblotting and quantified by densitometry.

Ca^{2+} Mobilization

Measurement of intracellular free Ca^{2+} was carried out using Indo-1 AM (acetoxymethyl) (2 μM; Molecular Probes, Invitrogen) as previously described (Garcia-Blesa et al., 2013). Calcium measurements were performed using a Synergy MX Multi-Mode Reader (Biotek) at 37°C. Cells were excited by light at a wavelength of 340 nm, and the fluorescence emitted at 405 and 485 nm was collected alternately per second. Calcium mobilization was evaluated by the ratio of 405/485 nm fluorescence signal.

Statistical Analysis

Western blots were densitometrically quantified, and statistics were performed with Microsoft Excel using a two-tailed *t*-test. Levels of significance $p < 0.05$ are presented as *.

RESULTS AND DISCUSSION

Generation of Lentiviral Transfectants of J. CaM2 Cells Expressing Wild-Type LAT and the LAT^{G131D} Mutant

To study the role of the conserved glycine residue preceding tyrosine 132 in LAT we generated lentiviral plasmids to express wild-type LAT or a LAT^{G131D} mutant in J.CaM2 cells, as previously described (Arbulo-Echevarria et al., 2016, 2018). This would allow us to verify the effects observed by Lo et al. (2019). in Jurkat cells in which CRISPR was performed to eliminate the *Lat* gene in both chromosomes, and also address other questions of interest. We designed lentiviral vectors containing the coding region of wild-type or the mutant LAT fused to a 6-His tag, followed by an IRES sequence and a GFP reporter (**Supplementary Figure S1A**). Lentiviral supernatants were generated and used for infection of J.CaM2 cells obtaining transfection levels always greater of 75% of cells, as measured by GFP expression (**Supplementary Figure S1B**). GFP levels were always similar in cells expressing wild-type LAT and the LAT^{G131D} mutant, although sometimes levels of GFP were slightly higher in J.CaM2 cells expressing wild-type LAT. To assure that any possible difference in the responses observed in cells expressing WT-LAT and LAT^{G131D} mutant was not due to differential expression of the TCR/CD3 complex, we analyzed by flow cytometry CD3 expression. As shown in **Supplementary Figure S1B**, CD3 expression was indistinguishable in J.CaM2 cells expressing WT-LAT and the LAT^{G131D} mutant. Next, to determine LAT levels in lentivirally transduced cells, Western blot analysis was performed with non-transduced JCaM2 cells or expressing WT-LAT or LAT^{G131D} mutant. As it can be seen in **Supplementary Figure S1C**, Western blot performed with the mAb LAT01 did not detect LAT expression in J.CaM2 cells transduced with the LAT^{G131D} mutant (**Supplementary Figure S1C**, left panel). This result was unexpected since these

cells had similar GFP expression levels than WT-LAT expressing cells. Western blots performed with an anti-6His mAb showed bands of similar intensity in both WT-LAT and LAT^{G131D} transduced cells (**Supplementary Figure S1C**, middle panel), supporting that the LAT^{G131D} mutant was expressed at similar levels than WT-LAT but was not recognized by the LAT01 mAb. Indeed, a different mAb (11B.12, **Supplementary Figure S1C**, right panel) showed similar reactivity in both types of cells. Therefore, it seems that the Gly to Asp amino acid substitution performed in the LAT^{G131D} mutant affects the epitope recognized by LAT01 mAb, and may constitute a way to differentiate wild-type LAT and the LAT^{G131D} mutant.

Mutation of Gly 131 to Asp of LAT Increases Intracellular Signaling

To verify whether the mutation of glycine 131 to aspartate reproduced the effects observed by Lo et al. (2019), we performed anti-CD3 stimulations in WT-LAT and LAT^{G131D} expressing J.CaM2 cells. As previously reported, the substitution of glycine at position 131 with an aspartate residue increased the kinetics and intensity of phosphorylation of LAT tyrosine 132, since phosphorylation 3 and 10 min after CD3 treatment induced an statistically significant increase of phosphorylation (**Figure 1A**). However, phosphorylation of tyrosine 171 was similar in both WT-LAT and LAT^{G131D} expressing J.CaM2 cells, confirming the specificity of the negative effect of the glycine residue in the phosphorylation of LAT Y132 (**Supplementary Figure S2A**).

Next, given the specific increase in phosphorylation of LAT Y132 after CD3 stimulation, we wondered if this would transduce enhanced downstream signals. Therefore, we analyzed the effect of replacing G131 by an aspartate residue on PLC-γ1 activation, which can be monitored by the phosphorylation of its tyrosine residue 783 (Wang et al., 1998). As can be seen in **Supplementary Figure S2B**, anti-CD3 treatment induced increased phosphorylation of PLC-γ1 in LAT^{G131D} expressing J.CaM2 cells at all the time points analyzed, supporting the view that the increased kinetics of LAT Y132 phosphorylation is enough to induce augmented downstream signals. Next we analyzed whether the mutation of G131 of LAT affected calcium influx generation. Indo-1AM labeled cells were stimulated with 1 μg/ml OKT3 mAb, and Ca^{2+} influx was analyzed. Interestingly, even at high doses of anti-CD3 (1 μg/ml) the calcium response in J.CaM2 cells expressing the LAT^{G131D} mutant was slightly higher than the one observed in WT-LAT expressing cells (**Figure 1B**). We also performed experiments with lower doses of OKT3 mAb, to corroborate previous results showing the difference in Ca^{2+} responses between T cells expressing LAT^{G131D} and WT-LAT were greater at low doses of the antibody. As it can be seen in **Supplementary Figure S2C**, stimulation with 0.5 μg/ml and 0.12 μg/ml of OKT3 showed greater differences between WT-LAT and LAT^{G131D} expressing cells. Therefore, our results confirm that glycine 131 acts as a negative regulator of LAT Y132 phosphorylation, and thus of TCR signaling.

To validate the negative role of G131 residue of LAT, we also analyzed Erk phosphorylation in WT-LAT and LAT^{G131D} expressing cells (**Figure 1C**). Again, mutation of glycine 131 to aspartate induced increased kinetics and intensity of Erk phosphorylation, with statistical significance at the 3 min time point (**Figure 1C**, lower diagram). Altogether, these data confirm that the LAT^{G131D} mutation releases the brake imposed on the TCR/CD3 signaling cassette, as previously observed by Lo et al. (2019).

Impact of Glycine 131 Substitution on LAT Cleavage and Protein Stability

Our group has previously demonstrated that LAT undergoes a proteolytic cleavage in T cells receiving proapoptotic stimuli (Garcia-Blesa et al., 2013; Klossowicz et al., 2013). Given that glycine in position 131 is close to one of the described cleavage points in LAT (aspartate 126), we decided to verify if the substitution of G131 by an aspartic acid residue modifies Fas-dependent LAT cleavage. Therefore, we treated lentivirally transduced J.CaM2 cells with an anti-Fas antibody for 4 h at 37°C. As it can be seen in **Figure 2A**, cleavage of both WT-LAT and LAT^{G131D} mutant generated two proteolytic fragments of the same electrophoretic mobility and similar intensity. Therefore, these results show that the LAT^{G131D} mutant is equally sensitive to Fas-mediated proteolytic cleavage as WT-LAT.

We have previously shown that a functional isoform of LAT originated from an intron six retention event, and that can be detected in human and other mammalian species at the RNA level, shows a shorter half-life than the canonical LAT isoform (Klossowicz et al., 2014). More recently we have described that mutation of a stretch of negatively charged residues, encoded by exon seven of human LAT, also affects LAT stability, since the substitution of this fragment with a stretch of non-charged amino acids significantly decreases LAT stability (Arbulo-Echevarria et al., 2018). Given that those LAT sequence modifications affected residues from position 113 to 126, we wondered if the substitution of glycine in position 131 of LAT would modify LAT stability. Consequently, we cultured J.CaM2 cells expressing WT-LAT or LAT^{G131D} in the presence of the translational inhibitor cycloheximide, and then cells were collected at specific time points and lysed. Surprisingly, Western blot analysis showed that the LAT^{G131D} mutant is degraded with slower kinetics than WT-LAT (**Figure 2B**, upper panel). Densitometric analysis of five independent experiments showed that LAT^{G131D} has increased stability in comparison with WT-LAT, with statistical significance after 4 h of cycloheximide treatment (**Figure 2B**, lower panel). This observation is in agreement with our previous reports about the role of this region on LAT turnover and could be, at least in part, responsible for the increase in downstream TCR-dependent signals. Overall, these results show that glycine 131 negatively influences the stability of LAT but it has no bearing on its sensitivity to Fas-mediated proteolytic cleavage.

Cells Expressing the LAT^{G131D} Mutant Produce Greater Amounts of IL-2 After CD3/CD28 Stimulation

Lo et al. (2019) have shown that mouse T cells lentivirally expressing a LAT mutant in which glycine 135 (mouse ortholog

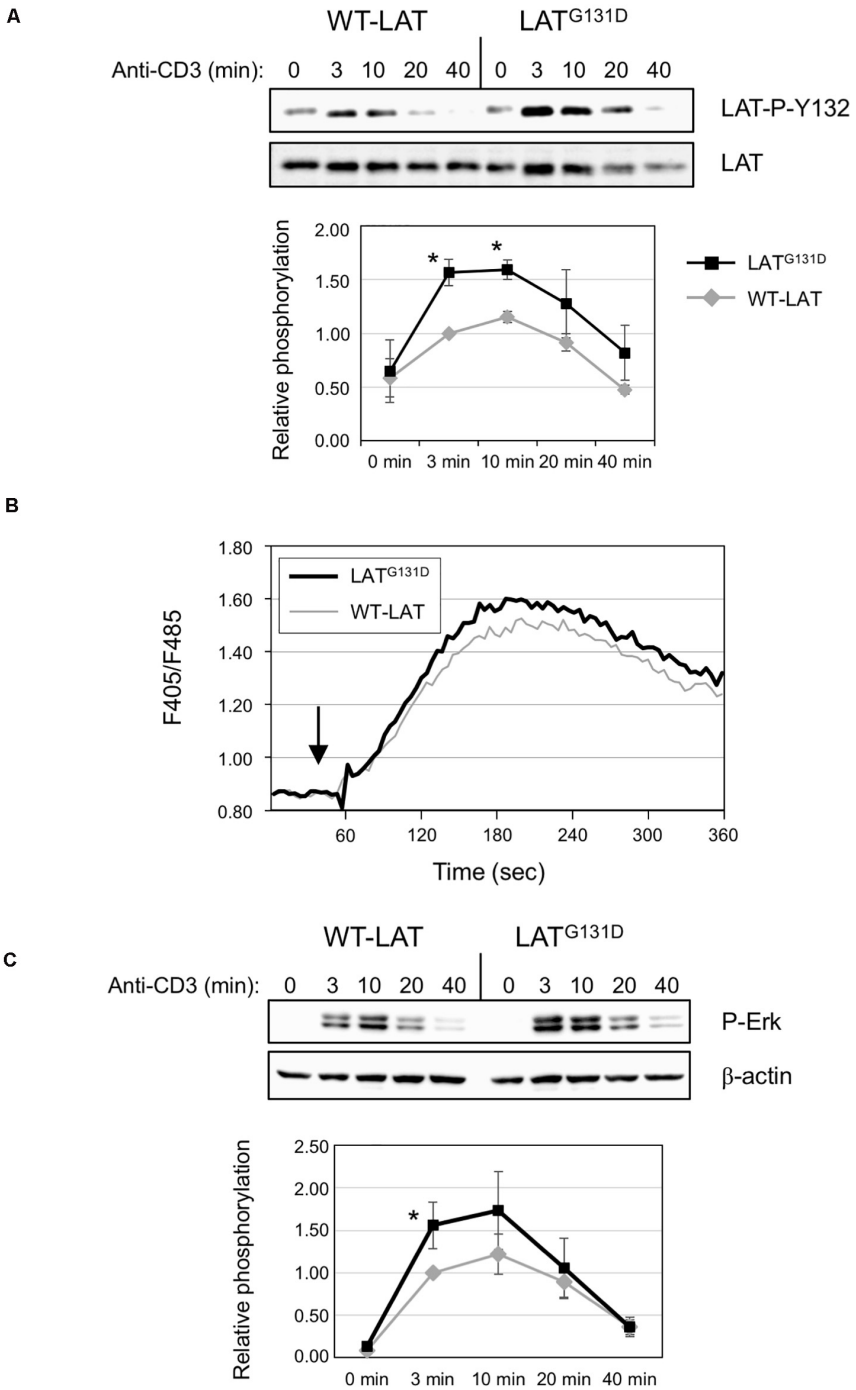

FIGURE 1 | Mutation of Gly 131 to Asp increases TCR-dependent intracellular signaling. **(A)** Immunoblots analyzing phosphorylation of LAT at tyrosine residue 132 in cells stimulated with soluble anti-CD3 were done with the phospho-specific antibody. Membranes were stripped and blotted with anti-LAT antibody (middle panel). The lower diagram represents the mean fold increase in phosphorylation in four independent experiments using J.CaM2 cells expressing WT-LAT (gray line) or the LAT^{G131D} mutant (black line). Phosphorylation levels were normalized to total LAT expression. Bars represent the standard error. The asterisk represents statistical significance. **(B)** J.CaM2 cells expressing WT-LAT or the LAT^{G131D} mutant were loaded with Indo-1AM and stimulated with OKT3 mAb (1 μg/ml) at the indicated time (black arrow). The intracellular Ca^{2+} concentration was determined at 37°C through the change in Indo-1AM fluorescence. The graphic represents the average of five experiments. **(C)** Whole-cell lysates were probed by Western blotting for the activation of Erk by using a mAb recognizing doubly phosphorylated on specific threonine and tyrosine residues on Erk (upper panel). Stripped membranes were blotted with anti-β-actin mAb to show equal protein expression (middle panel). Lower diagram represents the mean fold increase in Erk phosphorylation in four independent experiments using J.CaM2 cells expressing WT-LAT (gray line) or the LAT^{G131D} mutant (black line). Phosphorylation levels were normalized to β-actin expression. Bars represent the standard error. Asterisk represents statistical significance.

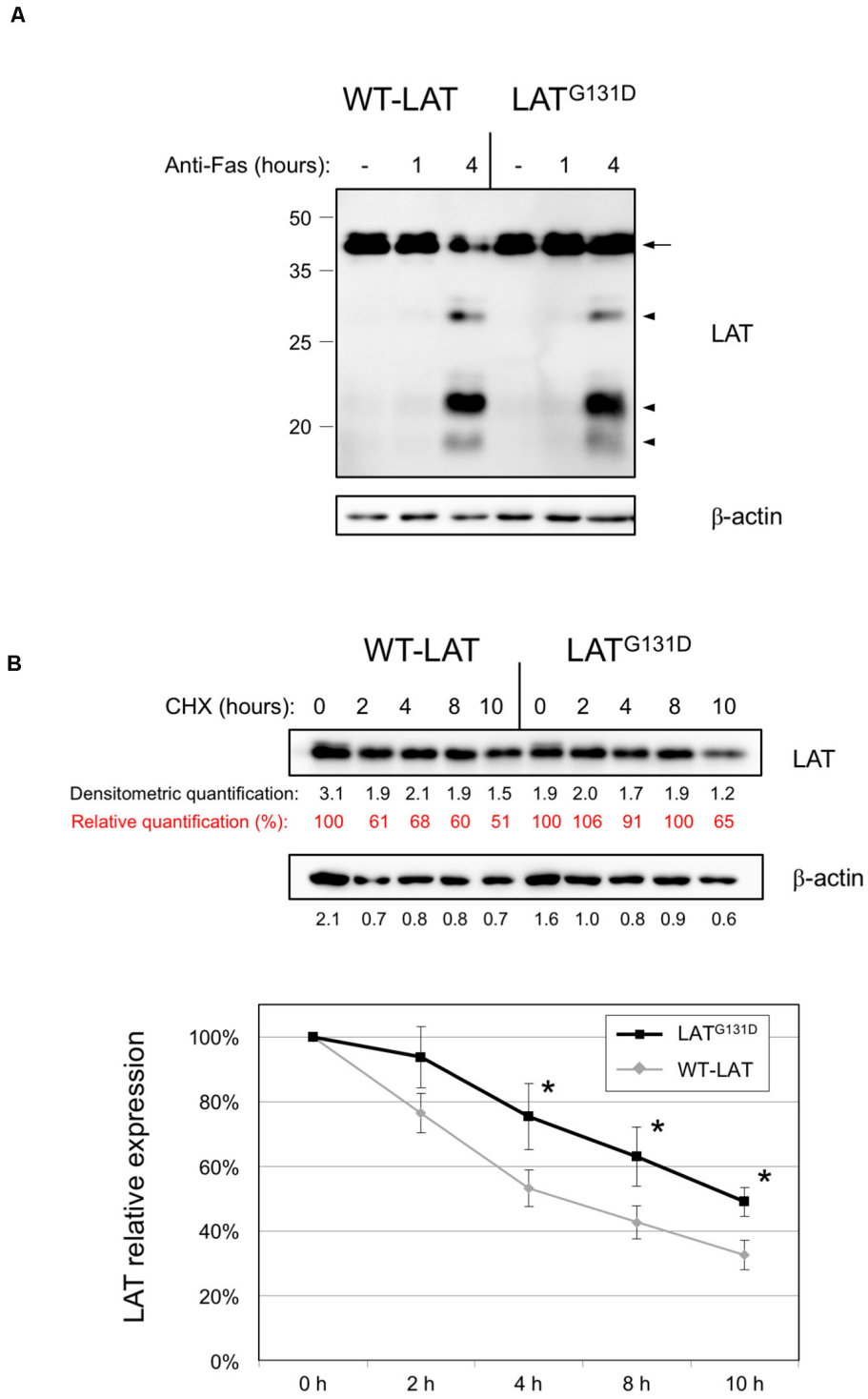

FIGURE 2 | Degradation of WT-LAT and LAT^{G131D} mutant proteins. **(A)** J.CaM2 cells expressing WT-LAT or the LAT^{G131D} mutant were treated for the indicated time in hours at 37°C with 100 ng/ml of anti-Fas antibody, and LAT cleavage was assessed in total cell lysates by Western blot with an anti-LAT antibody. Molecular masses in kDa are indicated adjacent to the Western blot. Arrow indicates the band corresponding to the whole LAT molecule, and arrowheads are indicative of the proteolytic fragments. **(B)** Cell lysates obtained from J.CaM2 cells expressing WT-LAT or the LAT^{G131D} mutant, previously treated with cycloheximide (CHX) for the indicated time in hours, and LAT (upper panel) or β-actin (middle panel) protein levels were analyzed by Western blot. Black numbers below each panel represent the quantification of corresponding bands. Red numbers below LAT panel represent the percentage of LAT expression relative to the one at 0 h of cycloheximide treatment. Densitometric analysis of six experiments was performed, and the relative expression of LAT was represented (lower panel). Bars represent the standard error. Asterisks represent statistical significance.

of human G131) had been substituted by an aspartate residue (LAT^{G135D} mutant) have a lower reactivity threshold to allow for IFN-γ production, and show augmented percentages of IFN-γ secreting cells. However, it remains to be determined the effect of this mutation on interleukin-2 (IL-2) secretion. IL-2 is an essential cytokine that allows controlling the differentiation and homeostasis of both pro- and anti-inflammatory T cells (Ross and Cantrell, 2018). Therefore, we cultured J.CaM2 cells expressing wild-type LAT or the LAT^{G131D} mutant for 24 and 48 h with anti-CD3/anti-CD28 beads. Supernatants were analyzed by ELISA, and the amount of IL-2 in supernatants from WT-LAT expressing cells after 24 h of CD3/CD28 stimulation was 15 ± 7 pg/ml (**Figure 3**), which constitutes a similar level of IL-2 production in stimulated J.CaM2 cells expressing LAT (Klossowicz et al., 2014). Interestingly, supernatants from J.CaM2 cells expressing the LAT^{G131D} mutant stimulated for 24 h contained increased levels of IL-2 levels with regard to WT-LAT expressing cells (37 ± 17 pg/ml, **Figure 3**), although this difference did not reach statistical significance, probably as the result of the low number of performed experiments (n = 4). Interestingly, analysis of supernatants from cells stimulated for 48 h with anti-CD3/anti-CD28 microbeads showed greater amounts of secreted IL-2 in both types of cells, with a statistically significant difference between WT-LAT and LAT^{G131D} expressing cells (30 ± 4 vs 71 ± 14 pg/ml, respectively; **Figure 3**).

However, although it has been previously shown that there is no basal expression of LAT in J.CaM2 cells, we previously reported that activation of protein kinase C (PKC) with PMA induces LAT re-expression at both mRNA and protein levels (Marek-Bukowiec et al., 2016). To rule out that endogenous LAT was not expressed, which may confound the observed results, we decided to stimulate the cells during one night with two doses of anti-CD3 immobilized to plastic, and verify by Western blot LAT expression. The endogenous form is smaller than the transfected LAT forms. As shown in **Supplementary Figure S3**, treatment with 2 or 10 μg/ml of OKT3 did not induce expression of the endogenous form of LAT, discarding any effect of endogenous LAT expression. Therefore, these results support a role of glycine 131 of LAT as a "brake" controlling not only early intracellular signals coming from the TCR but also late activation events that take place in fully activated T cells.

Effect of the Gly 131 to Asp Mutation in LAT Adaptor on Anergy Induction

It has been previously demonstrated that the integration of calcium signals with activation of other signaling pathways results in full activation of T cells, while unopposed calcium signaling leads to anergy (Macian et al., 2002; Baine et al., 2009). Anergy is an essential mechanism of peripheral tolerance, established when the TCR is engaged in the absence of a CD28-mediated costimulatory signals. Evidence indicates that calcium signaling is responsible for the establishment of anergy in T cells. Given that J.CaM2 cells expressing the LAT^{G131D} mutant show increased PLC-γ activation and Ca^{2+} responses (Lo et al., 2019), it was of interest to study the relationship of this mutation with anergy. J.CaM2 or Jurkat cells are not the best models to analyze the anergy potential, because these cell lines proliferate continuously in a TCR independent way. However, we have shown that lentivirally transduced J.CaM2 cells can secrete IL-2 in response to CD3/CD28 stimulation, allowing us to study if anti-CD3 pretreatment has any effect on CD3/CD28-mediated IL-2 production. To do so, J.CaM2 cells lentivirally transduced to express WT-LAT or the LAT^{G131D} mutant were

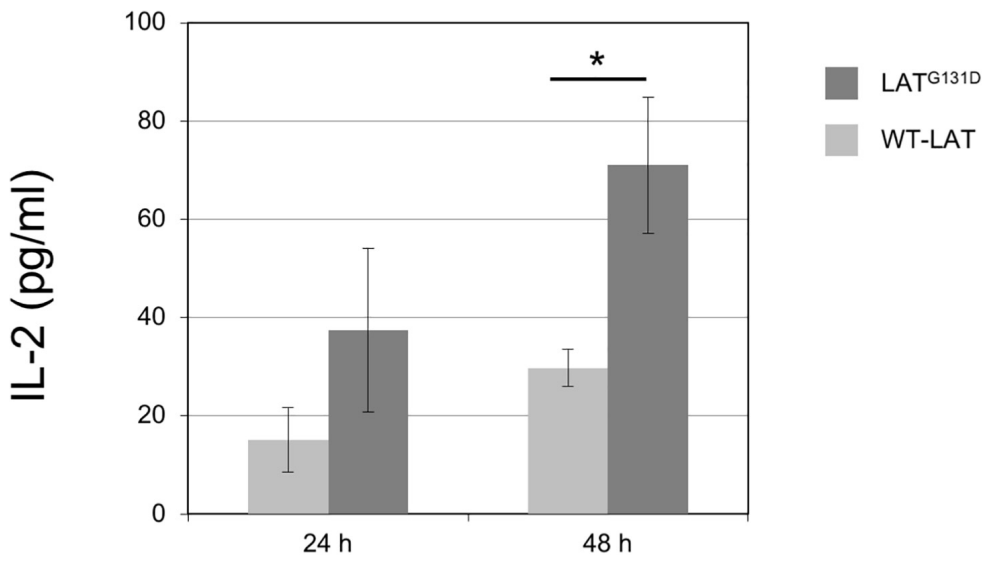

FIGURE 3 | Increased capacity of IL-2 production by J.CaM2 cells expressing the LAT^{G131D} mutant. J.CaM2 cells expressing human WT-LAT or the LAT^{G131D} mutant were stimulated with anti-CD3/CD28 microbeads for the indicated time in hours, and the amount of IL-2 in supernatants was measured by ELISA. The values presented are mean values of four (for 24 h incubation) or eleven (for 48 h) separate experiments. Bars represent the standard error. The asterisk represents statistical significance.

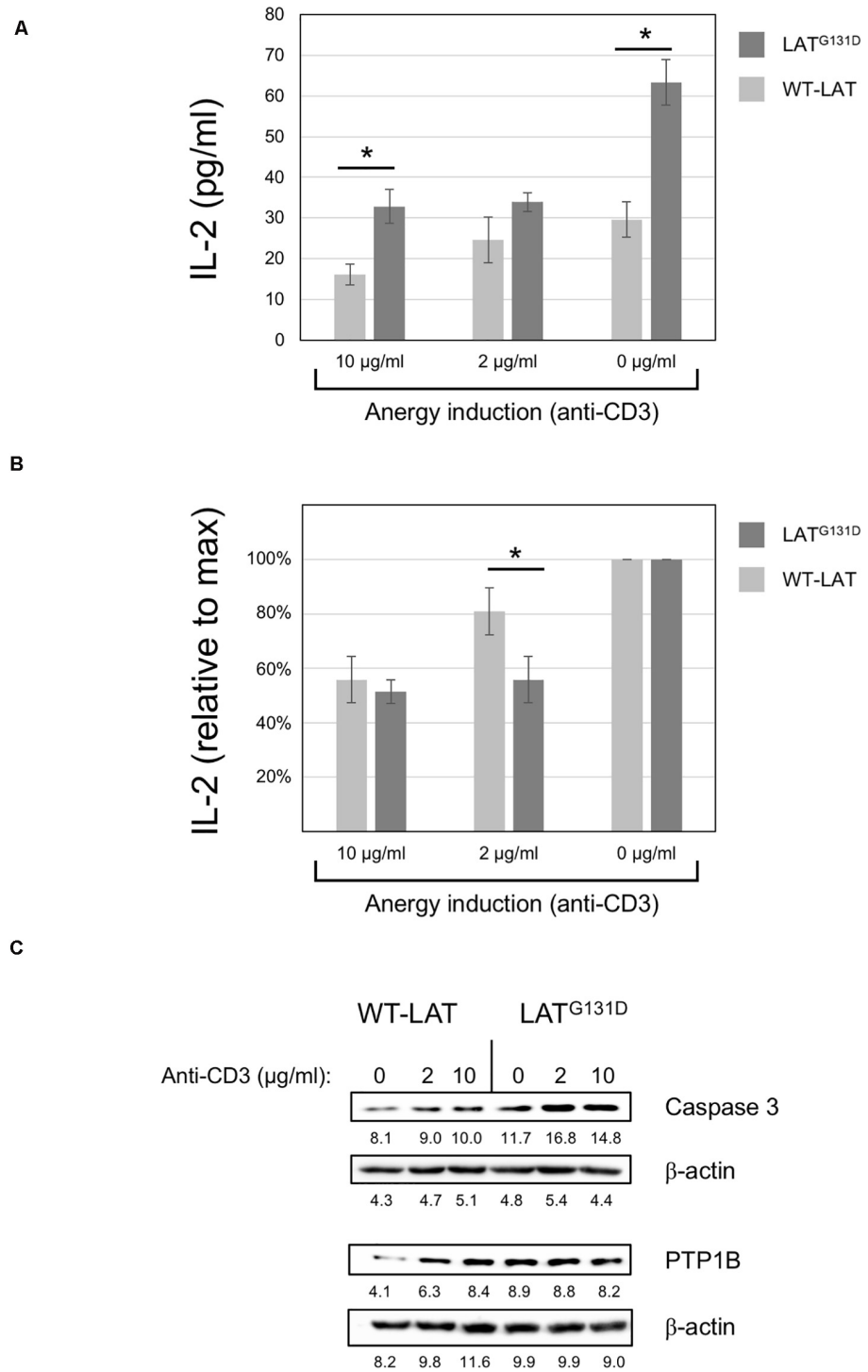

FIGURE 4 | Anergy induction in J.CaM2 cells expressing the LAT^{G131D} mutant. **(A)** Anergy was induced in J.CaM2 cells expressing human WT-LAT or the LAT^{G131D} mutant by culturing cells overnight on plates with the indicated doses of the immobilized anti-CD3 antibody. Recovered cells were then stimulated with anti-CD3/CD28 microbeads for 48, and the amount of IL-2 in supernatants was measured by ELISA. The values presented are the mean values of four independent experiments. Bars represent the standard error. **(B)** The relative effect of anti-CD3 treatment on IL-2 production in cells expressing WT-LAT or the LAT^{G131D} mutant. Maximal IL-2 production in cells treated without anti-CD3 mAb (0 µg/ml) for each type of cell was considered 100%, and the relative production of IL-2 was calculated for each cell type and condition. **(C)** Caspase 3 and PTP1B levels in cells treated overnight with the indicated doses of immobilized anti-CD3 antibody were analyzed by Western blot (upper panels). Stripped membranes were blotted with anti-β-actin mAb to show equal protein expression (lower panels). The numbers below each panel represent the quantification of corresponding bands. Representative images from one of the three experiments performed with similar results.

pretreated overnight with 2 or 10 μg/ml of immobilized anti-CD3 mAb, and then washed and stimulated with anti-CD3/CD28 microbeads for 48 h. IL-2 in the corresponding supernatants was then measured by ELISA. LAT^{G131D} expressing cells without anti-CD3 "anergizing" pretreatment (**Figure 4A**, 0 μg/ml of anti-CD3) produced 63 ± 6 pg/ml of IL-2, while WT-LAT expressing cells produced a statistically significant lower amount of IL-2 (30 ± 4 pg/ml). Overnight pretreatment of cells with 10 μg/ml of anti-CD3 reduced the amount of IL-2 produced by both types of cells by approximately half (16 ± 3 pg/ml for WT-LAT and 33 ± 4 pg/ml the LAT^{G131D}, **Figure 4A**). Interestingly, pretreatment of WT-LAT expressing cells with a lower concentration of anti-CD3 (2 μg/ml) induced a modest decrease on IL-2 production by WT-LAT expressing cells (from 30 ± 4 pg/ml to 25 ± 6 pg/ml, **Figure 4A**), while the same treatment in LAT^{G131D} expressing cells still reduced by approximately half IL-2 production (from 63 ± 6 pg/ml to 34 ± 2 pg/ml, **Figure 4A**). Data in **Figure 4A** were recalculated to show the relative effect on the maximal IL-2 production of anti-CD3 pretreatment. Therefore, IL-2 secretion by both cell types at 0 μg/ml of anti-CD3 pretreatment were considered 100 percent of IL-2, and the amounts of IL-2 at 10 and 2 μg/ml of anti-CD3 were recalculated. As it can be seen in **Figure 4B**, pretreatment with 2 μg/ml of anti-CD3 reduced the IL-2 production by LAT^{G131D} expressing cells from 100% to 54% ± 4, while the same treatment had a reduced effect on WT-LAT expressing cells (from 100 to 84% ± 19). Therefore, these data demonstrate that pretreatment of LAT^{G131D} expressing cells with anti-CD3 antibodies produces a relative reduction in IL-2 secretion significantly greater than in cells expressing WT-LAT. The up-regulation of genes coding for caspase 3 and the phosphatase PTP1B has been previously correlated with anergy (Dominguez-Villar et al., 2007). To confirm the prediction that LAT^{G131D} mutant could have a pro-anergic effect, we analyzed caspase 3 and PTP1B expression by Western blot in cell lysates obtained after overnight treatment with 2 and 10 μg/ml of anti-CD3. As it can be seen in **Figure 4C**, J.CaM2 cells expressing the LAT^{G131D} mutant show increased basal levels of both caspase 3 and PTP1B with regard to WT-LAT expressing cells. Moreover, anti-CD3 stimulation also induced higher caspase 3 levels in LAT^{G131D} than in WT-LAT expressing cells, which endorses the pro-anergic effect of the Gly to Asp mutation of the amino acid preceding Tyr132 in LAT.

Overall, our data support previous results recently published by Arthur Weiss and co-workers (Lo et al., 2019). We have verified that cells expressing the LAT^{G131D} mutant show a significant and specific increase in LAT-Y132 phosphorylation. As a consequence, PLC-γ activation, Ca^{2+} influx generation, and Erk phosphorylation are augmented in LAT^{G131D} expressing cells after CD3-mediated stimulation. Interestingly, we have shown that this mutation, which prevents the binding to LAT of the specific mAb LAT-01, increases the stability of this protein. The increased LAT protein stability provided by the G131D mutation is in line with our previous published data (Arbulo-Echevarria et al., 2018). We have analyzed the role of a negatively charged segment of amino acids in LAT, which is very close to Gly 131. The substitution of this segment with a sequence of non-charged residues significantly decreased LAT stability. Now, we have demonstrated that introducing a negatively charged residue increases LAT stability. This increase in LAT stability could be related, at least in part, to the observed increase in activation events triggered after TCR engagement in LAT^{G131D} expressing cells. More experiments should clarify whether this is related to the introduction of a negative charge or to the removal of the glycine residue. Last, we have shown that substitution of glycine 131 by an aspartate residue enhances IL-2 production. Lo et al. have previously shown that this mutation lowers the reactivity threshold to allow for an increased percentage of cells producing IFN-γ, with notable differences when cells were stimulated with low-affinity peptides (Lo et al., 2019). Here we demonstrate that full activation of J.CaM2 cells expressing LAT^{G131D} mutant with anti-CD3/CD28 microbeads induces statistically significant greater amount of secreted IL-2. Moreover, we have shown that these cells show an enhanced predisposition to CD3-mediated inhibition of IL-2 production, which could be related to anergy in primary cells. This could be of immunological relevance, since to our knowledge, this is the earliest signaling event described to be related to anergy. Although Jurkat and J.CaM2 cell lines are not the best models for anergy studies, until *in vivo* analysis can be performed, our approach is a straightforward attempt to predict if anergy is affected by mutation of Gly 131. Other groups have analyzed different aspects of anergy using Jurkat cells (Tzachanis et al., 2001; Howe et al., 2003; Sundstrom et al., 2005; Dominguez-Villar et al., 2007; Li et al., 2010; Hsu et al., 2014; Liu et al., 2016; Sanchez-Del Cojo et al., 2017; Wang et al., 2019), which gives support to our experimental approach. The greater relative inhibition of IL-2 secretion shown by J.CaM2 cells expressing the LAT^{G131D} mutant supports this hypothesis, as it does the increased levels of caspase 3 and PTP1B. Future analysis of anergy induction in knockin mice expressing the same mutant LAT isoform would be useful to confirm these data, and this would constitute an invaluable model with which to analyze the role of anergy in the maintenance of tolerance and its implications for autoimmune disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MA-E and EA designed the experiments. MA-E performed most of the experiments and interpreted results. IV-B and IN-S helped with cell culture, lentiviral transfections, and signaling experiments. MA-E, IV-B, FG-C, and AM provided input on study design and helped with manuscript writing. EA wrote the manuscript and directed the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.561503/full#supplementary-material

FIGURE S1 | Expression of WT-LAT and LAT^{G131D} in J.CaM2 cells. **(A)** Schematic representation of the lentiviral vectors used to express WT-LAT and the LAT^{G131D} mutant in J.CaM2 cells. **(B)** Histograms of GFP and CD3 expression in lentivirally transduced J.CaM2 cells expressing WT-LAT (green line) or LAT^{G131D} mutant (black line). Numbers in the histograms represent the percentage of positive cells. **(C)** Non transduced J.CaM2 and J.CaM2 cells expressing WT-LAT or the LAT^{G131D} mutant were lysed and LAT expression was analyzed by Western blot with the anti-LAT mAb LAT-01 (left upper panel), anti-6His (middle upper panel), and the 11B.12 anti-LAT mAb (right upper panel). Membranes were stripped and blotted with anti-β-actin mAb to show equal protein load (lower panels).

FIGURE S2 | Specific increase in TCR signaling in J.CaM2 cells expressing the LAT^{G131D} mutant. **(A)** Immunoblots analyzing phosphorylation of LAT at tyrosine residue 171 in cells stimulated with soluble anti-CD3 were done with phospho-specific antibody. Equal amounts of the same samples were run in parallel and analyzed for total LAT expression by Western blot (lower panel). Numbers below each panel represent quantification of corresponding bands. Representative images from one of the three experiments performed with similar results. **(B)** Western blot analysis of PLC-γ activation (upper panel). Membranes were stripped and blotted with anti-β-actin mAb to show equal protein load (lower panel). Numbers below each panel represent quantification of corresponding bands. Representative images from one of the three experiments performed with similar results. **(C)** J.CaM2 cells expressing WT-LAT or the LAT^{G131D} mutant were loaded with Indo-1AM and stimulated with the indicated concentrations of anti-CD3 mAb at the indicated time (black arrows). The intracellular Ca^{2+} concentration was determined at 37°C through the change in Indo-1AM fluorescence. Graphs represent the average of 3 and 5 experiments, for OKT3 concentrations of 0.5 and 0.125 μg, respectively.

FIGURE S3 | Stable expression of LAT after long-term CD3-stimulation. Immunoblots analyzing expression of LAT (upper panel) and β-actin (lower panel) in cells treated overnight with the indicated doses of immobilized anti-CD3 antibody. Molecular weights in kDa are indicated on the side of the upper panel.

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REVIEW
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Coreceptors and TCR Signaling – the Strong and the Weak of It

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The T-cell coreceptors CD4 and CD8 have well-characterized and essential roles in thymic development, but how they contribute to immune responses in the periphery is unclear. Coreceptors strengthen T-cell responses by many orders of magnitude – beyond a million-fold according to some estimates – but the mechanisms underlying these effects are still debated. T-cell receptor (TCR) triggering is initiated by the binding of the TCR to peptide-loaded major histocompatibility complex (pMHC) molecules on the surfaces of other cells. CD4 and CD8 are the only T-cell proteins that bind to the same pMHC ligand as the TCR, and can directly associate with the TCR-phosphorylating kinase Lck. At least three mechanisms have been proposed to explain how coreceptors so profoundly amplify TCR signaling: (1) the Lck recruitment model and (2) the pseudodimer model, both invoked to explain receptor triggering *per se*, and (3) two-step coreceptor recruitment to partially triggered TCRs leading to signal amplification. More recently it has been suggested that, in addition to initiating or augmenting TCR signaling, coreceptors effect antigen discrimination. But how can any of this be reconciled with TCR signaling occurring in the absence of CD4 or CD8, and with their interactions with pMHC being among the weakest specific protein-protein interactions ever described? Here, we review each theory of coreceptor function in light of the latest structural, biochemical, and functional data. We conclude that the oldest ideas are probably still the best, i.e., that their weak binding to MHC proteins and efficient association with Lck allow coreceptors to amplify weak incipient triggering of the TCR, without comprising TCR specificity.

Keywords: CD4, CD8, T-cell signaling, TCR triggering, antigen discrimination

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INTRODUCTION

Adaptive immune responses are initiated by T cells which continually patrol lymphoid and peripheral tissues for peptide, lipid or metabolite-derived antigens. Conventional T cells are activated through the binding of their αβ T-cell receptors (TCRs) to peptide-loaded major histocompatibility complex (pMHC) molecules on the surfaces of other cells. T-cell activation then leads to clonal expansion and the deployment of a battery of effector functions. T cells with distinct "helper" or "cytotoxic" activities were described as early as the 1960s and 70s (Bach et al., 1976). It was quickly established that these subsets could be distinguished by the mutually exclusive

expression of just two cell surface markers, CD4 and CD8: $CD4^+$ T cells aided antibody-producing B cells (Cantor and Boyse, 1977) while $CD8^+$ T cells directly killed infected targets (Shiku et al., 1975). However, experiments with T cell clones and blocking antibodies by Swain and others showed that expression of these markers did not correlate fully with effector function (Swain, 1983). Instead, it appeared that they had a more exclusive role in determining which class of MHC molecule was being recognized. CD4 and CD8 were first referred to as "coreceptors" by Janeway (1988), distinguishing them from simple "accessory molecules" based on emerging evidence that they physically associated with the TCR complex during T-cell activation, and in recognition of their especially large effects on T-cell responses. Parnes et al. (1989) subsequently confirmed that CD4 and CD8 bind to MHC class II (MHC-II) and MHC class I (MHC-I), respectively. The discovery that CD4 and CD8 are both associated with the TCR-phosphorylating Src-family kinase Lck further heightened their special status (Veillette et al., 1988, 1989; Barber et al., 1989; Rudd et al., 1989; Zamoyska et al., 1989).

Coreceptors are known to have important roles in driving the thymic development of $CD4^+$ or $CD8^+$ T cells, in effect by signaling to thymocytes depending on whether their TCRs bind to MHC-II or MHC-I, respectively (Tikhonova et al., 2012). Precisely how they contribute to the functions of peripheral T cells is still debated, however. TCR phosphorylation (i.e., triggering) can be induced by high-affinity ligands in the absence of coreceptors, but CD4 and CD8 significantly augment antigen sensitivity and are essential for responding to some ligands (Janeway et al., 1988; Hampl et al., 1997; Holler and Kranz, 2003). Early proposals were that coreceptors either initiated signaling by recruiting Lck to the TCR (Rudd, 1990; Janeway, 1992) or amplified signaling by stabilizing the ternary complex (Xu and Littman, 1993). Another possibility was that coreceptors cross-link TCR-pMHC complexes to produce receptor dimers (Irvine et al., 2002). In addition to these direct effects on receptor signaling, serial "scanning" for the small subset of coreceptors that are stably associated with Lck has been invoked as a form of kinetic proofreading (Stepanek et al., 2014).

In this review, we start by providing a context for how coreceptors work by discussing how T cells come into contact with antigen and how this leads to intracellular signaling. We then discuss new insights into the structure and behavior of CD4 and CD8 and consider the present status of each of the models of coreceptor function. Finally, we consider the roles of CD4 and CD8 in thymic development and antigen discrimination.

T-CELL ACTIVATION

Microvilli and Microclusters

T cells need to approach antigen-presenting cells (APCs) within a distance of ∼15 nm for TCRs and coreceptors to interact with pMHC. This presents a challenge for two main reasons. First, T cells are highly motile lymphocytes that form only transient contacts with APCs (Miller et al., 2002; Mandl et al., 2012; Cai et al., 2017). Second, leukocyte surfaces are covered in a dense glycocalyx barrier which sterically hinders the formation of close cell-cell contacts (Springer, 1990). However, it is becoming clear that both thymocytes and T cells interact with neighboring cells using numerous small, febrile membrane projections called filopodia or microvilli (**Figure 1A**; Majstoravich et al., 2004), potentially in order to overcome these obstacles. The flexibility and dynamics of these F-actin-enriched structures seem well-suited to extensive and rapid exploration of the surfaces of other cells in the search for antigens (Cai et al., 2017).

The recognition of a cognate TCR ligand leads TCR-coreceptor-pMHC interactions to initiate inside-out signaling to integrin-family adhesion molecules, resulting in a dramatic increase in the contact area. Ligand-engaged TCRs nucleate submicron regions called microclusters where cytosolic signaling proteins also accumulate (Bunnell et al., 2002; Campi et al., 2005). Sustained TCR signaling leads to large scale re-organization of TCR-pMHC and adhesive interactions into a radially symmetric structure called the immunological synapse (Grakoui et al., 1999), which arrests cell motility and allows for the delivery of effector functions (**Figure 1B**). Immunofluorescence imaging of T cells interacting with B cells or planar antigen-presenting substrates showed how this synaptic interface takes on a characteristic "bull's-eye" pattern of concentric rings referred to as supramolecular activation clusters (SMACs) (Monks et al., 1998). The canonical synapse consisted of three SMACs: the central (c)SMAC containing TCR/pMHC clusters, the peripheral (p)SMAC comprising adhesive LFA-1/ICAM-1 interactions, and the distal (d)SMAC marked by the presence of the large tyrosine phosphatase CD45 (Dustin et al., 1998; Monks et al., 1998; Freiberg et al., 2002).

This early view of the synapse has since been refined with advances in imaging technology revealing much about the complexity and dynamics of synapse formation. One principle that has emerged is that SMACs align with the different morphologies of the underlying F-actin networks; the dSMAC corresponds to a lamellipodium, the pSMAC to contractile lamella and the cSMAC to an F-actin-depleted secretory domain (Stinchcombe et al., 2001; Kaizuka et al., 2007; Yi et al., 2012; Fritzsche et al., 2017). Total internal reflection fluorescence microscopy, which selectively illuminates fluorophores close to the basal surface, has demonstrated that CD45 is relatively uniformly distributed across the synapse rather than being concentrated in the dSMAC as previously thought (Varma et al., 2006). The dSMAC also contains a substructure called the "corolla" which consists of sub-micron petal-like clusters of the CD2-CD58 adhesion pair and influences the distribution of important stimulatory/inhibitory molecules like CD28 and PD-1 (Demetriou et al., 2019). While TCR signaling was originally thought to be sustained in the cSMAC, it is now known to occur mainly in the dSMAC. TCRs are monovalent in their "resting" state, implying that the earliest signaling events are likely driven by this form of the complex (Brameshuber et al., 2018; Rossboth et al., 2018). Signaling TCRs then quickly form peripheral microclusters enriched in signaling proteins (Yokosuka et al., 2005) that perhaps enable sustained signaling. Microclusters frequently form at the tips of microvilli (Sage et al., 2012; Kumari et al., 2015; Jung et al., 2016) and migrate centripetally toward the cSMAC where signaling is eventually terminated (**Figure 1B**,

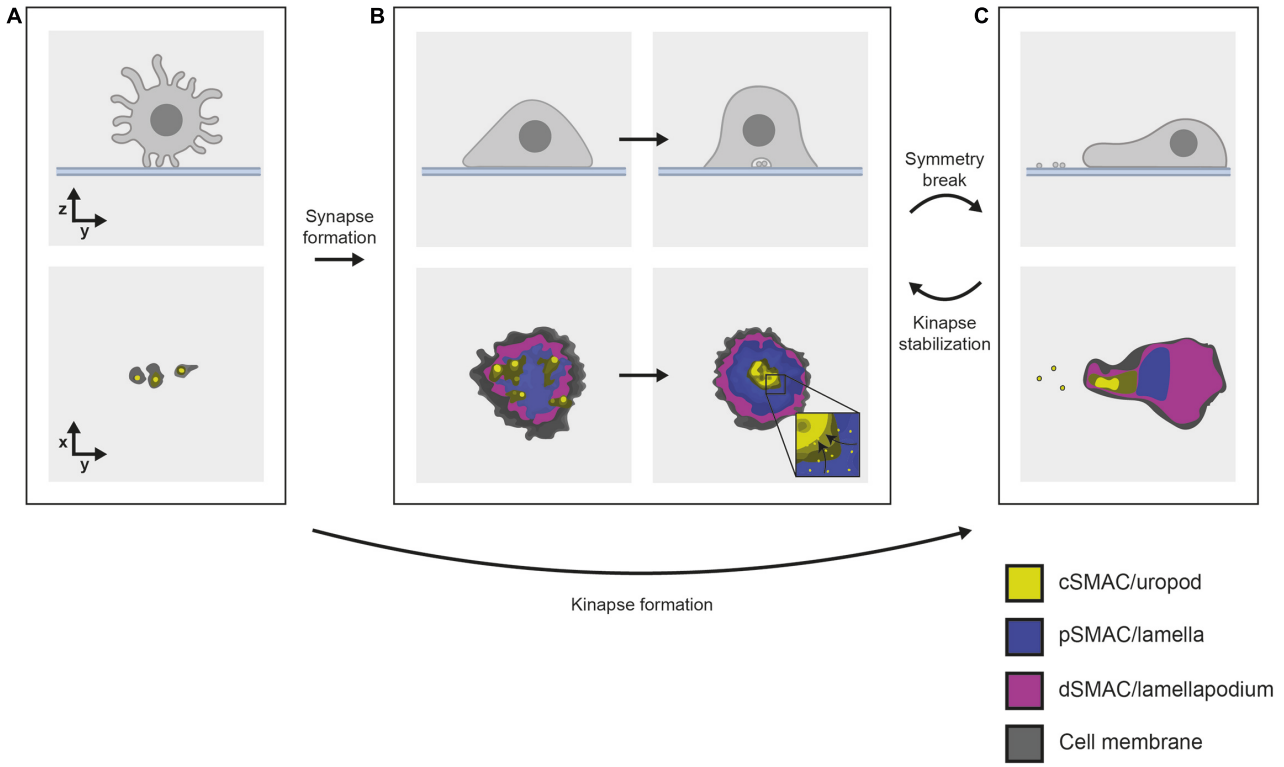

FIGURE 1 | (A) T cells interact with functionalized lipid bilayers using multiple microvilli, forming either **(B)** a radially symmetric immunological synapse or **(C)** an asymmetrical, motile kinapse. These structures consist of organized SMAC domains which correspond to the underlying actin networks, indicated by color. Effector vesicles/particles are indicated by small membrane-bound circles. The kinapse is the primary behavior adopted by most human T cells stimulated by antigen with the exception of $CD8^+$ memory T cells which are more likely to form stable synapses.

inset; Varma et al., 2006). The cSMAC is now thought to comprise a complex vesicular sorting structure (Choudhuri et al., 2014), which secretes extracellular vesicles or particles that deliver effector functions across the synapse in both $CD4^+$ (Saliba et al., 2019) and $CD8^+$ T cells (Balint et al., 2020).

Symmetry breaking of synapses allows motile "kinapses" to form that sustain extensive areas of close contact during cell movement (**Figure 1C**; Dustin, 2007; Sims et al., 2007). The kinapse is the default behavior of most human T cells during antigenic stimulation (Mayya et al., 2018). The maintenance of a symmetrical synapse is only a characteristic of $CD8^+$ effector T cells (Mayya et al., 2018) and appears to require WASP-dependent cytoskeletal tension (Sims et al., 2007; Kumari et al., 2020). TCR microclusters exhibit similar dynamics in synapses and kinapses, but remain stationary in the latter as the cell body moves past them, rather than moving centripetally (Beemiller et al., 2012).

The environments in which TCR- and coreceptor-binding to pMHC can initiate signaling thus include close contacts at microvillar tips, submicron microclusters within synapses/kinapses, and the much larger CD2 corolla, likely in that order. The organization and functions of each of these structures is a matter of intense investigation (Chang et al., 2016; Jung et al., 2016; Su et al., 2016; Demetriou et al., 2019). Although early imaging studies showed that CD4 and CD8 are recruited to the immunological synapse (Kupfer et al., 1987; Krummel et al., 2000; Zal et al., 2002), evidence for how they are organized within the smaller structures is only now beginning to emerge, including the suggestion that CD4 is pre-clustered at microvillar tips (Ghosh et al., 2020). But these environments are also highly dynamic, with remodeling on the order of seconds to minutes. Studies with a high spatial and temporal resolving power [e.g., imaging, spectroscopy and Förster resonance energy transfer (FRET) methods] will therefore be needed to understand the organization and evolution of TCR/pMHC/coreceptor interactions within these structures, and to understand how the very earliest stages of TCR signaling are influenced as a result.

TCR Signaling

The αβ TCR is expressed at the T-cell surface as a signaling-competent assembly with three CD3 dimers (CD3γε, CD3δε, and CD247/CD3ζζ), the full structure of which was recently determined by cryo-electron microscopy (Dong et al., 2019). The α and β subunits of the TCR heterodimer are structurally similar and each consist of variable and constant extracellular protein domains, a transmembrane helix and short cytoplasmic tails lacking any folded structure or known function beyond stabilizing the heterodimer at the membrane. TCR signaling is initiated by the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) located within the intracellular

tails of the CD3 subunits by Src-family kinases Lck and Fyn. Phosphorylated ITAMs then serve as docking sites for the SH2 domains of ZAP-70, a kinase whose activation is enhanced by Lck phosphorylation. ZAP-70, in turn, phosphorylates adaptor proteins LAT and SLP-76 which form signaling "scaffolds" to which downstream signaling proteins such as phospholipase C (PLC)-γ are recruited. PLC-γ catalyzes the production of second messengers diacetyl glycerol and inositol triphosphate which increase cytoplasmic Ca^{2+} levels and activate Ras and protein kinase C, initiating more signaling that ultimately promotes cell proliferation and differentiation (Smith-Garvin et al., 2009; Hwang et al., 2020). But what produces TCR phosphorylation in the first instance? Several theories have been proposed and these are broadly categorized as oligomerization-, conformational change- or segregation-based models, which have been expertly reviewed elsewhere (van der Merwe and Dushek, 2011; Mariuzza et al., 2020). Given that CD4 and CD8 bind the same pMHC ligand as the TCR, and are the only proteins known to be constitutively attached, at least to some degree, to the Src kinase Lck, the coreceptors must contribute to the earliest signaling events. The questions are: at what stage and how?

CD4 AND CD8 – AN OVERVIEW OF THEIR STRUCTURE AND FUNCTION

Structure and Binding Properties

Like the TCR, CD4 and CD8 both belong to the immunoglobulin superfamily (IgSF) insofar as they each have extracellular IgSF domains that are attached to a transmembrane segment and a short cytoplasmic tail (Leahy, 1995). Uniquely among receptors expressed by lymphocytes, the cytoplasmic tails of both coreceptors contain motifs that associate with membrane-anchored Lck (Veillette et al., 1988; Barber et al., 1989). CD4 and CD8 have few other structural similarities, however, suggesting that coreceptors may need simply (1) to bind MHC proteins and (2) to associate stably with Lck in order to perform their functions. CD4 is a monomer with four concatenated extracellular V- and C-set IgSF domains whereas CD8 is typically expressed as a disulfide-linked heterodimer of α and β subunits each comprising single extracellular V-set domains perched on top of a heavily O-glycosylated "stalk" (Li et al., 2013). The cytoplasmic regions of both coreceptors also contain membrane-proximal cysteines that are post-translationally palmitoylated (Crise and Rose, 1992; Arcaro et al., 2000).

A number of immune cell lineages express an alternate homodimeric form of CD8 consisting only of α-subunits. CD8αα binds MHC-I with a similar affinity to CD8αβ (Kern et al., 1999; Leishman et al., 2001) but it cannot fully substitute for CD8αβ as a coreceptor in T cells (Gangadharan and Cheroutre, 2004). Since the α-subunit associates with Lck (Turner et al., 1990) and the β-subunit is palmitoylated (Arcaro et al., 2001), CD8αα could in principle associate with two Lck molecules. There is evidence, however, that CD8β enhances the association of Lck with CD8α (Bosselut et al., 2000) implying that coreceptor palmitoylation is important for this interaction. Whether this is through post-translational co-trafficking through the ER (Shaw et al., 1989) or partitioning into membrane domains (He and Marguet, 2008) is unclear.

Coreceptors bind to MHC proteins with exceptionally low affinities – the CD4/MHC-II affinity, in particular, is among the weakest measured for any pair of interacting proteins (Jönsson et al., 2016). Surface plasmon resonance (SPR) assays, which are ideal for detecting weak protein-protein interactions, place the solution (3D) K_d value for the CD8αα/MHC-I interaction at ∼200 μM at 37°C (Wyer et al., 1999). Although SPR measurements of CD8αβ/MHC-I binding have not yet been conducted at 37°C, assays at room temperature suggest that CD8αα and CD8αβ have similar affinities for MHC-I (Kern et al., 1999; Leishman et al., 2001). SPR-based assays have thus far failed to reveal binding between soluble forms of CD4 and MHC-II, suggesting a lower limit of 2.5 mM for the 3D K_d value at 37°C (Jönsson et al., 2016). However, interactions between cell surface proteins are largely constrained to a two-dimensional (2D) plane and are therefore better described with 2D K_d values (i.e., the density of counter-receptors at which 50% of the receptor is bound) (Bell et al., 1984; Dustin et al., 1996; Zhu et al., 2007). Although no 2D K_d has yet been reported for CD8/MHC-I interactions, the 2D K_d for CD4/MHC-II binding was measured to be ∼5,000 molecules/μm^2 in a cell-bilayer contact using the rat CD2-CD48 adhesion pair to create a physiologically relevant membrane distance (Jönsson et al., 2016). One method of estimating the corresponding 3D K_d involves calculating a "confinement region" which takes into account the entropic and geometric constraints that arise from confining interactions to a plane (Dustin et al., 1997). The confinement region given by the 2D and 3D binding constants for CD2-CD48 produces a 3D K_d value of ∼5.1 mM for the CD4/MHC-II interaction, in agreement with the lower limit of 2.5 mM estimated using SPR (Jönsson et al., 2016). The remarkably low affinity of coreceptor/MHC binding has two important implications: (1) biologically important interactions may be undetectable using SPR assays, and (2) coreceptor/MHC interactions are unlikely to occur spontaneously at the cell surface (van der Merwe and Davis, 2003). Supporting the latter, biophysical studies show minimal binding of coreceptors to MHC, except in the presence of TCR (Huang et al., 2007; van der Merwe and Cordoba, 2011; Hong et al., 2015).

These very low affinities probably also explain why it took so long to crystallize the ternary TCR/pMHC/coreceptor complex. Indeed to produce crystals, Mariuzza et al. (2020) had to engineer an affinity-enhanced version of CD4 capable of forming a stable ternary complex (Yin et al., 2012). The structure revealed a distinctive V-shaped arch in which the TCR and CD4 are tilted ∼65° relative to the T-cell surface, apparently precluding any direct interaction between them. Once the complex had been solved it allowed the testing of various TCR triggering theories. On the basis of a dimerization site observed in the CD4 crystal lattice (Wu et al., 1997), CD4 homodimers have been proposed to contribute to T-cell activation through non-specific effects or by cross-linking MHC molecules to increase the avidity of TCR/pMHC binding (Moldovan et al., 2006). However, CD4 dimerization is incompatible with the geometry imposed by the ternary TCR/pMHC/CD4 structure

(Yin et al., 2012). The suggestion that MHC proteins are functional dimers (Brown et al., 1993) is also problematic because the claimed MHC dimerization site overlaps with the CD4/MHC-II binding site (Yin et al., 2012). Finally, TCR dimers have also been proposed to explain triggering (Kuhns et al., 2010). Unlike CD4 and MHC dimerization, this model is feasible because the proposed TCR dimerization site is located on a contiguous surface on TCRα outside of the TCR-pMHC-CD4 arch (Yin et al., 2012). However, a survey of 22 other TCR structures did not find similar dimerization sites (Wang and Reinherz, 2013) and conserved glycans in this region are thought to sterically preclude dimerization (Li et al., 2013). These observations highlight the need for orthogonal *in situ* approaches to validate protein-protein interactions inferred from structural studies. Using two fluorescence-based approaches, James et al. (2011) showed that CD4-Lck molecules are monovalent at the surfaces of live cells implying that coreceptors are likely to be functionally monovalent.

The structure of the ternary complex also suggested that the coreceptor would be positioned adjacent to the CD3 chains, whose location at that stage was unclear (Yin et al., 2012). The recent determination of the TCR/CD3 complex by Dong et al. (2019) confirms this arrangement, as a model made by superimposing the TCR/CD3 structure with the ternary complex shows that the CD3 chains are placed in the middle of the "arch," ideally positioned to be phosphorylated by CD4-Lck. Altogether, the structural data indicate that there is no contact between the coreceptor and the CD3 chain ectodomains, making it unlikely that the recruitment of CD4 or CD8 is directly enhanced by interactions involving their extracellular regions. As we discuss below, this makes their recruitment likely to be secondary to TCR triggering.

The binding of coreceptors to MHC in the absence of the TCR is proposed be a general mechanism of increasing T-cell/APC adhesion (Glatzová and Cebecauer, 2019). This, however, seems incompatible with their extremely low affinity for MHC. Adhesive interactions are not observed at physiological densities of these proteins, and their detection relies on over-expression of either the coreceptor or the MHC molecule (Doyle and Strominger, 1987; Norment et al., 1988). Binding assays also show that soluble CD4 tetramers do not bind detectably to MHC-II-expressing cells, but that very weak binding can be detected when CD4 is coupled to streptavidin-coated beads (∼50,000 CD4s per bead) (Jönsson et al., 2016), emphasizing their profoundly weak binding. T-cell/APC adhesion *in vivo* more likely depends on the much stronger interactions of "professional" adhesion proteins, such as the integrins (Shimaoka et al., 2002) and small adhesion molecules comprising the CD2 subset of the IgSF (Davis et al., 2003). What, then, would be the physiological relevance of a very weak, monovalent interaction if not to increase overall T-cell/APC adhesion? Computer simulations that take into account low affinity CD4/MHC-II interactions suggest that CD4-Lck recruitment would stabilize the TCR/pMHC interaction by only 2–20% and enhance TCR phosphorylation only 3-fold compared to free Lck in the membrane. In contrast, the recruitment of CD4-Lck to a pre-phosphorylated TCR results in a 30- to 40-fold increase in the rate of receptor phosphorylation compared to when CD4-Lck is recruited to an unphosphorylated TCR (Jönsson et al., 2016). On this basis it can be argued that coreceptors significantly enhance antigen-specific signaling only after it is initiated.

Lck and Coreceptor Occupancy

CD4 and CD8 both associate with Lck via a cytoplasmic "zinc clasp" formed by dicysteine motifs in the coreceptor tail and the Lck SH4 domain (Kim et al., 2003). Lck association is indispensable for coreceptor function as transgenic T cells expressing truncated "tailless" CD4 or CD8αβ molecules have severely diminished responses to *in vitro* stimulation (Zamoyska et al., 1989; Miceli et al., 1991). Supporting this contention, alignment of CD4 and CD8α sequences reveals that the "clasp" cysteines are very highly conserved across vertebrates (**Figure 2A**, highlighted in yellow). A LOGO analysis of the transmembrane helix and cytoplasmic tail of vertebrate CD4 sequences (**Figure 2B**) indicates that the "clasp" cysteines are more highly conserved than any other element, including the palmitoylation sites (Crise and Rose, 1992) and the glycine-rich transmembrane region (Parrish et al., 2015). In contrast, the extracellular MHC-binding sites are highly variable (Chida et al., 2011) presumably because the coreceptors had to accommodate a variety of MHC molecules (Sommer, 2005), which allowed diversity in the binding region to emerge. These observations emphasize that Lck association is an ancient and essential feature of coreceptors. Interestingly, all of the CD8α orthologs available from fish species lack the second cysteine residue in the "clasp" motif, where it is replaced where a histidine (**Figure 2A**, highlighted in blue). Histidine is the second most common Zn^{2+}-coordinating residue after cysteine (Dokmanić et al., 2008), reinforcing the notion that CD8α has to associate with Lck.

The coreceptor-Lck interaction was identified in the late 1980s by the Rudd and Schlossman groups who used co-immunoprecipitation (co-IP) assays to show that Lck was linked to CD4 and CD8 in T-cell lysates (Rudd et al., 1988; Barber et al., 1989). Preliminary observations indicated that the fraction of coreceptors associated with cytoplasmic Lck (referred to here as "occupancy") was high (Veillette et al., 1988) although this was not accurately measured. The first quantitative study was undertaken in the early 1990s by Carmo et al. (1993) who used radioactive antibody fragments to tag CD4. By labeling cell surface CD4 molecules prior to co-IP, the authors could carefully compare the amounts of radioactivity in anti-Lck "pulldowns" relative to anti-CD4 pulldowns, yielding a CD4-Lck occupancy of ∼80% (Carmo et al., 1993). Since this was consistent with the emerging idea that coreceptors recruit kinase activity to the TCR (Rudd et al., 1989; Janeway, 1992), the matter was considered settled. However, several recent studies are beginning to cast doubt on the assumption that coreceptors are wholly occupied by Lck. In 2016 and 2020, two groups reported unexpectedly small occupancy values using the co-IP method: 6% (Parrish et al., 2016) and 37% (Horkova et al., 2020) for the CD4-Lck interaction in single-positive T cells. Even lower values were reported for $CD8^+$ T cells and double-positive thymocytes (Horkova et al., 2020). Why would similar assays produce such drastically different occupancy values? One possibility is simply

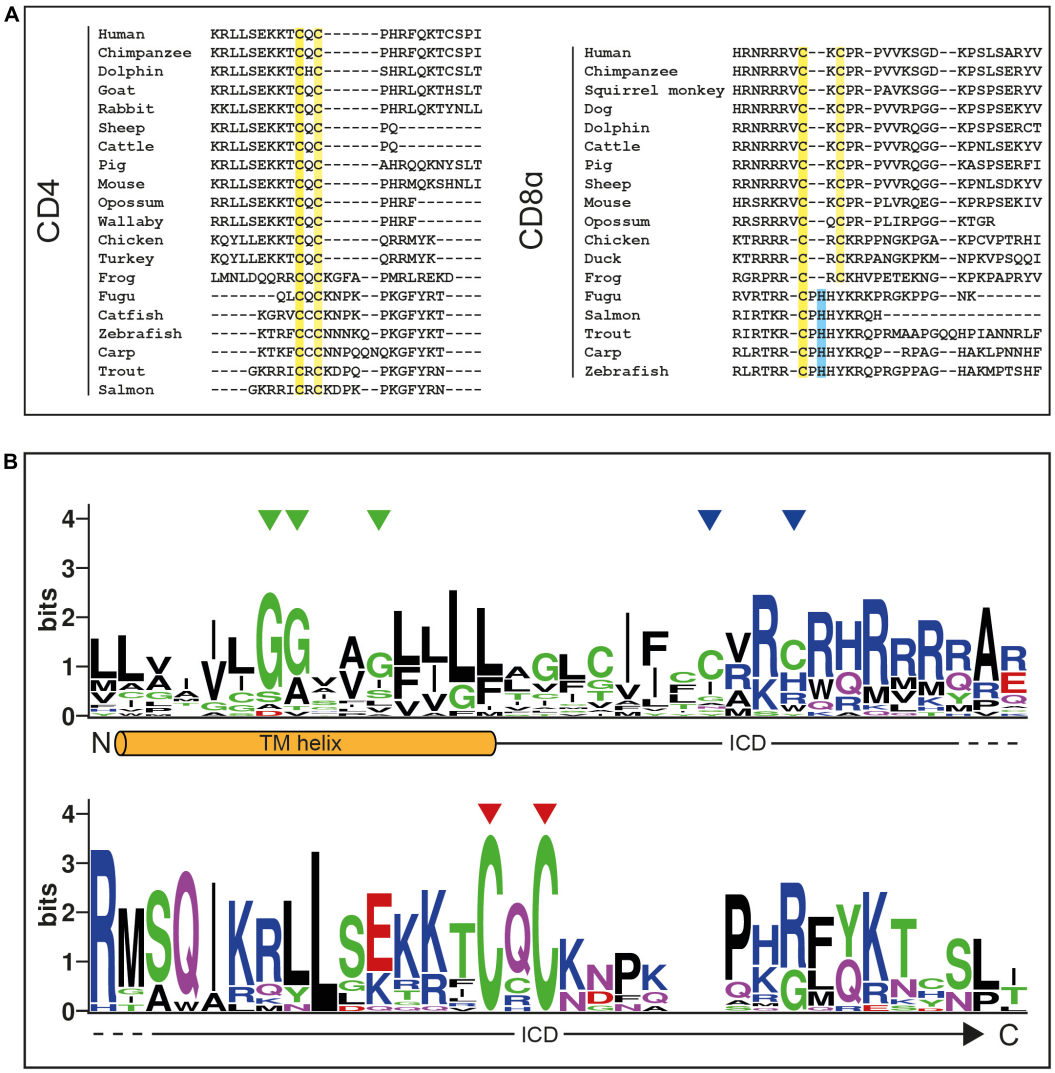

FIGURE 2 | The coreceptor "zinc clasp" is highly conserved. **(A)** MUSCLE alignment of C-terminal CD4 and CD8α sequences with clasp cysteines highlighted in yellow and histidines in equivalent positions highlighted in blue. Adapted from Chida et al. (2011). **(B)** A sequence LOGO of the CD4 transmembrane (TM) helix and intracellular domain (ICD). Green triangles indicate glycines in the conserved GGXXG motif, blue triangles indicate S-palmitoylation sites, and red triangles indicate clasp cysteines. Sheep CD4 was excluded from this analysis due to the presence of large insertions in this regions bearing no homology to any other species.

protocol (e.g., incubation periods, different controls, presence or absence of EDTA). Another is that the coreceptor/Lck "clasp" interaction is relatively weak. Assuming typical on-rates [10^5 M^{-1} s^{-1} (Schlosshauer and Baker, 2004)], the K_d values given by isothermal titration calorimetry (Kim et al., 2003) give k_{off} values of 0.04 s^{-1} for CD4-Lck and 0.09 s^{-1} for CD8-Lck, i.e., half-lives of ≈17 s and ≈8 s, respectively. This rapid decay suggests that additional interactions, for example involving the lipid modifications on CD4 (Crise and Rose, 1992), CD8β (Arcaro et al., 2000) and Lck (Paige et al., 1993), make important contributions to complex stability in mixed micelles, and these contributions are difficult to control for. Another limitation of the co-IP method is the risk of sampling interactions in intracellular compartments such as the ER and Golgi, and not just the plasma membrane. High resolution imaging approaches will likely be needed to settle the matter of occupancy, and to ascertain whether the bound and free states of Lck are also modulated as recently proposed (Wei et al., 2020).

THEORIES OF CORECEPTOR CONTRIBUTIONS TO TCR SIGNALING

The Lck Recruitment Model

The first and simplest proposal for coreceptor function was that CD4 and CD8 have the special role of delivering Lck to the ligand-bound TCR (**Figure 3A**; Rudd, 1990; Janeway, 1992). This idea incorporated three important experimental observations: (1) the TCR lacked intrinsic kinase activity but was phosphorylated upon ligand engagement (Samelson et al.,

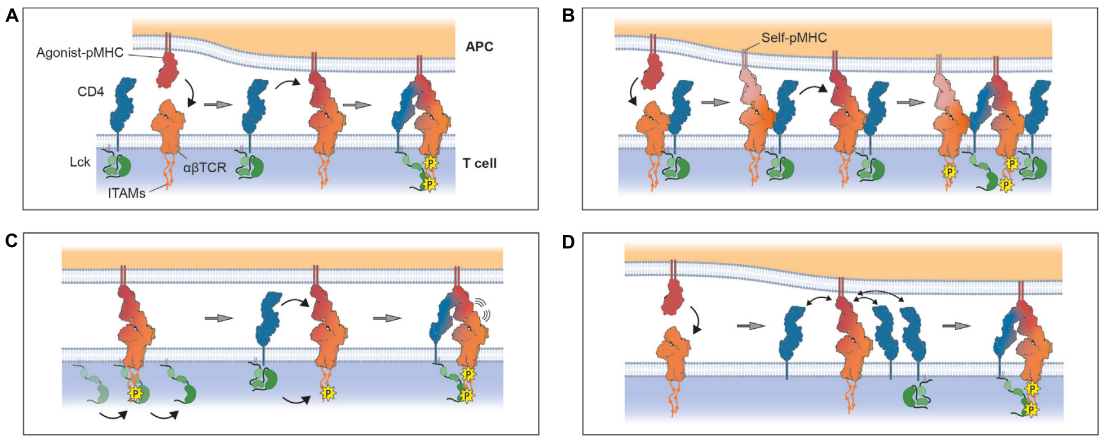

FIGURE 3 | Models for coreceptor function. **(A)** The Lck recruitment model: coreceptors recruit Lck to cognate pMHC-TCR complexes. **(B)** The pseudodimer model: coreceptors cross-link agonist-bound TCR to self-bound TCR. **(C)** The coreceptor recruitment model: ITAMs are incipiently phosphorylated by free Lck prior to recruitment of a coreceptor/Lck complex through SH2 domain-dependent interactions, e.g., between Lck and phosphorylated ITAMs. **(D)** The coreceptor scanning model: a cognate pMHC-TCR complex scans multiple "empty" coreceptors before encountering coreceptor-bound Lck. Gray arrows denote the passage of time. Only the zeta chain ITAMs are shown for simplicity. Protein models were generated from the crystal structures of TCR/CD3 (PDB ID: 6JXR), CD4 (PDB ID: 1WIQ), HLA-DR1 (PDB ID: 4I5B), and the ternary TCR-pMHC-CD4 complex (PDB ID: 3T0E).

1986), (2) coreceptor activity was highest when the MHC- and Lck-binding sites were simultaneously intact (Miceli and Parnes, 1991), and (3) coreceptors became physically associated with the TCR during T-cell activation (Dianzani et al., 1992). A compelling feature of this proposal was that it offered a simple explanation for TCR triggering, since only agonist ligands could form sufficiently stable TCR/pMHC complexes to permit coreceptor recruitment and TCR phosphorylation. However, it was later shown that TCR signaling could be coreceptor-independent (Locksley et al., 1993; Schilham et al., 1993) indicating that CD4 and CD8 are not essential for triggering. A prediction of the Lck recruitment model was that soluble pMHC monomers would trigger signaling, but this is now known not to occur (Boniface et al., 1998; Schott et al., 2002). Finally, given the poor ability of MHC-II molecules to recruit CD4 to the TCR (Jönsson et al., 2016), these observations indicate that the role of coreceptors is not to trigger *de novo* signaling by recruiting Lck to the TCR/pMHC complexes.

The Pseudodimer Model

When the structures of coreceptor-MHC complexes were first solved it was found, somewhat surprisingly, that coreceptors engage membrane-proximal regions of MHC molecules almost directly orthogonal to the TCR-binding site (Gao et al., 1997; Wang et al., 2001). The resulting topology was expected to prevent the TCR and the coreceptor from physically associating (van der Merwe and Davis, 2003), whereas it had been proposed that such an association could be important for signaling. One idea that could reconcile these arguments was the "pseudodimer" model (**Figure 3B**; Irvine et al., 2002) in which coreceptors are thought to bridge the gap between adjacent TCRs to form receptor pseudodimers. According to this idea, an agonist-bound TCR is stabilized by the recruitment of a second self-pMHC-bound TCR through a cross-linking coreceptor to create a geometry permissive for Lck-mediated phosphorylation (Krogsgaard et al., 2005). The main advantage of this hypothesis was that it provided an explanation for the observation that soluble, covalently linked pMHC dimers, consisting of an agonist-pMHC and a self-pMHC, could be shown to induce T-cell activation (Krogsgaard et al., 2007). However, structural studies offer little support for the notion that either affinity-matured (Yin et al., 2012) or native (Jönsson et al., 2016) CD4 associates physically with the TCR in the orientation required for pseudodimerization. This is less clear for CD8 because no structural information is available for the stalk region of the protein. However, glycosylation is thought to make the stalk rigid (Li et al., 2013) which could prevent CD8 bridging two TCRs. A second issue is that this idea, again, runs into the problem of the very low coreceptor/MHC affinity. Pseudodimerization, relying as it does on the interaction of the coreceptor with MHC proteins only, is unlikely to produce the levels of signaling enhancement typically observed in the presence of CD4 or CD8.

The Two-Step Coreceptor Recruitment Model

Xu and Littman introduced a new heterodoxy in 1993, proposing that the "delivery of a catalytically active Lck to the TCR complex is not the primary function of CD4." They suggested instead that the coreceptor function was modulated by TCR triggering, and not *vice versa*. In a series of remarkable experiments, Xu and Littman (1993) showed that the activity of a CD4-Lck chimera was (1) *abolished* by mutating its phosphotyrosine-binding SH2 domain, and (2) *increased* by deleting its kinase domain. Both results pointed to the dominant role not of the kinase domain of Lck in enhancing TCR triggering, but rather its SH2 domain. Xu and Littman (1993) interpreted the first of these results as implying that coreceptor function depended crucially on prior phosphorylation of the TCR. They proposed that the second, even

more troubling, of these observations could be explained by the absence of the C-terminal tyrosine of the kinase domain, which would normally become phosphorylated and block SH2 domain access. In support of these interpretations, biochemical studies had by then shown how TCR triggering enhances the binding of CD4 to the TCR (Mittler et al., 1989) and CD8 to MHC class I (O'Rourke et al., 1990).

Xu and Littman (1993) proposed a radical new, two-step mechanism of TCR triggering later referred to as the "coreceptor recruitment model" (van der Merwe and Cordoba, 2011; **Figure 3C**): (1) the ligand-engaged TCR complex is initially and partially phosphorylated by free Lck diffusing in the membrane, followed by (2) the recruitment of a coreceptor/Lck complex to the engaged TCR/pMHC, via bidentate interactions between the extracellular regions of the coreceptor and pMHC, and between the SH2 domain of Lck and phosphotyrosines in the cytoplasmic regions of the TCR. Direct evidence for a two-step process did not emerge until 2011, however, when Jiang et al., 2011 observed it directly using a bespoke mechanical adhesion frequency assay. By repeatedly bringing $CD8^+$ T cells into contact with red blood cells (RBCs) used as surrogate APCs and measuring the resulting RBC membrane deformation, Jiang et al. (2011) observed that bonds formed more frequently than expected for the simple sum of TCR/pMHC and pMHC/CD8 bonds, which was indicative of cooperative binding. They went on to show that the cooperative binding was induced and that it was blocked by kinase inhibitors. This data was interpreted as offering strong support for the proposal of Xu and Littman (Jiang et al., 2011; van der Merwe and Cordoba, 2011). Similar support for TCR-CD8 cooperation in binding pMHC were obtained for human T cells recognizing self-antigens (Liu et al., 2014). A potential mechanism for this two-step model was identified in 2014 when Gascoigne and colleagues, using FRET measurements, showed that free, coreceptor-unbound Lck catalyzes the initial phosphorylation of the ligand-engaged TCR, and that CD8 recruitment depends critically on the CD8-Lck "clasp" interaction (Casas et al., 2014).

But how do CD4 and CD8 increase T-cell sensitivity if TCR signaling and T-cell activation are not necessarily coreceptor-dependent (Locksley et al., 1993; Schilham et al., 1993)? These observations could be reconciled if it is first postulated that initial phosphorylation of the TCR is catalyzed inefficiently by free Lck but, in certain circumstances, e.g., for high affinity TCRs, this is enough to activate a cell. A second requirement would be that levels of incipient phosphorylation are sufficient for coreceptor recruitment, which then increases the initial signal. But how would enhanced signaling arise? Xu and Littman (1993) suggested that coreceptors contribute to the formation of a stable ternary signaling complexes and amplify an initially low level of TCR phosphorylation via the catalytic activity of Lck. Modeling studies are consistent with the second of these ideas and show that both coreceptors act primarily to shuttle Lck to the TCR (Li et al., 2004; Artyomov et al., 2010). But imaging and biophysical experiments show that CD4 has a negligible effect on the affinity and lifetime of TCR-pMHC complexes *in situ* (Huppa et al., 2010; Hong et al., 2015), although there is some evidence that CD8 has an additional contribution to complex stabilization (Wooldridge et al., 2005; Jiang et al., 2011). Importantly, the two-step coreceptor recruitment mechanism may ensure that coreceptor-mediated signal amplification is subservient to primary agonistic TCR signaling (Davis et al., 2003).

The ability of CD8, but not CD4, to increase the stability of the ternary complex has been attributed to the greater affinity of CD8 for MHC molecules (Artyomov et al., 2010). This raises the question of why CD4 and CD8 have such different affinities for MHC given their highly analogous functions. It might be that the contribution of each coreceptor is "tuned" to the physiological context in which they function. For example, CD8 may have to bind strongly to MHC-I because the targets of $CD8^+$ cytotoxic T lymphocytes (CTLs) are often infected or malignant somatic cells that do not express co-stimulatory molecules such as CD80/CD86 (McAdam et al., 1998), in contrast to the targets of $CD4^+$ helper T (Th) cells.

CORECEPTORS AND THE THYMUS

Thymic Development

Coreceptors are important for coupling the two principal T-cell effector functions of "help" and "killing" to MHC class. They ensure that, in the periphery, $CD4^+$ T-cells are only activated by pMHC-II on professional APCs while $CD8^+$ T-cells can respond to foreign or mutated peptides on all MHC-I-expressing somatic cells. This dichotomy is established during a complex developmental program in the thymus. Developing thymocytes express randomly generated TCRs that are tested against self-pMHC molecules. Weakly self-reactive thymocytes receive a survival signal, producing a pool of cells capable of recognizing host MHC proteins (positive selection), whereas strongly self-reactive thymocytes are deleted to avoid autoreactivity (negative selection). In addition, a limited number of strongly self-reactive thymocytes develop into regulatory T-cells that suppress harmful autoimmune responses and inflammation. Together, these processes drive the generation of mature, peripheral T-cells which are appropriately self-MHC-restricted and self-tolerant.

CD4 and CD8 are critically important for the maturation of MHC-restricted T cells, as illustrated by the failure of CD4- and CD8-deficient mice to generate $CD4^+$ Th or $CD8^+$ CTLs, respectively (Fung-Leung et al., 1991; Rahemtulla et al., 1991). But Lck itself must also play a central role since the simultaneous deletion of Lck and a closely related kinase called Fyn results in a complete failure to produce αβ T cells (van Oers et al., 1996). In 2007, Singer and colleagues proposed that CD4 and CD8 confer MHC restriction on developing T cells by sequestering Lck away from TCRs that, by chance, engage non-MHC thymic ligands that cannot also interact with the coreceptors (Van Laethem et al., 2007). According to Xu and Littman's (1993) two-step signaling mechanism, however, T cells encountering these ligands would be expected to develop if their TCRs bound strongly enough for free Lck to produce sufficient signaling to negotiate positive and negative selection. These predictions were borne out when Van Laethem et al., 2007 showed that mice lacking CD4, CD8, MHC-I and MHC-II (so called "quad-deficient" mice) produced a diverse repertoire

of αβTCR-expressing, MHC-unrestricted T cells. Singer and colleagues then established the binding specificities of two such TCRs, and found that the TCRs bound the surface protein CD155 in a manner similar to antibodies, i.e., to distinct conformational epitopes, with nanomolar affinity, and without any involvement of MHC proteins (Tikhonova et al., 2012; Lu et al., 2020). Whether or not MHC selection in the thymus is entirely dependent on Lck sequestration by the coreceptors, or evolutionary pressures on the germline have also encoded a set of "rules of engagement," will likely continue to be debated (Garcia, 2012; Van Laethem et al., 2012). But how would naturally occurring MHC-unrestricted T cells develop if signaling is normally coreceptor-dependent? The Singer group have also shown that the timing of coreceptor expression is carefully controlled allowing, for example, signaling in γδTCR-expressing thymocytes to be triggered by free Lck before CD4 or CD8 are expressed (Van Laethem et al., 2013).

Coreceptor Scanning as a Discriminative Mechanism

Signaling by the TCR needs to reach sufficient levels to activate a mature T cell, but also be sensitive to the "quality" of a ligand, especially in the service of thymocytes that must discriminate between the self-ligands mediating positive and negative selection. Palmer and colleagues have proposed a role in this for coreceptors in the form of a processive "coreceptor scanning" mechanism (**Figure 3D**; Stepanek et al., 2014). They suggest that TCR-pMHC complexes would likely have to rapidly engage or "scan" several CD4/CD8 proteins before encountering Lck, because coreceptor occupancy is very low (less than 10%) according to their measurements. The delay between pMHC binding and Lck recruitment, they argued, would allow the TCR to translate small differences in affinity into large differences in response, providing a long sought-after explanation for kinetic proofreading in T-cell activation (McKeithan, 1995). "Coreceptor scanning" is reminiscent of an earlier signaling mechanism called the "occupancy model" in which Lck activity is regulated by altering coreceptor occupancy (Rudd, 1990). However, these mechanisms are effectively refinements of the Lck recruitment model, and therefore suffer from the same general problems as this theory. First, the very low affinity of coreceptor/MHC interactions means that the recruitment of coreceptor-bound Lck, regardless of occupancy, would already be very inefficient. Second, Lck is generally expressed in excess of either CD4 or CD8 (Takada and Engleman, 1987; Davis et al., 1998; Hui et al., 2017; Voisinne et al., 2019; Felce et al., 2020) and the free Lck would be expected to work against low coreceptor occupancy unless it is actively kept low. A third problem is that the work of Xu and Littman (1993) shows that Lck recruitment is dependent on its SH2 domain, i.e., that it requires prior TCR phosphorylation, making discrimination at the point of coreceptor recruitment redundant. Finally, it is unclear how discrimination would be protected from physiological variations in coreceptor expression levels (Itoh et al., 2005; Xiao et al., 2007) which might affect the coreceptor/Lck coupling equilibrium.

How might T cells or thymocytes discriminate between agonists and non-agonists if not through kinetic proofreading mechanisms such as "coreceptor scanning"? One proposal is mechanotransduction, in which the TCR and the coreceptor together are intrinsically capable of differentiating between ligands on the basis of the type of molecular bonds they form (Hong et al., 2018). Another is that antigen discrimination is an emergent property of a signaling mechanism constrained by T-cell topography, i.e., one relying only on receptor dwell-time at phosphatase-depleted regions of contact between T cells and APCs (Fernandes et al., 2019).

CONCLUSION

The marking of distinct T-cell subsets by CD4 and CD8 thrust the coreceptors into the limelight from the very outset. But it is now more than 40 years since their special status became apparent, first as coreceptors forming ternary complexes with the TCR and pMHC strongly enhancing T-cell responses (Janeway, 1988, 1992), and then as potent amplifiers of signaling, acting secondarily to TCR triggering (Xu and Littman, 1993). In the ensuing period we have learnt a great deal about the structures and interactions of CD4 and CD8, but there are still many unanswered questions in this area of T-cell biology, including:

1. What is the true occupancy level of coreceptors and does it change during thymic development or between T-cell subsets?
2. Do we fully understand why coreceptor/MHC interactions are so unusually weak? And are there physiologically relevant situations in which they are enhanced (Owen et al., 2016)?
3. Once the bidentate binding to TCR/pMHC is established, what is the mechanism by which coreceptors enable orders-of-magnitude signal amplification?
4. Why are CD4 and CD8 palmitoylated and is it linked to membrane heterogeneity?
5. Are CD4 and CD8 organized within microvilli, microclusters and the corolla or are they randomly distributed across the cell surface, and how does this change in the course of activation?
6. Finally, do CD4 and CD8 play a role in the numerous other leukocytes in which they are expressed (Gibbings and Befus, 2009; Kadivar et al., 2016)?

AUTHOR CONTRIBUTIONS

AM wrote the initial draft and generated sequence alignments. All authors made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Coordinating Cytoskeleton and Molecular Traffic in T Cell Migration, Activation, and Effector Functions

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Dynamic localization of receptors and signaling molecules at the plasma membrane and within intracellular vesicular compartments is crucial for T lymphocyte sensing environmental cues, triggering membrane receptors, recruiting signaling molecules, and fine-tuning of intracellular signals. The orchestrated action of actin and microtubule cytoskeleton and intracellular vesicle traffic plays a key role in all these events that together ensure important steps in T cell physiology. These include extravasation and migration through lymphoid and peripheral tissues, T cell interactions with antigen-presenting cells, T cell receptor (TCR) triggering by cognate antigen–major histocompatibility complex (MHC) complexes, immunological synapse formation, cell activation, and effector functions. Cytoskeletal and vesicle traffic dynamics and their interplay are coordinated by a variety of regulatory molecules. Among them, polarity regulators and membrane–cytoskeleton linkers are master controllers of this interplay. Here, we review the various ways the T cell plasma membrane, receptors, and their signaling machinery interplay with the actin and microtubule cytoskeleton and with intracellular vesicular compartments. We highlight the importance of this fine-tuned crosstalk in three key stages of T cell biology involving cell polarization: T cell migration in response to chemokines, immunological synapse formation in response to antigen cues, and effector functions. Finally, we discuss two examples of perturbation of this interplay in pathological settings, such as HIV-1 infection and mutation of the polarity regulator and tumor suppressor adenomatous polyposis coli (Apc) that leads to familial polyposis and colorectal cancer.

Keywords: TCR, signaling molecules, actin, microtubules, molecular transport, HIV-1, immunological synapse, polarity regulators

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INTRODUCTION

Dynamic compartmentation of receptors and signaling molecules is key for T cells to sense environmental cues, trigger membrane receptors, and transduce and fine-tune intracellular signals controlling T cell migration, activation, and effector functions. This molecular compartmentation is ensured by the interplay between the plasma membrane, cytoskeleton networks, and intracellular organelles.

At the plasma membrane, dynamic assemblies of lipids and proteins form nano- to micro-scale domains that may become platforms for receptor signaling (i.e., cholesterol- and sphingolipid-enriched membrane domains or lipid rafts). These domains may facilitate either segregation or

interaction between receptors (e.g., chemokine receptors and T cell receptors [TCRs]) and signaling molecules, conditioning their state of activation and preventing or facilitating receptor triggering and signaling (Lillemeier et al., 2006; Viola and Gupta, 2007; Simons and Gerl, 2010; Swamy et al., 2016). In addition, specific membrane phosphoinositides, transiently generated by enzymatic activation during chemokine receptor or TCR signaling, form different domains that target signaling effectors (e.g., Pleckstrin homology (PH) domain-containing proteins) at sites of receptor stimulation (Courtney et al., 2018).

The cortical actin cytoskeleton contributes to plasma membrane organization by generating areas of differential mobility of lipids and proteins. Thus, membrane-associated cytoskeletal fences shape the lateral distribution of membrane components involved in cell adhesion or receptor activation (Sako and Kusumi, 1995), adding a level of membrane organization cooperative with lipid microdomain partitioning. Furthermore, actin dynamics contribute to cell reorganization in response to chemokine or antigen stimulation needed for T cell migration, activation, and effector functions (Viola and Gupta, 2007; Nicolson, 2014; Niedergang et al., 2016). Although cortical actin and plasma membrane domains are often considered two-dimensional entities, three-dimensional membrane-cytoskeletal structures, such as microvilli, may form sensing exploratory extensions displaying receptor signaling components and adhesion molecules located within flexible subcellular areas distant from the cell body (Singer et al., 2001; Cai et al., 2017; Ghosh et al., 2020).

Several cellular organelles, including the Golgi apparatus and the endosomal and lysosomal compartments, continuously exchange with the plasma membrane. They contribute to lipid and protein sorting to subcellular areas involved in cell migration, activation, or secretion (Bretscher and Aguado-Velasco, 1998; Griffiths et al., 2010; Niedergang et al., 2016). Moreover, the endoplasmic reticulum (ER) and mitochondria contribute not only to protein synthesis and metabolism but also to T cell signaling (Quintana and Hoth, 2012).

Microtubules are crucial for intracellular transport and subcellular localization of molecules, vesicles, and organelles. They form a network that interacts with the nucleus, the cortical actin cytoskeleton, the plasma membrane, and various organelles, including endo-lysosomal compartments, the ER, and the Golgi apparatus. Microtubules coordinate the localization of proteins and organelles by means of their associated molecular motors, dynein, and kinesins. In this way, they ensure the dynamic relocalization of a variety of cellular components during T cell migration, activation, and effector functions (Vicente-Manzanares and Sanchez-Madrid, 2004; Niedergang et al., 2016; Martin-Cofreces and Sanchez-Madrid, 2018).

Intermediate filaments are the third major element of the cytoskeleton displaying different stabilities and mechanical properties from actin and microtubules. They cooperate with actin and microtubules in cellular architecture being important for cell polarization during migration, nuclear positioning, cellular mechanics, and cell adhesion-mediated mechano-transduction in various cell types (Etienne-Manneville, 2018). Their role in T cell biology remains poorly explored. In circulating T cells, vimentin intermediate filaments display a spherical pattern that relocalizes to a juxtanuclear area in chemokine-induced polarized cells. T cell rigidity (Brown et al., 2001), lymphocyte adhesion, transendothelial migration, and homing depend on intact intermediate filaments (Nieminen et al., 2006). In regulatory T cells (Tregs), vimentin intermediate filaments contribute to PKCθ localization at the distal pole of TCR-stimulated cells and to the control of Treg activity (McDonald-Hyman et al., 2018). In addition, vimentin regulates apoptosis in T cells during inflammation (Su et al., 2019). Septins are an additional component of the cytoskeleton in eukaryotic cells. These GTP-binding proteins assemble into hetero-oligomers that further associate forming higher order structures (e.g., filaments, bundles, and circles; Mostowy and Cossart, 2012). Recently, they have been shown to regulate several aspects of T cell biology, including signaling, differentiation, and cell division (Lassen et al., 2013; Sharma et al., 2013; Mujal et al., 2016). In particular, their role in regulating amoeboid T cell motility has been recently characterized (Tooley et al., 2009). Septins have been shown to regulate cortical rigidity and membrane dynamics. Their knockdown in T cells results in membrane blebbing and abnormal structure of both the leading edge and the uropod. These defects make T cell motility uncoordinated and poorly persistent (Tooley et al., 2009). The interplay of intermediate filaments and septins with actin and microtubules in T cells is not well defined, and it will not be further discussed in this review.

Among the most striking features of T cells is their capacity to rapidly change shape and profoundly reorganize their cellular interior leading to differential cell polarization in response to chemokine or antigenic stimuli (**Figure 1**). These cues induce coordinated changes in actin and microtubule cytoskeletons, membrane receptors, adhesion molecules, and various organelles that prepare the T cell to migrate in response to chemokines or to generate a signaling platform, the immunological synapse, in response to antigenic stimulation. Chemokine stimulation makes T cells to adopt a bipolar organization, with a lamellipodium at the migration front and a protruding uropod in the back differing in shape, cytoskeleton, and membrane component organization (**Figures 1A,B**). Such a remodeling prepares T cells to adhere and migrate through lymphoid organs and inflamed peripheral tissues (del Pozo et al., 1996). In turn, the encounter of T cells with antigen-presenting cells displaying cognate peptide antigen–major histocompatibility complex (MHC) complexes at their surface stabilizes the interaction between the two cells and triggers the formation of a highly organized and dynamic cell–cell interface named the immunological synapse (**Figure 1C**). Actin and microtubule cytoskeletons reorganize at the immunological synapse, together with TCR, co-stimulatory receptors, signaling molecules, and adhesion receptors. In addition, several organelles, such as the Golgi apparatus, the endosomal compartment, and the mitochondria, polarize to the immunological synapse releasing their cargo or retrieving membrane receptors and signaling molecules (**Figures 1D,E**). Altogether, the reorganization of molecular components at the immunological synapse ensures the control of T cell activation and effector functions (Niedergang et al., 2016).

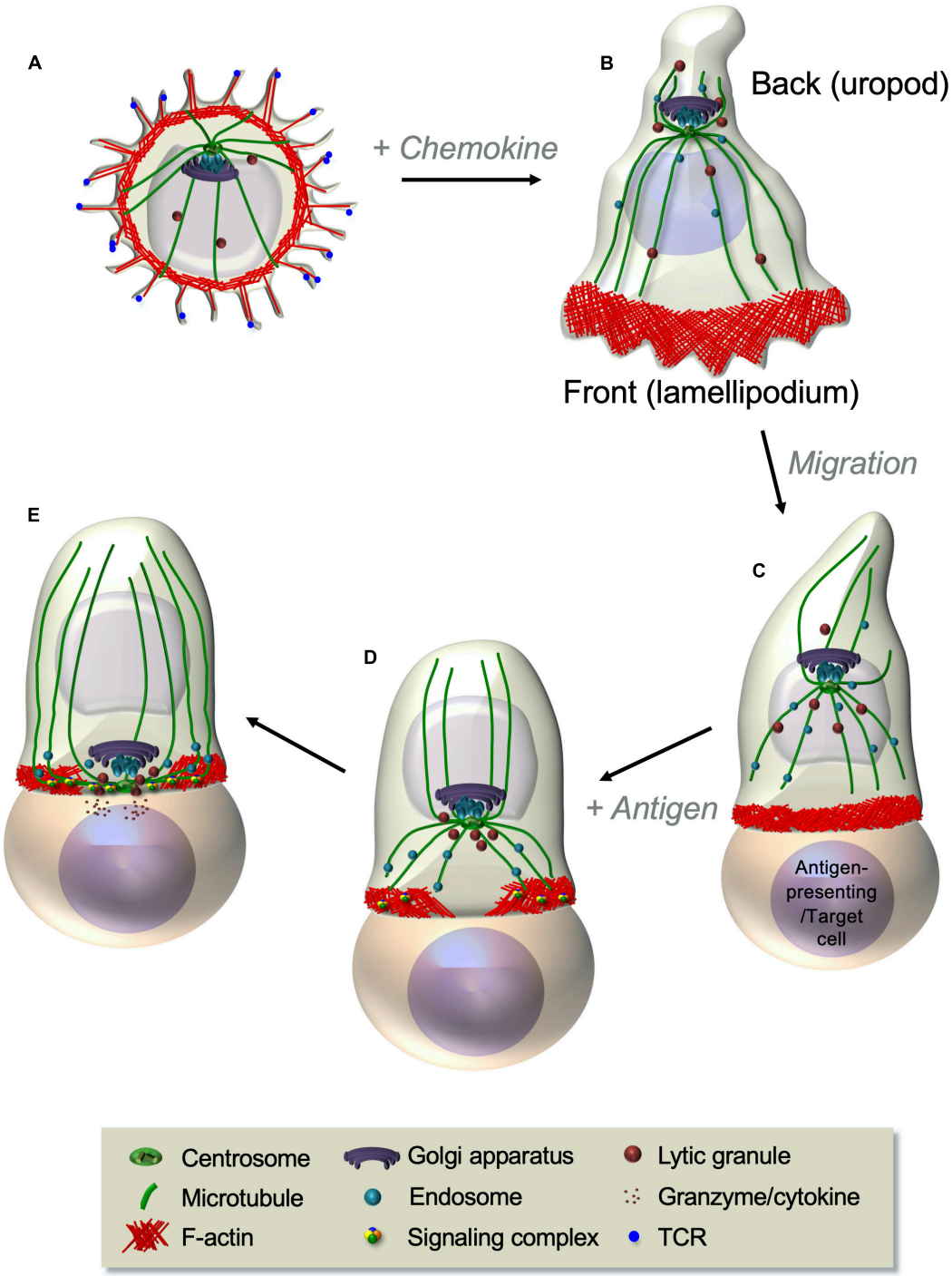

FIGURE 1 | T cell polarization during T cell migration and immunological synapse formation. **(A,B)** Resting T cells, displaying microvilli at their surface **(A)**, polarize and start migrating in response to chemokines. A lamellipodium in which robust actin cytoskeleton dynamics takes place appears at the front edge, whereas a protrusion, named the uropod, forms at the back **(B)**. The centrosome is positioned between the nucleus and the uropod (see also **Figure 3**). Following chemokine gradients, T cells migrate through lymphoid organs or peripheral tissues where they meet antigen-presenting cells or target cells expressing their cognate antigen in complex with MHC proteins. **(C–E)** Upon antigen recognition, TCR signaling induces the coordinated polarization of actin and microtubule cytoskeletons. This is characterized by strong actin polymerization at the cell–cell contact site and the reorganization of the microtubule network that moves the centrosome toward the contact site **(C)**. Centrosome-associated organelles, such as the Golgi apparatus, endosomes, or lytic granules, move together with microtubules toward the contact site. Actin reorganizes while the T cell spreads at the contact site, forming a peripheral F-actin-enriched ring and a central F-actin poor area, where the centrosome and microtubule-associated organelles approach the cell–cell interface **(D)**. A final cytoskeleton-coordinated reorganization of the contact area generates the immunological synapse, where a concentration and dynamic clustering of TCRs, signaling and adhesion molecules, and co-signaling receptors occurs, thus ensuring sustained and controlled TCR signaling (further developed in **Figure 4**). In effector T cells, this is an area where cytokines or lytic granules are secreted **(E)**.

The tight interplay between receptors and their signaling machineries, the actin and microtubule cytoskeleton, and intracellular molecular transport enables T cells to perform their functions, namely, sense environmental cues, polarize, migrate and patrol through lymphoid organs, recognize cognate antigen, and get activated to accomplish clonal expansion and differentiation into helper, regulatory, or cytotoxic T cells. Finally, it allows T cell effector functions, such as polarized secretion of cytokines to help B cells, and cytotoxic granules to eliminate infected or transformed cells. Various pivotal proteins facilitate the interplay between membrane, cytoskeletal, and organelle components. Among them, membrane–cytoskeleton linkers, such as the ezrin–radixin–moesin (ERM) family of proteins, talin, and several polarity regulators, play important roles at the different stages of T cell migration and immunological synapse formation (Krummel and Macara, 2006; Lasserre and Alcover, 2010; Garcia-Ortiz and Serrador, 2020).

Ezrin–radixin–moesin proteins bind plasma membrane components, such as phosphatidylinositol (4,5)-bisphosphate (PIP_2) and transmembrane proteins, *via* their N-terminal FERM domain, and the cortical actin cytoskeleton *via* its threonine-phosphorylated C-terminal domain (**Figure 2**). Thus, ERMs help localizing membrane proteins at particular subcellular areas in various cell types (Arpin et al., 2011). T cells express ezrin and moesin that are important for confining TCRs and some of its signaling proteins to microvilli (Jung et al., 2016; Ghosh et al., 2020) and several adhesion proteins (i.e., intercellular adhesion molecules [ICAMs] and P-selectin glycoprotein ligand [PSGL])

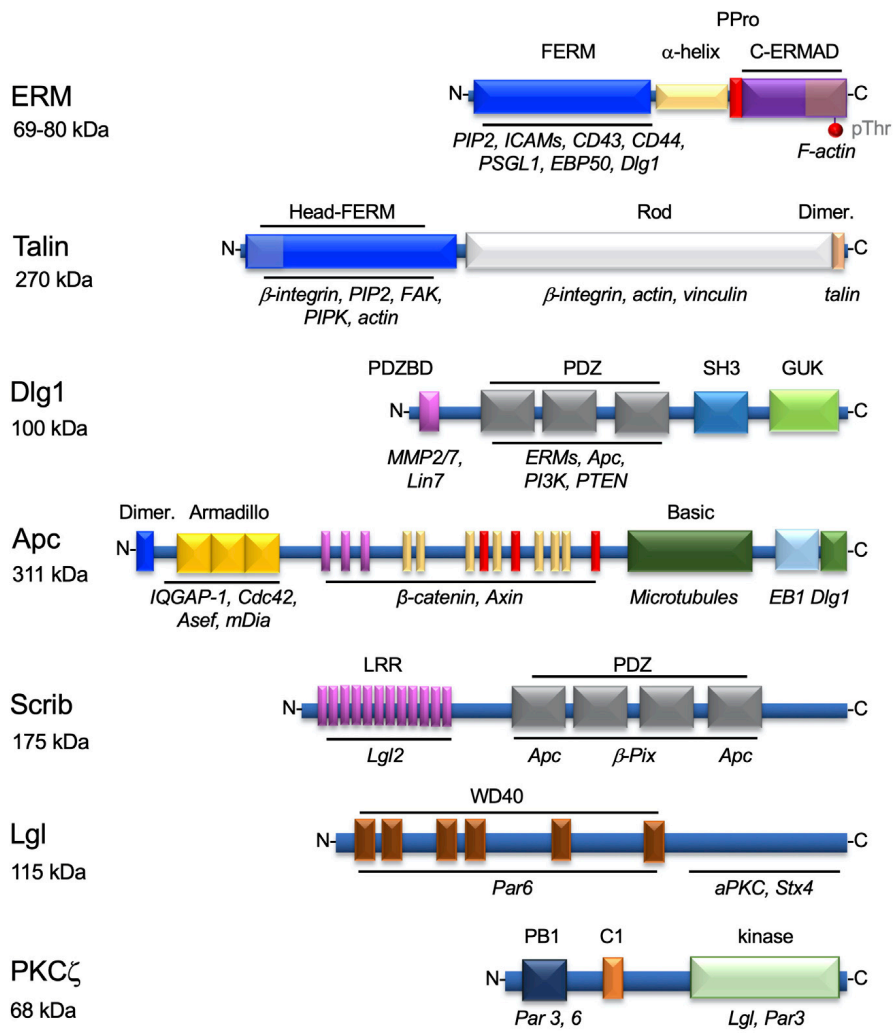

FIGURE 2 | Proteins involved in the interplay between the plasma membrane components and the cytoskeleton in T cells. Structural organization of proteins regulating the interplay between membrane components and the actin and microtubule cytoskeletons. The modular domains involved in their interactions with lipids or other proteins are highlighted. Each domain, named on top, is shown in a different color and its interacting molecules depicted below in italics. ERMs and talin are mostly involved in the localization of adhesion proteins to particular areas of the plasma membrane, as the uropod (ERMs), or the immunological synapse periphery (talin). Dlg1, Apc, Scrib, Lgl, and PKCζ are polarity regulators involved in T cell migration and/or immunological synapse formation. For ERM, the phosphorylatable regulatory threonine residue (pThr) in the C-terminal domain is also shown. Molecular weights in kDa are show below each protein name.

to the uropod of migrating cells (Serrador et al., 1997, 1998, 2002). They can also link cortical actin with membrane rafts (Itoh et al., 2002). Finally, ezrin and moesin are key for immunological synapse formation and function (Allenspach et al., 2001; Delon et al., 2001; Roumier et al., 2001; Itoh et al., 2002; Faure et al., 2004; Shaffer et al., 2009; Lasserre et al., 2010). Other proteins also ensure the interplay between the plasma membrane and the actin cytoskeleton. For instance, talin and vinculin anchor adhesion proteins of the integrin family to the cortical actin cytoskeleton in areas of the cell in contact with integrin ligands in migrating cells and at the periphery of the immunological synapse (Jankowska et al., 2018; **Figure 2**).

Polarity regulators are multifunctional proteins displaying a variety of protein–protein interaction domains. These domains (e.g., PDZ domains) ensure interactions between polarity regulators themselves and with cytoskeleton components, cytoskeleton regulators (e.g., Cdc42), and membrane–cytoskeleton linkers, such as ERMs (**Figure 2**). Polarity regulators act in complexes. Several of them, such as Scribble, Dlg1, Lgl, PKCζ, Crumbs, PAR, and adenomatous polyposis coli (Apc), have been shown to control T cell polarization during migration, immunological synapse formation, or activation (Xavier et al., 2004; Ludford-Menting et al., 2005; Krummel and Macara, 2006; Real et al., 2007; Round et al., 2007; Bertrand et al., 2010; Lasserre et al., 2010; Aguera-Gonzalez et al., 2017).

In this review, we summarize the available knowledge on how the interplay between membrane receptor dynamics and signaling, the cytoskeleton, and intracellular vesicular compartments modulates three main aspects of T cell biology: T cell migration, immunological synapse formation in response to antigen stimulation, and effector functions. Finally, we describe two examples of perturbation of this interplay in pathological settings, i.e., HIV-1 infection and mutation of the polarity regulator and tumor suppressor Apc in familial polyposis and colorectal cancer.

CYTOSKELETON INTERPLAY IN REGULATING T CELL POLARIZATION AND MIGRATION

T cells are activated in lymph nodes, where they acquire the expression of specific tissue-homing receptors, such as adhesion and chemokine receptors, that sense information from the environment and lead T cell trafficking. Driven by the presence or the absence of these signals, T cells leave central lymphoid organs and undergo bloodstream navigation reaching peripheral lymph nodes or inflamed tissues. Their spherical shape facilitates the blood flux to push them forward. Moreover, the presence of thin protrusions on their surface, named microvilli, where some chemokine receptors, such as CXCR4, and adhesion molecules, such as L-selectins, are concentrated (Berlin et al., 1995; Singer et al., 2001) promotes sensing of the environment and the attachment necessary for them to slow down navigation and dock at a destination site. Once T cells have adhered to the blood vessel wall, chemokine stimulation induces the transient collapse of microvilli, and integrin activation leads to firm arrest, lymphocyte polarization, and transmigration through the vascular endothelial cell layer (Brown et al., 2003; Nijhara et al., 2004).

In the tissues, T cells modify their shape and adopt a different motility based on adhesion and on contact with the surrounding cells and the extracellular matrix. This allows them to migrate through tissues of different architecture and to interact with antigen-presenting cells (Moreau et al., 2018). This plasticity is fine-tuned by cytoskeleton structures, whose dynamics and interplay with molecular adaptors, such as cell polarity regulators, is essential for processes required for efficient T cell migration, including polarization, adhesion, and vesicle trafficking.

T cell polarization, an inherent requirement for migration, implies the formation of specialized subcellular areas, a lamellipodium at the leading edge and a uropod at the trailing edge (**Figure 3**). The leading edge, being enriched in chemokine receptors, guides the displacement, whereas the adhesive uropod supports cell–cell interactions. T cell migration relies on the mechanical cyclicity of lamellipodium extension and uropod retraction (Lauffenburger and Horwitz, 1996; Sánchez-Madrid and del Pozo, 1999).

Cytoskeleton Rearrangements Shaping T Cell Polarization

The Rho family GTPases Cdc42, Rac1, and RhoA regulate actin and microtubules specialized dynamics at the front and the rear by transducing signals from surface receptors (Rougerie and Delon, 2012; Saoudi et al., 2014). At the cell front, chemokine stimulation induces the activation of Cdc42 and Rac1/2 *via* the phosphorylation of their guanine nucleotide exchange factors (GEFs), such as Vav1. These, in turn, engage several actin-binding proteins, trigger actin nucleation, and modulate the stability of filamentous actin (F-actin)-rich protrusions (reviewed in Dupre et al., 2015). In particular, Cdc42 and Rac1/2 induce the extension of filopodia and lamellipodia, respectively (Ridley et al., 2003; Dupre et al., 2015). Their function involves the activation of the WASP and WAVE proteins, followed by the activation of the Arp2/3 effector complex, that ensure actin polymerization and branching necessary for lamellipodium extension. Thus, defects in the Arp3 subunit are sufficient to affect the lamellipodium formation and the migratory behavior of CD8 T cells (Obeidy et al., 2020). In addition, the RhoA–ROCK pathway-dependent stimulation of actomyosin contraction is both responsible for the actin retrograde flow, on which lamellipodium extension and migration persistence rely on (Maiuri et al., 2015; Moreau et al., 2018), and essential for the detachment from the substrate (Alblas et al., 2001).

The precise control of microtubule organization in migrating lymphocytes is not fully understood, but their disassembly by nocodazole treatment disrupts cell polarity (Takesono et al., 2010). Events of microtubule growth and catastrophe may occur as described in other cell types (Hui and Upadhyaya, 2017). Interestingly, while migrating astrocytes or fibroblasts orient their centrosome between the nucleus and the front lamellipodium, migrating lymphocytes have their centrosome

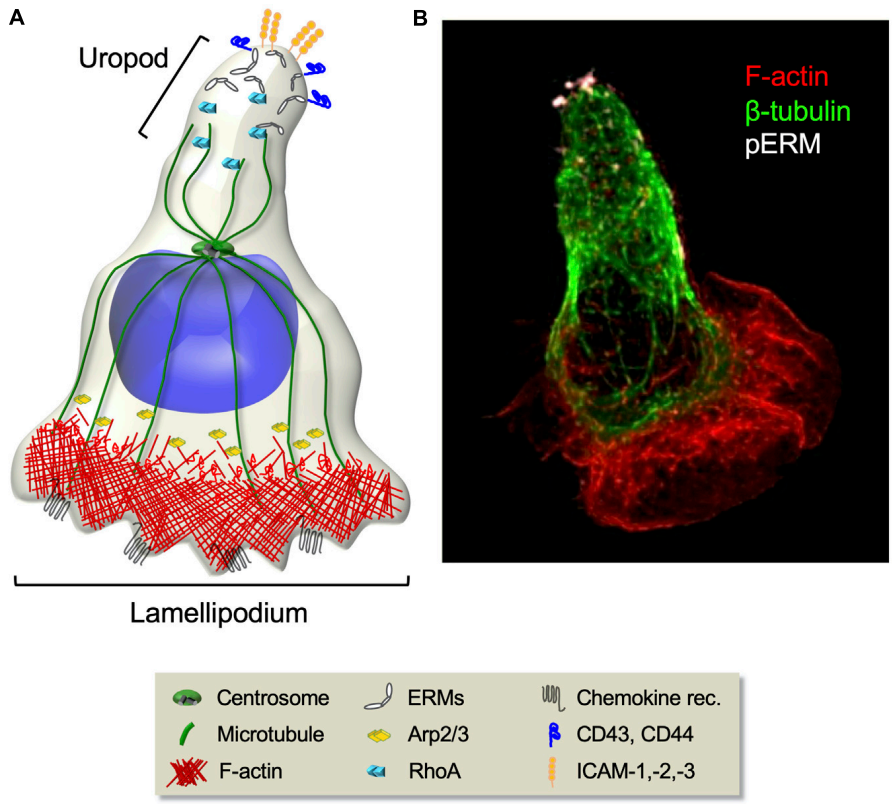

FIGURE 3 | Cytoskeleton rearrangements during T cell migration. **(A)** Schematic representation of migrating T cell polarization involving the orchestrated rearrangement of both the actin and the microtubule cytoskeletons. At the cell front, chemokine stimulation induces the activation of the Arp2/3 effector complex that leads to actin polymerization and branching necessary for lamellipodium extension. At the rear, RhoA-dependent phosphorylation of ERM proteins induces their selective segregation to the uropod, where they recruit transmembrane adhesion molecules. **(B)** Fluorescence confocal microcopy image of a CEM T cell polarized in response to the chemokine SDF-1. F-actin (red), microtubules (green), and phosphorylated ERMs (white) are shown.

behind the nucleus (Ratner et al., 1997; Serrador et al., 1997; Lee et al., 2004; **Figure 3**). This positioning likely reflects some functional peculiarities of lymphocytes that need to be dissected.

In astrocytes and other non-leukocyte cell types, microtubule plus-end growth at the leading edge contributes to the lamellipodium activity by participating to the F-actin–membrane protrusion formation (Etienne-Manneville, 2004, 2013). Thus, microtubules drive vesicle exocytosis necessary for membrane extension (Bretscher, 1996), and their growth favors the increase in Rac1–GTP amounts, promoting the Rac1 signaling cascade (Liao et al., 1995; Waterman-Storer et al., 1999). In turn, Rac1/PAK1 activation may promote microtubule growth by inhibiting the microtubule-destabilizing protein Op18/stathmin (Wittmann et al., 2004). These features have only been partly described in T cells or leukocytes.

Microtubules contribute to RhoA activation at the rear of T cells. This involves the RhoGEF H1, which is sequestered by microtubules (Meiri et al., 2012), and the subsequent activation of the RhoA–ROCK pathway and phosphorylation of myosin light chain, which induces uropod contraction (Chang et al., 2008; Kaverina and Straube, 2011; Yoo et al., 2012). The RhoA–ROCK pathway also contributes to activate the formin mDia, an actin nucleator that regulates peripheral actin flow (Otomo et al., 2005). Hence, microtubule dynamics in the front contributes to the persistence of the actin flow (Park and Doh, 2015), whereas microtubule stability at the rear is required for myosin light chain-dependent uropod contraction, providing the mechanical force necessary for effective cell locomotion.

Ezrin–radixin–moesin proteins, which ensure interactions between cortical actin and membrane components, are key for chemokine-induced T cell polarization. Chemokines induce transient ERMs de-phosphorylation, dissociation from the plasma membrane and the actin cytoskeleton, and release of GEF proteins that in turn activate Rac1 and Cdc42. This supports F-actin polymerization at the protrusive leading edge (Hao et al., 2009; Garcia-Ortiz and Serrador, 2020). Then, RhoA-dependent re-phosphorylation of ERMs induces their selective segregation to the uropod, where they recruit adhesion molecules, such as ICAM-1, -2, -3, CD44, and PSGL-1 (**Figure 3**). Ezrin and moesin FERM domains interact with a consensus sequence in the intracellular region of these adhesion molecules. Phosphorylated ERMs constitute a functional polar cap in the rear pole *via* their cooperation with lipid raft-associated flotillins (Lee et al., 2004; Martinelli et al., 2013) where they re-activate

RhoA and myosin, modulating contractility at the uropod in a positive feedback loop.

Membrane–Cytoskeleton Interactions During T Cell Adhesion and Migration

Cell membrane components participate to adhesion and migration, acting as sensors of the environment and converting external signals into biochemical messages for the cell. Lipid rafts of different composition redistribute during T cell polarization in response to chemokines, being enriched in the ganglioside GM3 at the leading edge and in GM1 in the uropod (Gomez-Mouton et al., 2001). This contributes to the spatial segregation of chemokine receptors or adhesion molecules and to their interaction with cytoskeleton structures and/or signaling complexes, thus influencing their spatiotemporal activation (reviewed in Dustin et al., 2004; Manes and Viola, 2006). While front GM3-enriched rafts mainly concentrate chemokine receptors, such as CXCR4 and CCR5, GM1-enriched rafts colocalize with the adhesion protein CD44 at the uropod, where ERM-associated flotillins are found as well (Gomez-Mouton et al., 2001). Integrin activation depends on their localization in ganglioside GM1-containing rafts (Gomez-Mouton et al., 2001). Integrins are present not only in the uropod of polarized T cells but also in a larger zone in contact with their ligands (Gomez-Mouton et al., 2001; Leitinger and Hogg, 2002; Smith et al., 2005). Indeed, integrins move laterally within lipid rafts, and their activation state may result in the localization in different cell compartments, including the leading edge (Hogg et al., 2003; Hyun et al., 2009).

Integrins represent the main class of adhesion molecules responsible for interactions with both the extracellular matrix and neighboring cells. They are heterodimeric proteins whose activation relies on their reversible conformational changes triggered by surface receptors, including the TCR and chemokine receptors, or by their own binding to multivalent ligands (reviewed by Baker and Koretzky, 2008; Abram and Lowell, 2009). In addition, both lipid raft microenvironment and cytoskeleton interactions shape integrin activation by controlling single hotspots of integrins in the membrane and their clustering in larger plasma membrane domains (Stewart et al., 1998; Leitinger and Hogg, 2002; Cairo et al., 2006; van Zanten et al., 2009). Integrin clustering selectively provides higher avidity for ligands (Stewart and Hogg, 1996; van Kooyk et al., 1999), although it does not change their affinity (Kim et al., 2004; Luo et al., 2005). It results from the TCR-mediated signaling (Abram and Lowell, 2009) and may be negatively regulated by GTPases. Indeed, inhibition of the RhoA–ROCK pathway induces clustering of lymphocyte function-associated antigen-1 (LFA-1), followed by the induction of adhesion to its ligand, ICAM-1 (Rodriguez-Fernandez et al., 2001).

Integrin activation state in turn influences the composition of the surrounding environment, thus impacting the downstream signaling and enabling cytoskeleton remodeling (Schwartz, 2010; Byron et al., 2015). Whereas active β1 integrins are mainly found in complexes with actin and microtubule-associated proteins, such as talin and kindlin, inactive integrins form complexes with molecules involved in adhesion and cytoskeleton organization (Rho and Ras GTPase family members) or in membrane trafficking (Arf and Rab GTPases) in K562 leukemic cells, which may resemble to T cells for their adhesion pattern (Byron et al., 2015).

Interestingly, the link between integrins and the cytoskeleton is bidirectional, and their functions are reciprocally modulated (Vicente-Manzanares et al., 2009). For instance, LFA-1 activation during cell migration is modulated by physical forces on its β subunit applied by the actin cytoskeleton (Nordenfelt et al., 2016). Moreover, the inhibition of actin polymerization by cytochalasin D prevents the formation of new nascent adhesions (Choi et al., 2008), whereas microtubule regrowth after nocodazole washout correlates with adhesive structure disassembly (Kaverina et al., 1998; Ezratty et al., 2005).

T cell adhesion to the substrate and subsequent changes on the physical properties of their membranes are also sensed by BAR domain-containing proteins that translate these signals into cytoskeleton remodeling. Substrate attachment of the adhesive uropod of neutrophils induces a membrane curvature critical for the activation of the SRGAP2 BAR protein, in keeping with the notion of phospho-ERMs asymmetrical segregation at the uropod during T cell migration (Ren et al., 2019). Hence, the rear membrane curvature would be responsible for the activation of specific BAR proteins and then kinases, determining the local phosphorylation of ERMs and their membrane binding at the rear (Ren et al., 2019). It is noteworthy that the BAR protein CIP4, involved in membrane deformation during endocytosis, is also crucial for integrin-dependent activation of WASP. Indeed, T cells from CIP4$^{-/-}$ mice present defects in adhesive interactions, impairing transmigration across endothelial cell monolayers (Koduru et al., 2010).

Intracellular Traffic in T Cell Adhesion and Migration

Intracellular trafficking may promote the polarization of motile lymphocytes by allowing the dynamic turnover of membrane and the delivery of cargos, such as chemokine or cytokine receptors and integrins, to specific subcellular localizations. Cargos are transported along actin and microtubule structures *via* myosin, kinesin, and dynein molecular motors, respectively, and may be associated with vesicles. Integrins continuously cycle between the plasma membrane and endosomal compartments (Paul et al., 2015). Clustering of integrins in lipid rafts may contribute to their internalization and recycling, possibly facilitating integrin targeting at the leading edge (Hyun et al., 2009) where they would establish adhesion during migration. These processes are poorly elucidated in T cells, and most of the information is on LFA-1. In the uropod of T cells migrating on ICAM-1, LFA-1 undergoes a caveolar endocytosis, which is regulated by G-protein-coupled receptor, mediated for instance by Gαq/11 (Svensson et al., 2012). Partitioning into lipid rafts is likely pivotal for LFA-1 to undergo a caveolae-dependent endocytic pathway (Upla et al., 2004; Fabbri et al., 2005). Moreover, inhibition of the small GTPases Rab13, a key regulator of intracellular membrane trafficking, could reduce LFA-1-dependent adhesion on ICAM-1 and the formation of

micro-adhesion rings of LFA-1 at the contact site with antigen-presenting cells (Nishikimi et al., 2014), essential for T cell activation (Hashimoto-Tane et al., 2016) (see section "Actin–Microtubule Interplay Shaping T Cell Effector Functions").

T CELL SENSING OF ANTIGEN CUES, TCR TRIGGERING, AND IMMUNOLOGICAL SYNAPSE FORMATION

Topological Distribution of the TCR

Once in the lymph nodes or in peripheral tissues, T cells scan antigen-presenting cells searching for cognate peptide–MHC complexes. The localization of the TCR and some of its proximal signaling molecules on microvilli may enhance the sensing capacity of T cells.

Mapping TCRs localization relative to the 3D membrane topology demonstrated that TCRs are segregated on the tips of microvilli in fixed resting and effector T cells (Jung et al., 2016). CD3ε follows the same distribution than TCR, and both proteins significantly colocalize with L-selectin, further confirming their localization at microvilli tips. This approach has been recently extended to analyze the distribution of additional membrane receptors and signaling proteins in human effector T cells and the Jurkat T cell line (Ghosh et al., 2020). It has been shown that the majority of the CD3ζ subunit, the co-receptor CD4, and the adhesion protein CD2 are localized to microvilli. The protein kinase Lck and the adaptor LAT are also enriched in microvilli, although a significant fraction of these molecules is found outside these structures. This observation agrees with Lck and LAT being partially associated with intracellular vesicular compartments (Soares et al., 2013; see also "Alterations of T Cell Cytoskeleton and Molecular Traffic in Pathological Settings" section). On the contrary, the protein tyrosine phosphatase CD45, which inhibits TCR/CD3 complex phosphorylation, is segregated from the TCR, hence mostly excluded from microvilli (Razvag et al., 2018; Ghosh et al., 2020).

The structural integrity of microvilli requires an intact actin cytoskeleton, and the confinement of proteins into microvilli is dependent on membrane–cytoskeleton linker proteins of the ERM family (Ghosh et al., 2020). Indeed, phosphorylated ERMs are concentrated into microvilli where they co-localize with F-actin and TCRs. Additionally, overexpression of a dominant-negative form of the ERM member ezrin in Jurkat T cells results in the disappearance of membrane protrusions and redistributions of microvilli-associated proteins throughout plasma membrane. These modifications correlate with a reduction of TCR-dependent signaling, as measured by the inhibition of the phosphorylation of ERK kinases (Ghosh et al., 2020).

Studies of microvillar dynamics in live T cells indicated that most microvilli undulate and move laterally, allowing a faster and more efficient scanning of the antigen-presenting cell surface (Cai et al., 2017). Microvillar dynamics is slowed down once the contact with the antigen-presenting cells is stabilized, likely as a consequence of TCR engagement by peptide–MHC complexes and integrin activation. Further analyses demonstrated that signaling complexes containing the TCR and the ZAP70 protein kinase colocalized in areas corresponding to microvillar tips (Cai et al., 2017), suggesting that the geometry and dynamics of signaling protein complexes or pre-existing "protein islands" described before (Lillemeier et al., 2010) are actually influenced by membrane 3D topology.

Collectively, these data indicate that concentration of TCRs, associated co-receptors, and signaling proteins at microvilli tips plays a critical role in antigen recognition and early activation steps. Indeed, this organization and the mobility of membrane protrusions would allow a "topological scan" of antigen-presenting cell surface, increasing speed and efficiency of antigen search (Cai et al., 2017). Moreover, focusing TCR and its signaling machinery to microvilli increase the avidity of interaction of antigen receptors with peptide–MHC complexes and facilitate early signal transduction. However, at later steps of activation, ERMs dephosphorylation may lead to microvilli resorption (Ghosh et al., 2020), thus favoring mixing of signaling proteins with TCRs and centripetal movement of signaling complexes, followed by their internalization and/or dissociation.

It is worth noting that microvilli might also have additional functions, such as the recently described generation of extracellular organelles or "immunological synaptosomes," through a mechanism similar to trogocytosis (Kim et al., 2018). These entities may carry various signals to the antigen-presenting cells (e.g., TCR/CD3 complexes, co-stimulatory proteins, and cytokines) and are probably related to the extracellular vesicles previously detected at the center of the immunological synapse (Choudhuri et al., 2014).

Immunological Synapse Formation

The early consequence of a productive TCR engagement by its cognate antigen displayed on the surface of an antigen-presenting cell is twofold. First, the T cell stops or slows down its movement, and then it starts polarizing toward the antigen-presenting cell. These initial events, driven by rearrangements of the actin and microtubule cytoskeletons, result in the formation of the immunological synapse. This specialized interface allows the communication between the two cells involved, ensuring efficient TCR signal transduction leading to T cell activation, clonal expansion, and differentiation.

The immunological synapse is characterized by intensive F-actin polymerization at the interface with the antigen-presenting cell. Once the synapse is stabilized, F-actin clears from the center of the synapse leaving an actin-rich peripheral ring (Bunnell et al., 2001; Ritter et al., 2015). Microtubules also reorganize at the immunological synapse, some irradiating from the centrosome and oriented toward the periphery of the synapse where some appear to anchor and bend (Kuhn and Poenie, 2002; Lasserre et al., 2010; Aguera-Gonzalez et al., 2017). Concomitantly, the centrosome translocates toward the center of the synapse, beneath the plasma membrane, within a minute after F-actin clearance (Geiger et al., 1982; Kupfer et al., 1986; Stinchcombe et al., 2006; Ueda et al., 2011; Ritter et al., 2015; **Figure 4**). The exact molecular mechanism moving

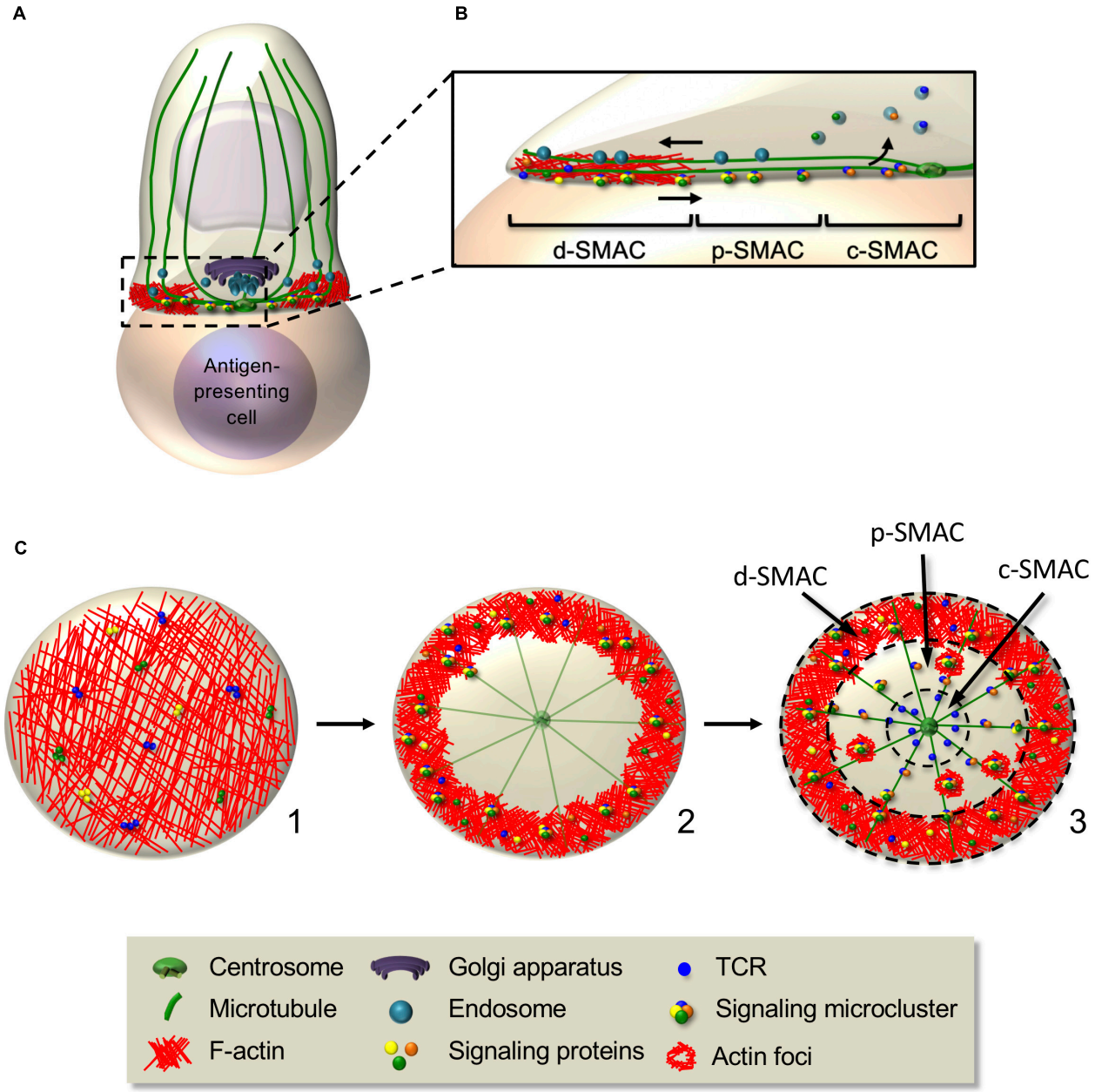

FIGURE 4 | Interplay between actin and microtubule cytoskeleton controls signaling microcluster dynamics at the immunological synapse. **(A,B)** Schematic representation of cellular and molecular rearrangements leading to immunological synapse formation. This involves actin and microtubule rearrangements and organelle polarization, driving to the generation of dynamic signaling microclusters. The latter form in the d-SMAC, at the periphery of the immunological synapse **(B)**, then centripetally move to the center in an actin- and microtubule-dependent manner. Some molecules (e.g., the TCR) coalesce in the center generating the c-SMAC, whereas others are internalized or disassembled in the p-SMAC during their centripetal movement. **(C)** *En-face* view of an immunological synapse showing the kinetics of its reorganization. F-actin, which is initially disseminated throughout the synapse (1), redistributes and concentrates in the peripheral area, whereas microtubules adopt a radial organization from the centrosome to the periphery (2). Signaling microclusters form at the synapse periphery and move centripetally (2,3), first by retrograde actin waves, then by the microtubule-based motors dynein. Adhesion rings (not shown) and F-actin foci transiently surround signaling microclusters, reminiscent of "micro-synapses" (3). Dashed circles in (3) separate d-SMAC, p-SMAC, and c-SMAC.

the centrosome to the synapse is still not clear. Interaction of microtubules with the actin cortex at the synapse periphery *via* ezrin and Dlg1 appears to facilitate centrosome polarization (Lasserre et al., 2010). A process of microtubule bending at the synapse periphery mediated by the motor dynein has been proposed to facilitate microtubule tension and centrosome docking close at the synapse center (Kuhn and Poenie, 2002). Decreased F-actin polymerization at the centrosome could also

allow its detachment from the nucleus and its translocation, as shown in B cells (Obino et al., 2016). In turn, microtubules and the centrosome could control the F-actin remodeling at the synapse, as centriole depletion impairs actin clearance (Tamzalit et al., 2020). By converging toward the centrosome at the center of the synapse, microtubules guide polarized transport of vesicular components and organelles, such as the Golgi and several endosomes and secretory lysosomes (Kupfer and Dennert, 1984; Das et al., 2004; Chemin et al., 2012). The clearance of F-actin at the center of the synapse may be related to the localization of this secretory machinery. For instance, nitric oxide synthase-mediated post-translational modifications of actin may remodel the actin cytoskeleton by controlling polymerization/depolymerization (Garcia-Ortiz et al., 2017).

T cells can form simultaneously multiple synapses, integrating signal from several antigen-presenting cells, but polarize their cytokine secretory machinery mainly toward the one displaying the strongest stimulus (Depoil et al., 2005). In some instances, the T cell does not completely stop and forms asymmetric and not stabilized synapses, while it continues to move over the antigen-presenting cell. In this case, the cell–cell contact zone is called immunological kinapse. As in migrating cells, T cell presents an F-actin-rich lamellipodium, and the centrosome and vesicular components are localized at the uropod (reviewed by Fooksman et al., 2010). Interestingly, kinapses still permit durable interactions and TCR signal integration (Skokos et al., 2007; Moreau et al., 2012; Mayya et al., 2018). T cells may cycle between synapse and kinapse *in vitro* and *in vivo*, depending on the stimulation level, which may facilitate T cell interaction with several antigen-presenting cells (Sims et al., 2007; Moreau et al., 2015).

TCR Signaling Drives Cytoskeleton Reorganization

The initial TCR signaling occurring during the immunological synapse formation proceeds through serial reactions to control cytoskeleton reorganization. TCR-associated CD3 subunits are phosphorylated in their cytoplasmic regions on tyrosine-containing signaling motifs named immunoreceptor tyrosine-based activation motifs (ITAMs) (Barber et al., 1989; Reth, 1989). ITAM phosphorylation by Lck, a membrane-associated protein kinase of the Src family, induces ZAP70 recruitment to CD3 and its activation (Iwashima et al., 1994). Then, ZAP70 phosphorylates LAT, which in turn recruits SLP76 (Finco et al., 1998; Yablonski et al., 1998b; Zhang et al., 1998). Centrosome and microtubule repositioning requires efficient recruitment and activation of all these proteins (Lowin-Kropf et al., 1998; Kuhné et al., 2003; Tsun et al., 2011). Phosphorylated SLP76 binds the GEF Vav and the adaptor protein Nck (Wu et al., 1996; Wunderlich et al., 1999). The second signal received by T cells through the co-stimulatory molecule CD28 also allows the recruitment of Nck and Vav that bind to CD28 and can be activated in a TCR-independent manner upon CD28 engagement (Acuto et al., 2008). Vav activates the Rho family GTPases Rac1 and Cdc42 that together with Nck recruit and activate WAVE2 and WASP. As in migrating cells, WAVE2 and WASP then stimulate Arp2/3 ensuring actin polymerization and branching (Blumenthal and Burkhardt, 2020). Interestingly, WASP, together with PKCθ, controls the conversion of kinapses into synapses, as $WASP^{-/-}$ T cells cannot reform symmetric stable interaction with stimulatory surfaces after a cycle of migration (Sims et al., 2007). Vav, Rac1, Arp2/3, and formins have been involved in centrosome translocation, likely regulating the interplay between actin and microtubule networks (Ardouin et al., 2003; Gomez et al., 2007; Randzavola et al., 2019). TCR-induced signaling recruits at the synapse and activates actin cytoskeleton regulators involved in its polarization, cortical reorganization, and maintenance, such as dynamin 2, the cortactin homologue HS1, and the polarity regulator Dlg1 (Gomez et al., 2005, 2006; Round et al., 2005). Finally, clathrin accumulation at the synapse recruits the actin-polymerization machinery, indicating a relationship between the endocytic machinery and actin dynamics (Calabia-Linares et al., 2011).

Initial TCR triggering modifies the membrane phospholipid composition that controls F-actin organization at the synapse. LAT recruits PLCγ1, which metabolizes PIP_2, generating the second messengers diacyl glycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3), that respectively activate PKCs and calcium release from intracellular stores. CD28 recruits the phosphoinositide-3-kinase (PI3K), which converts PIP_2 into phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Both PIP_2 and PIP_3 regulate F-actin localization at the immunological synapse. Indeed, F-actin depletion from the center of the synapse correlates with a reduction of PIP_2 at the plasma membrane (Ritter et al., 2015; Gawden-Bone et al., 2018), whereas generation and maintenance of the actin-rich ring is controlled by the annular accumulation of PIP_3 at the synapse periphery (Le Floc'h et al., 2013).

DAG plays an important role in centrosome polarization (Quann et al., 2009; Liu et al., 2013; Chauveau et al., 2014). The mechanisms involved in microtubule anchoring at the synapse periphery and centrosome reorientation are complex and regulated by various effectors, underscoring the interplay between actin and microtubule cytoskeleton. These include membrane–microfilament linkers, such as ezrin, molecular motors, such as dynein, and polarity regulators, such as Dlg1 and Apc (Combs et al., 2006; Stinchcombe et al., 2006; Gomez et al., 2007; Martin-Cofreces et al., 2008; Bertrand et al., 2010; Lasserre et al., 2010; Liu et al., 2013; Aguera-Gonzalez et al., 2017). However, the interplay between these effectors is poorly understood.

Signaling Complexes Assembly and Regulation by the Cytoskeleton

Early TCR and co-stimulatory molecule signaling is responsible for bringing the actin polymerization machinery, regulators of its organization, and the centrosome and microtubules to the immunological synapse (**Figure 4**). However, a positive feedback loop exists since actin and microtubule cytoskeletons are in turn necessary for maintaining TCR signaling. They regulate the spatiotemporal organization of the signaling machinery, not only

reinforcing and sustaining signaling but also driving TCR signal downregulation (Nguyen et al., 2008; Lasserre et al., 2010).

Initially, TCRs, signaling and adhesion molecules, as well as cytoskeleton structures, are not uniformly distributed at the plasma membrane, possibly reflecting their distribution in microvilli (Jung et al., 2016; Cai et al., 2017; Ghosh et al., 2020). Then, they coalesce into concentric supramolecular activation clusters (SMACs) (Monks et al., 1998; Grakoui et al., 1999). A central-SMAC (c-SMAC) is enriched in TCR and associated proteins, such as CD3, co-signaling receptors, such as CD2 and CD28, inhibitory receptors, such as CTLA-4 and PD1, and their downstream signaling proteins (reviewed in Dustin and Choudhuri, 2016). Surrounding the c-SMAC, the peripheral SMAC (p-SMAC), containing integrins, such as LFA-1, and its cytoskeleton linkers as talin (Monks et al., 1998; Grakoui et al., 1999), stabilizes the synapse (Comrie et al., 2015). Finally, the distal SMAC (d-SMAC) contains large proteins, such as the protein tyrosine phosphatase CD45 (Davis and van der Merwe, 2006; Cordoba et al., 2013). The d-SMAC also corresponds to the peripheral actin ring and is enriched in microtubule linkers (e.g., IQGAP-1 and ezrin) (Roumier et al., 2001; Watanabe et al., 2004; Stinchcombe et al., 2006; Lasserre et al., 2010). This SMAC-type organization was mostly observed *in vitro* on stimulatory surfaces made of planar bilayers displaying ICAM-1 and MHC–peptide antigen molecules or using B cells as antigen-presenting cells. Indeed, when reducing the concentration of antigenic peptide or costimulatory molecules or studying different physiological conditions (e.g., T cells in different differentiation states and/or interacting with different antigen-presenting cells), the spatiotemporal pattern is highly diverse (reviewed in Thauland and Parker, 2010). For instance, in the case of the asymmetrical contacts formed in kinapses, the molecular organization at the uropod is reminiscent of the c-SMAC (reviewed in Dustin, 2008).

Upon initial TCR triggering, Lck, ZAP70, SLP76, and LAT are recruited at the plasma membrane close to the area of TCR stimulation (see "Alterations of T Cell Cytoskeleton and Molecular Traffic in Pathological Settings" section). Some of these molecules (e.g., TCR and LAT) are pre-clustered in separate stable domains before TCR stimulation that mix upon TCR engagement (Lillemeier et al., 2010; Beck-Garcia et al., 2015). Studying immunological synapse formation using activating planar bilayers as surrogate antigen-presenting cells and live cell TIRF microscopy revealed that once at the plasma membrane, these signaling molecules nucleate into dynamic microclusters in the d-SMAC where they are phosphorylated (Lee et al., 2002) and rapidly engage into a centripetal movement (Bunnell et al., 2002; Campi et al., 2005; Yokosuka et al., 2005; Varma et al., 2006; Kaizuka et al., 2007). The F-actin-rich ring acts as a scaffold for microcluster assembly and stabilization (Campi et al., 2005), whereas the microtubules seem to be dispensable for microcluster formation but needed for their centripetal movement (Lasserre et al., 2010; Hashimoto-Tane et al., 2011; **Figures 4B,C**).

Signaling microclusters have been shown to be surrounded by adhesion molecules similar to the p-SMAC and by F-actin enrichments, called foci (Kumari et al., 2015; Hashimoto-Tane et al., 2016; **Figure 4C**). The adhesion ring formation depends on LFA-1 signaling and actin dynamics, whereas actin foci are regulated by WASP (Kumari et al., 2015; Hashimoto-Tane et al., 2016). These observations suggest the existence of transient "micro-synapses" within the immunological synapse with similar structure but at a smaller scale. They likely provide scaffolds for TCR and signaling molecules clustering, promoting efficient signaling (Pageon et al., 2016).

Impairing actin cytoskeleton meshwork alters microcluster formation and TCR signaling. For instance, TCR and SLP76 microclusters do not form in T cell treated with latrunculin-A that depolymerizes F-actin (Campi et al., 2005; Babich et al., 2012). Furthermore, impairing F-actin dynamics, with jasplakinolide that stabilizes filaments, alters the centripetal movement of SLP76 microclusters, which cannot reach the c-SMAC (Babich et al., 2012). Accordingly, downstream events, such as calcium flux, NFAT1 activation, and interleukin (IL)-2 transcription, are also altered by actin inhibitors, although with differential effects depending on the dose used (Nolz et al., 2007). Similarly, to the events taking place in T cell migration, F-actin continuously pulls forces on the plasma membrane and the antigen-presenting cell due to contraction dependent on the molecular motor myosin II. Additionally, actin polymerization pushes forces and drives the retrograde flow of the actin network.

Microtubules have been recently involved in the regulation of these forces. Indeed, T cell treated with nocodazole displayed more sustained actin flow on activating planar bilayers (Hui and Upadhyaya, 2017). Together, these forces stabilize the actin cytoskeleton meshwork, allow the formation of the integrin-rich p-SMAC, and maintain the radial symmetry of the immunological synapse (Campi et al., 2005; Nguyen et al., 2008; Ilani et al., 2009; Hashimoto-Tane et al., 2011; Husson et al., 2011; Babich et al., 2012; Comrie et al., 2015). Mechanical forces and waves of actin polymerization also initiate the centripetal movement of signaling microclusters toward the p-SMAC (Campi et al., 2005; Yokosuka et al., 2005; Nguyen et al., 2008; Ilani et al., 2009; Yi et al., 2012; Comrie et al., 2015; Murugesan et al., 2016) and their segregation into the c-SMAC where signaling terminates (Lee et al., 2003; Varma et al., 2006; Kumari et al., 2012). Some signaling molecules (e.g., SLP76, LAT, and ZAP70) are downregulated in the p-SMAC, before reaching the c-SMAC (Yokosuka et al., 2005; Lasserre et al., 2011), whereas the TCR is downregulated in the c-SMAC by internalization (Lee et al., 2002, 2003; Varma et al., 2006; Vardhana et al., 2010) or by accumulation into extracellular vesicles (Choudhuri et al., 2014; Saliba et al., 2019).

Impairing the microtubule cytoskeleton alters microcluster centripetal movement (Bunnell et al., 2002; Lasserre et al., 2010; Hashimoto-Tane et al., 2011). For instance, SLP76 microclusters do not move to the c-SMAC in T cells silenced for ezrin or the polarity regulators Dlg1 and Apc that display altered microtubule network organization at the synapse (Lasserre et al., 2010; Aguera-Gonzalez et al., 2017). Likewise, perturbing the microtubule-associated molecular motor dynein impairs centripetal TCR microcluster movement

(Hashimoto-Tane et al., 2011). In addition, knockdown of the microtubule end-binding protein 1 (EB1) alters TCR dynamics at the immunological synapse and downstream signaling (Martin-Cofreces et al., 2012).

Importantly, impairing or slowing down microcluster movement toward the center of the synapse correlates with enhanced T cell signaling (e.g., higher level of phosphorylated LAT at the synapse and higher activation of Erk1/2), indicating that microcluster dynamics is linked to TCR signal downregulation (Mossman et al., 2005; Nguyen et al., 2008; Lasserre et al., 2010; Hashimoto-Tane et al., 2011). The molecular mechanisms involved in signaling complex deactivation and their relationship with microcluster centripetal movement are not fully understood. Several mechanisms may coexist at the synapse, including tyrosine dephosphorylation in the c-SMAC by the presence of the CD45 phosphatase (Varma et al., 2006) or post-translational modifications of signaling complexes facilitating their disaggregation (Lasserre et al., 2011).

In conclusion, while dynamic F-actin first initiates the formation of signaling microclusters, it subsequently leads to signaling molecule deactivation by targeting them to the c-SMAC, in close cooperation with the microtubule network. Therefore, a fine-tuned interplay between both cytoskeletons is key for sustaining TCR signaling and for conditioning its intensity and duration.

Vesicle Traffic Controls TCR Signaling and the Cytoskeleton

Targeting of organelles and intracellular vesicular compartments to the immunological synapse regulates T cell signaling and effector functions, as well as participates to the communication between T cells and antigen-presenting cells. Indeed, TCR–CD3 and two of its proximal signaling molecules, Lck and LAT, not only are localized at the plasma membrane, in part in microvilli, but also are present in endosomal and Golgi compartments. These molecules partition differently between plasma membrane and intracellular compartments, and their targeting to the immunological synapse is uniquely regulated. Targeting of vesicles carrying CD3ζ, Lck, and LAT to the immunological synapse follows TCR triggering and the formation of early microclusters containing phosphorylated forms of these proteins (Blanchard et al., 2002a; Ehrlich et al., 2002; Bonello et al., 2004; Balagopalan et al., 2013, 2018). This is consistent with the role of plasma membrane pools of these molecules in the initial signal triggering and of vesicular pools in signal amplification by fueling additional signaling molecules to the immunological synapse.

TCR–CD3 components exchange between the plasma membrane and recycling endosomes. Interestingly, although part of the same multi-subunit TCR–CD3 complex, the CD3ζ chain has a different turnover, and it is more concentrated in the endosomal compartment than in other subunits (reviewed in Alcover et al., 2018). Clustering of TCR–CD3 complexes at the synapse is maintained by microtubule-dependent polarized vesicle traffic (Blanchard et al., 2002a; Das et al., 2004; Soares et al., 2013) and controlled by several regulatory proteins. These include intraflagellar transport proteins and the microtubule-binding protein EB1, which interact with microtubules and TCR–CD3 components (Finetti et al., 2009; Martin-Cofreces et al., 2012), several Rab GTPases, and vesicle fusion regulators, such as the SNAREs VAMP-3, SNAP-23, syntaxin-4, and the calcium sensor synaptotagmin-7 (Das et al., 2004; Patino-Lopez et al., 2008; Finetti et al., 2009, 2015; Soares et al., 2013; Onnis et al., 2015). Altered expression of some of these regulators results in reduced TCR signaling and T cell activation (Finetti et al., 2009; Martin-Cofreces et al., 2012). Intraflagellar transport proteins are key for the formation of the primary cilium, a sensory structure present in many cell types. Although T cells lack primary cilia, they use the same molecular machinery, including IFT20, IFT57, and IFT88 proteins, to transport TCR–CD3 complexes to the synapse (Finetti et al., 2009). Likewise, T cells express and utilize SNARE proteins involved in vesicle fusion in other secretory cellular systems (Sudhof and Rizo, 2011) to control polarized traffic to the immunological synapse (Das et al., 2004; Soares et al., 2013; Finetti et al., 2015). Proteins controlling actin polymerization and branching, such as ARPC2 (Zhang et al., 2017) or WASH (Piotrowski et al., 2013), can also modulate TCR endosomal trafficking and its polarization, thus affecting T cell homeostasis and function.

Lck is partly associated with endosomes, and contrary to CD3ζ and LAT, its plasma membrane pool is bigger than the endosomal one (Soares et al., 2013). Intracellular Lck is mainly localized in the $Rab11^+$ recycling endosomal compartment (Soares et al., 2013; Bouchet et al., 2017). It constitutively recycles between the plasma membrane and pericentrosomal endosomes, and it is targeted to the immunological synapse soon after TCR engagement *via* endosomal polarization (Ehrlich et al., 2002; Anton et al., 2008). The Rab11 effector FIP3 (Rab11 family interacting protein-3) controls Lck subcellular localization, its clustering at the immunological synapse, and its signaling functions. FIP3 links Rab11 with microtubule molecular motors, such as dynein and kinesin, and with components of the exocyst complex controlling endosomal traffic (Horgan and McCaffrey, 2009). Interestingly, FIP3-mediated Lck localization conditions both basal and TCR-mediated phosphorylation of Lck substrates and intracellular calcium (Bouchet et al., 2017). Moreover, perturbing Lck endosomal localization by FIP3 silencing impairs constitutive CD3ζ phosphorylation and leads to increased total amount of CD3ζ and higher TCR–CD3 cell surface expression (Bouchet et al., 2017). This is consistent with the described effect of Lck-mediated phosphorylation on CD3ζ turnover (D'Oro et al., 2002). Therefore, Lck endosomal localization is key for a variety of Lck functions. Interestingly, Unc119, an adapter protein that activates Rab11 and recruits the actin-based molecular motor myosin 5B, controls Lck traffic in an opposite manner than FIP3. Unc119 also associates to CD3 and CD4 and facilitates Lck activation (Gorska et al., 2004; Gorska et al., 2009). Unc119A cooperates with the ciliary ARL-3 GTPase and its GEF ARL-13B to transfer active Tyr394-phosphorylated Lck to the immunological synapse (Stephen et al., 2018). Lck is associated with membrane rafts (Rodgers and Rose, 1996; Drevot et al., 2002). In this context, Lck localization is also regulated by MAL (Anton et al., 2008, 2011), a small tetraspanin associated

with membrane rafts and controlling their polarized intracellular traffic (Martin-Belmonte et al., 2003). Finally, the late endosomal transporter CD222 regulates Lck localization, intracellular traffic, and activation (Pfisterer et al., 2014). Interestingly, these different Lck traffic regulators seem to balance the anterograde (MAL, Unc119, and CD222) and retrograde (FIP3) Lck transport, key to regulate Lck function in T cell activation.

LAT cycles between the plasma membrane, endosomes, and the Golgi. The intracellular LAT compartment is polarized to the immunological synapse concomitantly with those of CD3ζ and Lck. Particular amino acid residues control LAT association to intracellular vesicle pools and its targeting to the synapse (Bonello et al., 2004). Intracellular LAT contributes to the synapse as a second wave, following the formation of microclusters derived from plasma membrane LAT (Bonello et al., 2004; Balagopalan et al., 2018). Several intracellular traffic regulators control LAT localization. Some are common with CD3ζ, such as flagellar transport proteins (Vivar et al., 2016), or vesicle docking and fusion regulators, such as the SNARE VAMP7 or the calcium sensor synaptotagmin-7 (Larghi et al., 2013; Soares et al., 2013). In addition, LAT undergoes retrograde transport from the plasma membrane and endosomes to the Golgi under the control of the Rab6 GTPase, the tSNARE syntaxin-16, and the golgin GMAP210 (Carpier et al., 2018; Zucchetti et al., 2019), which together facilitate LAT delivery to the immunological synapse and subsequent T cell activation. It is likely that a continuous traffic between the plasma membrane and endosomal and Golgi compartments takes place and is modified upon T cell contact with antigen-presenting cells. However, the spatiotemporal organization, sequence of events, and regulation of these events are still ill defined.

The mechanisms described above are thought to target TCR–CD3 complexes and Lck and LAT signaling molecules to the immunological synapse, fueling the formation of signaling microclusters at the plasma membrane. After their dynamic trip within microclusters, TCRs and some of its proximal signaling molecules may be internalized and either recycled back to the plasma membrane to participate in additional cycles of signaling, restored in the vesicular compartment, or degraded to downregulate TCR signaling. This may be modulated by post-transcriptional modifications, such as phosphorylation and ubiquitination (Cenciarelli et al., 1992; D'Oro et al., 1997; Valitutti et al., 1997; Wang et al., 2001; Bonello et al., 2004; Balagopalan et al., 2007, 2011; Huang et al., 2010; Ivanova and Carpino, 2016). Worth noting, the existence of a transient endosomal/Golgi compartment where signaling may continue has been inferred from the presence of active kinases and phosphorylated signaling molecules associated with intracellular compartments after TCR engagement (Luton et al., 1997; Yudushkin and Vale, 2010; reviewed in Alcover et al., 2018; Saveanu et al., 2019; Evnouchidou et al., 2020).

As described above, Rac1 and Cdc42 GTPases transduce TCR signals driving actin cytoskeleton remodeling during immunological synapse formation. These molecules were shown to be associated with vesicles in other cellular types (Phuyal and Farhan, 2019). Interestingly, we observed that a minor fraction of Rac1 in T cells colocalizes with Rab11$^+$ recycling endosomes, whereas most of the Rac1 protein seems to be associated with the plasma membrane or diffused in the cytosol. Interestingly, perturbing recycling endosome dynamics by overexpressing the Rab11 effector FIP3 concentrates Rac1 in pericentrosomal endosomes, whereas FIP3 silencing disperses endosomal Rac1 all over the cytoplasm. Importantly, FIP3 silencing releases the tight control of Rac1 on the actin cytoskeleton, inducing T cell overspreading on stimulatory surfaces (i.e., anti-CD3-coated) or on poly-L-lysine-coated surfaces. Moreover, FIP3-silenced cells form larger and asymmetrical immunological synapses. These shape changes could be due, at least in part, to a reduction of T cell rigidity. Therefore, Rac1 association and traffic *via* Rab11 endosomes is key to balance basal *versus* TCR-stimulated actin cytoskeleton rearrangements, perhaps by the differential compartmentalization of Rac1 and its regulatory molecules, such as the GEFs Vav1 or Tiam1. Finally, Rac1 endosomal traffic is required for the regulation of T cell activation leading to cytokine production (Bouchet et al., 2016, 2018).

Vesicle traffic to the synapse may also be involved in the termination of T cell activation, as the inhibitory receptor CTL4, which competes with CD28 co-stimulatory receptor, is also associated with an endo-lysosomal vesicular compartment, which is released at the synapse in a LYST-regulated manner (Linsley et al., 1996; Shiratori et al., 1997; Barrat et al., 1999; Iida et al., 2000).

Finally, T cells forming immunological synapses produce extracellular microvesicles containing TCRs, CD40L, ICOS, and tetraspanins (Blanchard et al., 2002b; Choudhuri et al., 2014; Saliba et al., 2019), as well as RNA and DNA (Mittelbrunn et al., 2011; Torralba et al., 2018). Extracellular vesicle protein and nucleic acid components undergo a process of molecular sorting, since extracellular vesicles are enriched in some components while lacking others (Villarroya-Beltri et al., 2013; Yanez-Mo et al., 2015; Saliba et al., 2019). They accumulate at the synaptic cleft, by a budding process regulated by ESCRT proteins (Choudhuri et al., 2014), where they may play a dual role: first, to reduce TCR cell surface expression to control T cell activation and second, to contribute to dendritic cell priming and maturation and B cell help. This may occur in two ways, by binding MHC–peptide antigen or stimulatory molecules on antigen-presenting cells, such as CD40 or ICOSL (Saliba et al., 2019), and by fusing and transferring their microRNA or DNA content (Mittelbrunn et al., 2011; Torralba et al., 2018).

Therefore, a complex balance of exchanges between the plasma membrane and intracellular vesicular compartments, involving the TCR, several signaling molecules, and an array of traffic regulatory proteins ensures TCR signal transduction and actin cytoskeleton remodeling. Distinct spatiotemporal localization of these various proteins may ensure the fidelity of TCR triggering and sustained T cell activation. Finally, the production of extracellular vesicles plays a key role on antigen-presenting cells regulation contributing to dendritic cell priming and maturation or B cell help. Importantly, some of these mechanisms may be altered by pathogen infections or specific genetic disorders. For instance, HIV-1 hijacks these processes to ensure viral replication and transmission and escape from the immune system (see "Alterations of

T Cell Cytoskeleton and Molecular Traffic in Pathological Settings" section).

Role of the Cytoskeleton in Signaling to the Nucleus

One of the consequences of antigen stimulation is the nuclear translocation of several transcription factors, such as nuclear factor of activated T cells (NFAT), nuclear factor kappa B (NFkB), and activator protein 1 (AP1), that play a central role in T cell activation, differentiation, and effector functions. Recent work has highlighted the involvement of the cytoskeleton in controlling this step, particularly in the case of NFAT.

The NFAT family of transcription factors encompasses five different members, two of them being expressed in T cells: NFAT1 (NFATc2 or NFATp) and NFAT2 (NFATc1 or NFATc) (Muller and Rao, 2010). A third member, NFAT4 (NFATc3 or NFATx), is preferentially expressed in thymocytes (Oukka et al., 1998). The expression of these factors may be differentially regulated: for instance, NFAT1 is constitutively expressed in T cells, whereas NFAT2 is induced upon T cell stimulation (Northrop et al., 1994; Lyakh et al., 1997).

In unstimulated T cells, NFAT transcription factors are phosphorylated on a series of serine residues that expand over the nuclear localization signal. Phosphorylation prevents NFAT nuclear translocation, ensuring cytoplasmic localization in resting T cells. NFAT activation is initiated by TCR-induced PLCγ1-dependent production of IP3 and consequent release of Ca^{2+} from ER stores (reviewed in Hogan et al., 2003). Low Ca^{2+} concentration in the ER lumen triggers the multimerization on ER membranes of the single transmembrane domain protein STIM that contacts the pore-forming ORAI proteins on the plasma membrane. As a result, Ca^{2+} influx from the extracellular space is stimulated (Zhang et al., 2005; Prakriya et al., 2006; Penna et al., 2008). The rise of intracellular Ca^{2+} leads to the rapid activation of the Ser/Thr-specific phosphatase calcineurin that binds to and dephosphorylates cytosolic NFAT proteins, leading to their nuclear import (Hogan et al., 2003). Once in the nucleus, NFAT usually acts together with other transcription factors. For instance, it interacts with AP1, FOXP3, or GATA family members (Macian et al., 2001; Monticelli et al., 2004; Wu et al., 2006) and functionally cooperates with NFkB to regulate the transcription of multiple cytokine genes (e.g., IL-2, IL-4, interferon gamma [IFNγ], and IL-17), transcription factors (e.g., FOXP3), or other receptors (e.g., CD25 and CTLA-4) (Muller and Rao, 2010). Notably, NFAT can also act alone to induce CD8 T cell exhaustion (Martinez et al., 2015).

Inactivation of NFAT and its nuclear export depends on the activity of multiple kinases, such as casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3), and the dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK), that phosphorylate specific motifs in the conserved N-terminal regulatory region (Okamura et al., 2004; Gwack et al., 2006). These kinases have been found to be constitutively associated with NFAT in a large cytoplasmic RNA–protein scaffold complex, which also contains the GTPase IQGAP and the noncoding RNA *NRON* (Sharma et al., 2011). Dephosphorylation of NFAT requires the dissociation of this complex and results in masking the nuclear export sequence (NES) in NFAT, exposing its nuclear localization sequences (NLS), as well as promoting its transcriptional activity (Okamura et al., 2000).

Once the NLSs are exposed, NFAT may reach nuclear pore complexes by simple diffusion in the cytoplasm, before its import into the nucleus. However, several data suggest a potential implication of the microtubule cytoskeleton in this process. Initial findings in neuroblast cells showed that treatments altering tubulin polymerization, such as decreasing cellular zinc or exposure to colchicine or vinblastine, prevent NFAT transport to the nucleus (Mackenzie and Oteiza, 2007). Further studies revealed that NFAT nuclear translocation depends on importin-β and requires tubulin acetylation (Ishiguro et al., 2011). Interestingly, our recent work (Aguera-Gonzalez et al., 2017) has revealed that endogenous NFATc2 forms discrete clusters juxtaposed to microtubules in unstimulated T cells. These clusters move closer to the immunological synapse surface at early time points after activation and then progressively move to the perinuclear region. Moreover, NFAT clusters progressively move away from microtubules, correlating with NFAT shuttling to the nucleus (Aguera-Gonzalez et al., 2017). Hence, these data suggest that the association of NFAT with the microtubule network could facilitate concentration of this transcription factor around the nucleus and/or its interaction with nuclear pores. In agreement with a functional link between NFAT and the microtubules, we have also observed that knockdown of several proteins that control the appropriate organization of the microtubule network, such as the polarity regulators Apc and Dlg1 and the actin–cytoskeleton linker ezrin, impairs NFAT nuclear translocation and transcriptional activity (Lasserre et al., 2010; Aguera-Gonzalez et al., 2017; Juzans et al., 2020). Altogether, these data underscore the involvement of microtubules in driving NFAT nuclear localization.

The role of actin cytoskeleton in NFAT activation is less clear. Indeed, treatment of cells with actin polymerization inhibitors has been shown to affect the Ca^{2+}/NFAT pathway; however, the effects were positive or negative depending on the cell type, dose, and/or stimulation protocol (Rivas et al., 2004; Mackenzie and Oteiza, 2007). This is likely due to the multiple roles of actin that is implicated in regulating T cell/antigen-presenting cell interactions, receptor triggering, and signaling complex dynamics at the immunological synapse (see "T Cell Sensing of Antigen Cues, TCR Triggering, and Immunological Synapse Formation" section). Several regulators of the actin cytoskeleton have been implicated in NFAT activation. These include the actin nucleators WASP and WAVE2 (Silvin et al., 2001; Nolz et al., 2006), the Ser/Thr kinase PAK1 (Yablonski et al., 1998a), the GTPase RhoG (Vigorito et al., 2003), and the GEF SLAT (Becart et al., 2008). However, in most cases, these proteins do not affect directly NFAT but act on upstream signaling proteins and/or Ca^{2+} influx. On the other hand, the aforementioned role of ezrin, which binds to actin and can act in concert with Dlg1 to organize the microtubule network at the immunological synapse (Lasserre et al., 2010), suggests that proper crosstalk between actin and microtubule cytoskeletons is required for NFAT nuclear translocation. Importantly, ezrin and Dlg1 may also control

NFAT activation *via* Dlg1 interaction with the p38 MAP kinase, indicating an influence of these cytoskeleton crosstalk regulators in TCR signaling (Round et al., 2007; Lasserre et al., 2010).

It is worth noting that both actin and microtubule cytoskeletons are involved in the reorganization of the ER and mitochondria in activated T cells, which is key to regulate TCR-induced Ca^{2+} signaling (reviewed in Babich and Burkhardt, 2013). As mentioned above, the ER has to move toward the plasma membrane in order to allow the contact between STIM oligomers and ORAI and trigger extracellular Ca^{2+} influx. This movement may directly involve microtubules (Grigoriev et al., 2008). On the other hand, the mitochondria have to be repositioned close to membrane Ca^{2+} channels to buffer Ca^{2+} concentration locally and keep these channels active (Ishii et al., 2006; Baixauli et al., 2011; Quintana et al., 2011; Quintana and Hoth, 2012). Polarization of both ER and mitochondria in this setting depends on the coordinated action of actin and microtubules and associated molecular motors (Babich and Burkhardt, 2013).

Finally, another structural link between the nucleus and immunological synapse modulating T cell functions involves A-type lamins. These proteins that belong to the intermediate filaments family and form the nuclear lamina have been shown to indirectly connect with actin and microtubules and affect T cell activation. Indeed, lamin A defective T cells have impaired actin and microtubule dynamics, altered signaling, and a lower ability to form immunological synapses (Gonzalez-Granado et al., 2014).

ACTIN–MICROTUBULE INTERPLAY SHAPING T CELL EFFECTOR FUNCTIONS

The cooperation between actin and microtubules is also key for T cell effector functions occurring at the synapse, such as lytic granule release or polarized cytokine secretion.

Lytic Granule Release

When cytotoxic T cells recognize target cells, lytic granules rapidly move along microtubules, cluster around the moving centrosome, and then polarize with it at the immunological synapse. Centrosome translocation and actin clearance at the synapse have been proposed to be key for lytic granule docking and fusion at the membrane and target cell killing (Stinchcombe et al., 2006; Ritter et al., 2015; **Figure 5**), although this seems not to be the sole mechanism (Bertrand et al., 2013; Tamzalit et al., 2020). Conversely, actin recovery terminates cytotoxic granule release (Ritter et al., 2017). At the plasma membrane, the centrosome defines a precise secretory domain next to the c-SMAC, concentrating perforin and granzymes in the synaptic cleft (Stinchcombe et al., 2001, 2006). Perforin and granzymes then induce target cell apoptosis. Interestingly, granule movement involves multiple molecular motors. Initially, dynein-dependent retrograde transport on microtubules brings lytic granules to the centrosome (Stinchcombe et al., 2006). Then, granules travel to the immunological synapse, together with the centrosome, and may be positioned close to the plasma membrane by microtubule-based anterograde movement-dependent kinesin motors (Kurowska et al., 2012) or just by the sole proximity of the centrosome to the synapse (Stinchcombe et al., 2006).

The alteration of any of these steps results in impaired lytic granule release. Indeed, the deficiency of several polarity and cytoskeleton regulators impacts both cytoskeleton and centrosome translocation. For instance, silencing of Dlg1 or Apc results in impaired F-actin remodeling, microtubule disorganization, and impaired centrosome and CD3 polarization at the synapse (Round et al., 2005; Lasserre et al., 2010; Humphries et al., 2012; Aguera-Gonzalez et al., 2017; Juzans et al., 2020). Therefore, Dlg1 and Apc modulate CTL immunological synapse formation and function, consequentially influencing both the lytic granule delivery to the synapse and the ability to kill target cells (Silva et al., 2015; Juzans et al., 2020). In addition, the impairment of actin regulators, such as WASP or the Arp2/3 complex, results in altered target cell elimination (De Meester et al., 2010; Randzavola et al., 2019). However, this does not affect lytic granule secretion, assessed by Lamp1 cell surface expression, but impair immunological synapse symmetry and stability (De Meester et al., 2010; Houmadi et al., 2018; Randzavola et al., 2019). Actin dynamics is thus necessary for efficient killing, while apparently not essential for lytic granule release. However, we have recently shown that Lamp1 cell surface measurement could not be sensitive enough to discriminate small secretion differences (Juzans et al., 2020).

Interestingly, several mechanisms of CTL killing may exist, and plasticity could be an attribute of cytotoxic immunological synapses. On the one hand, a mechanism has been described involving centrosome and cytotoxic granule polarization to a well-structured immunological synapse in which actin and microtubule dynamics orchestrate lytic granule delivery to target cells. On the other hand, various examples challenge this rule, questioning the importance of centrosome docking. For instance, the polarity regulator PKCζ is required for centrosome polarization in CD8 T cells, but not for efficient lytic granule release and target cell killing (Ludford-Menting et al., 2005; Bertrand et al., 2013). Its potential role in actin reorganization at the synapse has not been addressed to date, but it has been shown to control F-actin dynamics in migrating T cells (Real et al., 2007; Crespo et al., 2014). Lytic granule translocation to the cytotoxic synapse may occur in the absence of centrosome polarization, and CTLs may simultaneously kill several target cells (Wiedemann et al., 2006; Bertrand et al., 2013). Conversely, human B cells, by inducing weak CD2 signaling, may trigger non-polarized granule exocytosis by the CTLs, although the centrosome is at the synapse (Kabanova et al., 2016; Zurli et al., 2020). Finally, centriole deletion has no effect on lytic granule polarized secretion, but reduces killing efficiency by impairing lytic granule biogenesis and actin-induced forces at the synapse (Tamzalit et al., 2020).

Altering cytoskeleton organization and the interplay between cortical actin and microtubules affects synapse symmetry and stability (Ludford-Menting et al., 2005; Lasserre et al., 2010; Juzans et al., 2020). For instance, we have recently shown

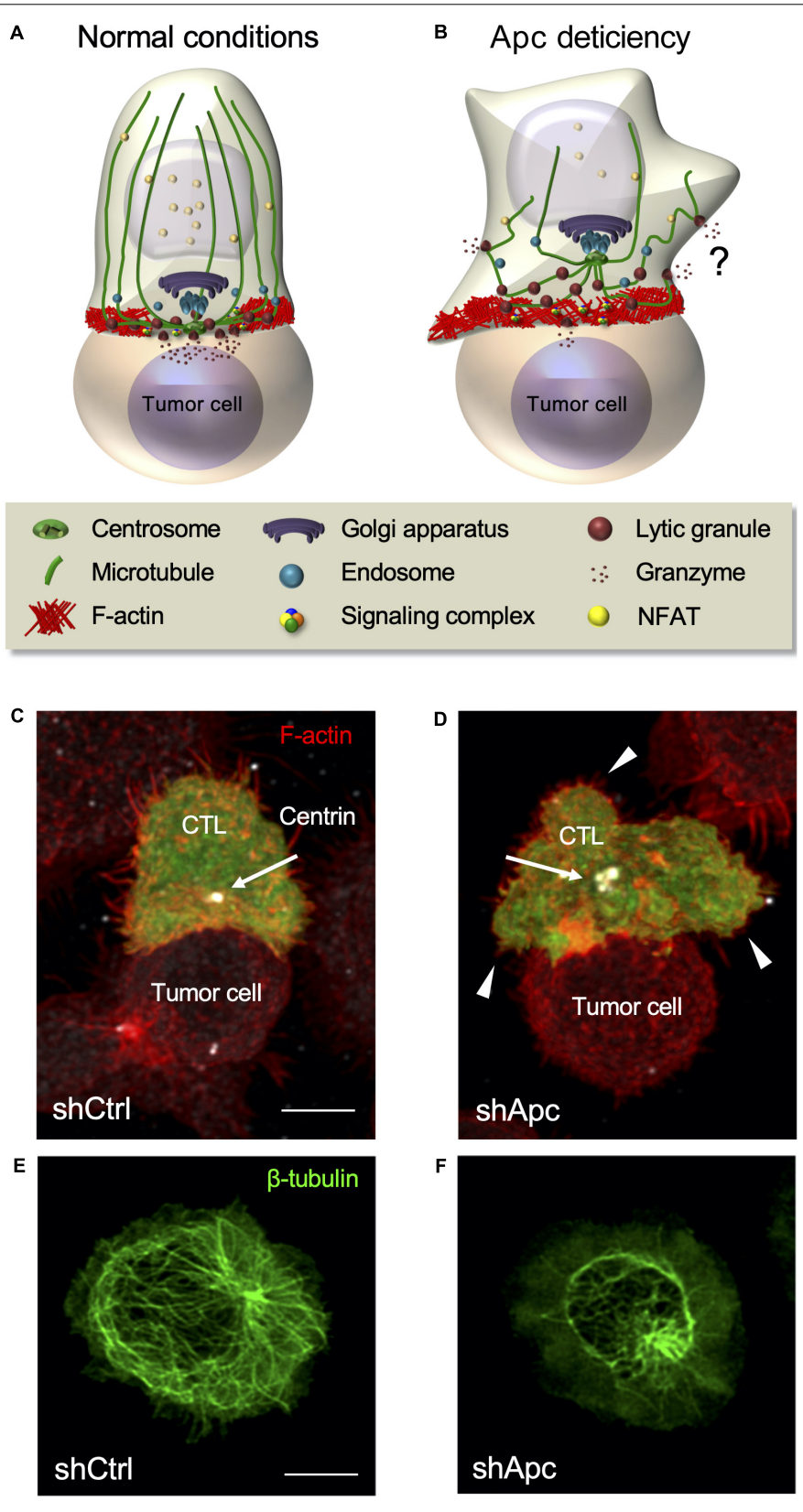

FIGURE 5 | Continued

FIGURE 5 | Defects of the polarity regulator and tumor suppressor Apc impair CTL function. **(A,B)** Schematic representation of CTL polarization leading to lytic granule release and killing of a tumor target cell **(A)**. Apc defects impair actin and microtubule reorganization at the immunological synapse, NFAT nuclear translocation, centrosome polarization, immunological synapse symmetry and stability, and lytic granule polarized release leading to tumor target cell killing **(B)**. Granule release is not completely hampered, and it might occur in an unpolarized manner. **(C,D)** Fluorescence confocal microscopy of a control **(C)** or Apc-silenced **(D)** human CTL encountering a tumor target cell coated with an anti-CD3 Ab. Arrows point to the centrosome and arrowheads to large membrane protrusions. Control CTL appears symmetric with the centrosome close to the center of the synapse, whereas Apc-deficient CTL appears dissymmetric with large membrane protrusions and the centrosome distant from the synapse. **(E,F)** Fluorescence confocal microscopy of control **(E)** or Apc-silenced **(F)** human CTLs stimulated anti-CD3-coated coverslips to form immunological pseudo-synapses. Alteration of the microtubule network is evident in Apc-silenced compared with control cells. Confocal images are from Juzans et al. (2020). Bar = 5 μm.

that Apc silencing results in altered synapse shape, symmetry, and stability (Juzans et al., 2020; **Figures 5A–D**). Interestingly, cytotoxic synapses may not require to be fully formed and stable to efficiently kill. Indeed, CTLs exhibit low TCR stimulation threshold to induce lytic granule release compared with the one required for efficient TCR signal transduction to the nucleus (Faroudi et al., 2003). Killing may occur with only three TCR–pMHC interactions, whereas stable synapse formation requires at least 10 interactions (Purbhoo et al., 2004). Finally, the formation of a mature synapse with typical SMAC pattern and CD2 enrichment is not always necessary for efficient cytotoxic granule release (Faroudi et al., 2003; Depoil et al., 2005; O'Keefe and Gajewski, 2005). Therefore, synapse stability may not be necessary for killing, but could increase its efficiency. Indeed, lytic granule release has been spatiotemporally correlated with the forces exerted by CTLs against target cell surfaces. These forces, due to the pushing–pulling action of the actin cytoskeleton, increase target cell membrane tension, which in turn enhances the perforin pore-forming activity (Basu et al., 2016; Tamzalit et al., 2019).

Therefore, actin and microtubule cytoskeleton mechanical properties are crucial for efficient target cell killing, but the extracellular environment may also play a key role. Since the conflicting results described above were most (if not all) obtained *in vitro*, it is possible that the *in vivo* requirements for an effective immune response are more stringent. Indeed, *in vivo*, CTLs act in a crowded environment of healthy or pathological tissues that generate strong forces and interact simultaneously with several cells. Detailed analyses *in vivo* will help to better understand cytotoxicity mechanisms in health and diseases (Boulch et al., 2019).

Cytokine Secretion

As perforin and granzymes, cytokine secretion involves the Golgi apparatus and the transit through secretory vesicles. Both Golgi and vesicles polarize with the centrosome and facilitate cytokine secretion at the immunological synapse, on a time scale of hours rather than minutes as for lytic granules (Kupfer et al., 1991, 1994; Huse et al., 2006). Interestingly, cytokine polarization is under the control of PKCζ, which is not involved in lytic granule secretion (Bertrand et al., 2010, 2013). Additionally, CD4 T cells can release cytokines in a multidirectional manner (Huse et al., 2006). This could facilitate the dispersion of local signals and the recruitment of target cells. Polarized and multidirectional pathways involve different molecular effectors and could depend on the secreted cytokine, e.g., IL-2, IL-10, and IFNγ are released at the synapse, whereas IL-4 and tumor necrosis factor alpha (TNFα) multi-directionally (Huse et al., 2006). However, this distinction may not be strict, since other authors reported polarized release of IL-4 and TNFα (Depoil et al., 2005; Hivroz et al., 2012). Different experimental setups may explain these differences, suggesting that *in vivo*, cytokine polarization may depend on the stimuli.

The importance of actin clearance from the center of the synapse has been much less addressed for cytokine secretion than for lytic granule release. The impairment of actin dynamics and clearance in Cdc42-silenced or WASP-deficient CD4 T cells significantly decreases IFNγ secretion, without altering its production (Morales-Tirado et al., 2004; Chemin et al., 2012). Interestingly, Cdc42 silencing also inhibits TNF secretion. However, in the setup used by the authors, TNFα is polarized at the synapse and does not appear to be secreted in a multidirectional manner (Chemin et al., 2012; Hivroz et al., 2012). As for lytic granule release (Ritter et al., 2015), impaired actin clearance from the secretion site could act as a physical barrier restraining the access of vesicles to the plasma membrane (Chemin et al., 2012). In addition, in WASP-deficient cells, disorganization of the cis-Golgi morphology appears to take place and could contribute to impaired secretion (Morales-Tirado et al., 2004). Interestingly, impaired actin clearance induced by Apc silencing in CTLs that correlates with reduced lytic granule release does not alter IFNγ nor TNF secretion (Juzans et al., 2020). Hence, the effects of Apc silencing on F-actin appear less significant than those of Cdc42 or WASP deficiency, and Apc may be replaced by another polarity regulator.

The microtubule cytoskeleton seems to play a specific role in the polarized secretion of cytokines. Indeed, nocodazole or vinblastine treatment, which impairs microtubule polymerization and centrosome polarization, alters IFNγ and IL-2 concentrations at the synapse and their polarized secretion. These cytokines are then secreted in a multidirectional manner, likely due to Rab relocalization (Huse et al., 2006; Ueda et al., 2015). On the contrary, nocodazole treatment of CD4 T cells has no effect on multidirectional secretion of TNFα (Huse et al., 2006). Therefore, microtubules would be crucial for cytokine-specific targeting at the synapse but not for their release. Importantly, their alteration could reorient cytokine polarized secretion to a multidirectional one (Huse et al., 2006; Ueda et al., 2015).

The expression of a truncated mutant of ezrin lacking F-actin binding domain that inhibits cortical interaction with the plasma membrane and microtubules leads to defective production of

IFNγ and IL-2, but not of TNF (Allenspach et al., 2001). This suggests that the microtubule role and their interplay with the actin are more significant for cytokine secretion in a polarized manner. However, little is known on the actin and microtubule cytoskeleton interplay in cytokine secretion.

Similarly, to what has been observed for lytic granule release, cytokine secretion may not require a well-structured immunological synapse. Indeed, IFNγ production is poorly correlated with extensive TCR clustering in CD4 T cells and depends on the stimulation conditions (Blanchard et al., 2004). However, IFNγ production still requires higher antigen stimulation of CTLs than lytic granule release (Valitutti et al., 1996; Faroudi et al., 2003). Moreover, *in vivo* secretion could be less stringent. Indeed, naive CD4 T cells interact successively with several antigen-presenting cells and undergo synapse–kinapse cycles, promoting IL-2 and IFNγ production (Celli et al., 2005; Sims et al., 2007). Therefore, CD4 T cells could form fewer stable synapses than expected. Interactions with several targets may provide signal integration and facilitate amplification of the immune response or target cell elimination. Furthermore, polarized secretion could still induce signal spreading as the immunological synapse does not spatially restrict IFNγ secretion by CTLs, allowing IFNγ bystander activity important to alter tumor environment (Sanderson et al., 2012; Hoekstra et al., 2020; Thibaut et al., 2020).

ALTERATIONS OF T CELL CYTOSKELETON AND MOLECULAR TRAFFIC IN PATHOLOGICAL SETTINGS

As mentioned above, infection of T cells by specific pathogens or genetic alterations may result in dysregulation of the cytoskeleton, endosomal trafficking, and/or their crosstalk, thus impairing TCR signaling, T cell activation, and effector functions. Two examples are described below, i.e., HIV-1 infection of T cells and inherited mutations of the Apc gene in familial adenomatous polyposis.

HIV-1 Subverts the Interplay Between Endosomal Traffic, TCR Signaling, and Actin Cytoskeleton

HIV-1 infects CD4 T cells hijacking T cell physiology to produce new viral particles and spread to other cells. Viral infection eventually leads to chronic infection and the production of viral reservoirs that escape host immune control. HIV-1 encodes several "accessory" proteins mediating the subversion of various cellular processes. Among these proteins, Nef is key for *in vivo* viral replication and AIDS pathogenesis. Nef is expressed soon upon infection and has pleiotropic effects in T cells, modifying the intracellular environment to enhance virus replication, while reducing host immunity (Fackler et al., 2007). Nef expression subverts endosomal traffic, actin cytoskeleton regulators, and T cell signaling effectors in infected T cells. As a consequence, HIV-1 infection: (i) modifies cell surface expression of several T cell molecules, including CD4, CD28, and MHC class I and II (Pereira and Dasilva, 2016); (ii) reduces actin accumulation at the synapse and alters related features, such as T cell shape, membrane protrusions, cell spreading, and T cell motility (Fackler et al., 1999, 2000; Haller et al., 2006; Rauch et al., 2008; Nobile et al., 2010; Stolp et al., 2010, 2012; Lehmann et al., 2011); and (iii) modulates T cell activation by affecting various signaling pathways, including those controlling activation and apoptosis (Fackler et al., 2007; Abraham et al., 2012; Markle et al., 2013).

The action of Nef on actin cytoskeleton occurs at different levels and appears to affect different stages of the virus life cycle, including virus entry and viral particle assembly, and egress from infected cells and transmission to other cells (Stolp and Fackler, 2011; Bracq et al., 2018). In addition, Nef modifies some intracellular vesicle traffic pathways and as a consequence cellular processes depending on protein transport (Pereira and Dasilva, 2016). Interestingly, Nef perturbs endosomal recycling and hijacks Lck and Rac1 endosomal traffic leading to their concentration in partially overlapping intracellular compartments, thus preventing the formation and signaling function of the immunological synapse (**Figure 6**; Thoulouze et al., 2006; Del Rio-Iniguez et al., 2018). Nef also limits the communication between LAT and SLP76 adaptors, reducing their capacity to form signaling complexes at the immunological synapse (Abraham et al., 2012). Hence, HIV-1 infection interferes with a key intracellular regulatory hub that ensures the interplay between vesicle traffic, T cell signaling, and actin cytoskeleton remodeling (Bouchet et al., 2016, 2017). Moreover, by concentrating Lck in recycling endosomes, HIV-1 may generate an endosomal signaling compartment, which concentrates Lck in its active form (phosphorylated on Tyr394, see **Figure 6**), together with tyrosine phosphorylated (i.e., active) species of other signaling molecules, such as CD3ζ, ZAP70, SLP76, and Vav1. In contrast, LAT, associated with different endosomes than Lck, is not concentrated in this compartment. The Nef-induced endosomal compartment likely generates T cell activation signals since a concomitant upregulation of early activation and cytokine genes was observed in the absence of TCR stimulation (Pan et al., 2012; Del Rio-Iniguez et al., 2018). Indeed, impairing the formation of the Nef-induced Lck compartment, by interfering with the endosomal transport regulator FIP3, prevented the upregulation of Nef-induced genes (Del Rio-Iniguez et al., 2018). Interestingly, Nef also extensively sequesters Rac1 in an intracellular compartment partially overlapping with that of Lck. In this manner, Nef modulates Rac1-dependent actin cytoskeleton remodeling and reduces T cell spreading. Thus, by hijacking the endosomal traffic of Lck and Rac1, Nef modulates signaling and actin cytoskeleton-mediated processes in infected T cells (Del Rio-Iniguez et al., 2018).

Therefore, HIV has evolved to subtly modify several regulatory cellular processes at key points of their crosstalk *via* the expression of the viral protein Nef. This may contribute to active steps of virus cycle leading to its replication (Fackler et al., 2007; Pan et al., 2012). It

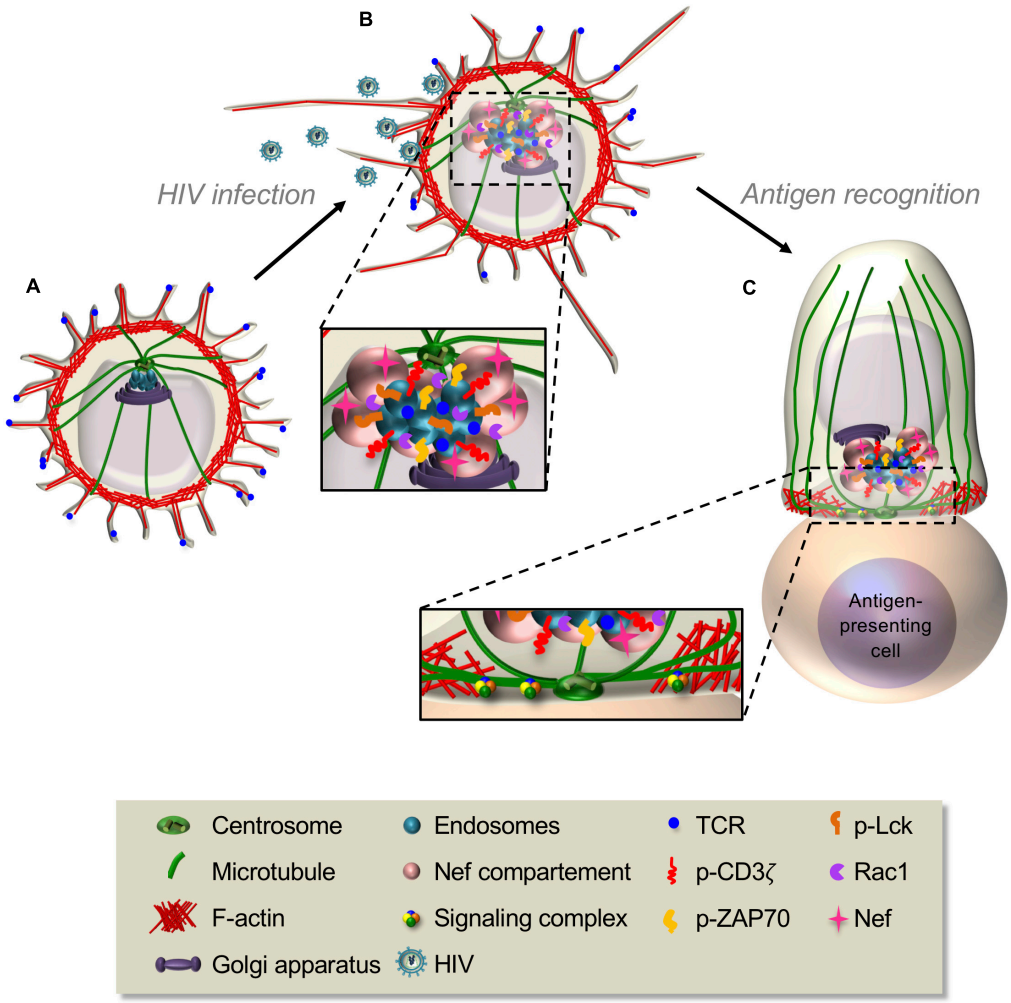

FIGURE 6 | HIV-1 subverts endosomal traffic, TCR signaling, and actin cytoskeleton. **(A,B)** Upon HIV-1 infection, the expression of the viral protein Nef induces pleiotropic effects in the infected T cell, including changes in actin cytoskeleton dynamics and intracellular vesicle traffic. Actin cytoskeleton changes are likely responsible for cell shape modifications (e.g., infected T cells produce less ruffles and longer filopodia), whereas hijacking of endosomal traffic drives changes in the expression of several cell surface molecules and the concentration close to the centrosome of active phosphorylated forms of several proximal TCR signaling molecules, including Lck (p-Lck), the CD3ζ subunit (p-CD3ζ), the tyrosine kinase ZAP-70 (p-ZAP-70), the adapter SLP76, and the cytoskeleton regulators Vav1 and Rac1 (only some depicted here and not depicted in **(A)** because their pattern is diffuse). This Nef-induced "endosomal signaling compartment" (depicted in light blue), which partially overlaps with a larger intracellular compartment containing Nef (in pink), appears to drive the expression of several activation genes, independently of TCR engagement. **(C)** Nef-induced perturbation of signaling molecules and actin cytoskeleton leads to the generation of defective immunological synapses that display fewer signaling complexes and contain a separate endosomal signaling compartment that impairs the TCR signaling cascade (compare with **Figure 1E**).

could also be important for inducing latency of infected T cells that favors virus reservoirs and avoids host immune control.

The Tumor Suppressor Apc in T Cell Physiology and Pathology

Apc is a cell polarity regulator and tumor suppressor whose mutations are associated with familial adenomatous polyposis and colorectal cancer development. Patients suffering from familial polyposis develop hundreds to thousands of polyps in their colon and/or rectum that finally turn into carcinomas if not removed by surgery (Lesko et al., 2014).

Thanks to its multiple binding domains (**Figure 2**), this large (310-kDa) protein is involved in several cell functions. The central region of Apc contains short peptide motifs that bind the transcriptional co-activator β-catenin and three sets of SAMP (Ser-Ala-Met-Pro) domains that bind Axin to regulate β-catenin (Rubinfeld et al., 1993). Due to its involvement in a protein complex controlling β-catenin degradation, Apc is mostly known for its implication in the Wnt/β-catenin signaling pathway that is essential during embryonic development and crucial for intestinal epithelium homeostasis.

Apc N-terminal portion contains a dimerization domain and an armadillo repeat domain. The latter interacts with many

cytoskeleton regulators as the actin and microtubule regulator IQGAP-1, the Rho GTPase Cdc42, the Rho GTPase regulator Asef, or kinesin regulators (Kawasaki et al., 2000; Jimbo et al., 2002; Watanabe et al., 2004; Sudhaharan et al., 2011). The C-terminal portion contains domains binding other cytoskeleton regulators, including the microtubule plus-end binding protein EB1 and Dlg1, but also a basic domain directly interacting with microtubules and modulating their elongation and stability and cell polarity (Munemitsu et al., 1994; Nakamura et al., 2001). This basic domain stimulates F-actin nucleation and filament bundling (Moseley et al., 2007; Okada et al., 2010). Finally, Apc has been shown to interact directly and indirectly with nuclear pore and nuclear transport proteins and apoptosis- or mitosis-related proteins (Nelson and Nathke, 2013).

Although Apc involvement in familial adenomatous polyposis and colorectal cancer has been extensively investigated, most studies concern how and why the epithelium is altered to form premalignant lesions, without questioning if Apc mutations could also alter immunosurveillance processes. Some studies conducted in Apc mutant mice have shown altered control of inflammation by Tregs (Akeus et al., 2014; Chae and Bothwell, 2015). These cells present impaired expression and/or activity of transcription factors key for their effector function regulation, such as FoxP3 and Gata-3, and as a consequence, their differentiation and production of anti-inflammatory cytokines, such as IL-10, are decreased (Gounaris et al., 2009; Aguera-Gonzalez et al., 2017). Interestingly, some studies showed that Apc mutant Tregs start to produce the pro-inflammatory cytokine IL-17 (Gounaris et al., 2009; Chae and Bothwell, 2015), described to promote tumor progression (Chae et al., 2010; Chae and Bothwell, 2015).

Variable alterations of T cell development and survival were observed in mouse models according to the extent and timing of Apc defects. For instance, conditional deletion of Apc in CD4 T cells induced Wnt pathway activation and apoptosis of mature cells leaving the thymus, resulting in lymphopenia (Wong et al., 2015). Likewise, thymocyte-specific Apc loss leads to extensive thymic atrophy due to a blockade of T cell development at the double negative stage (Gounari et al., 2005). Conversely, we observed in $Apc^{Min/+}$ mice, bearing a heterozygous mutation in the Apc gene, increased lymphocyte numbers in the spleen and lymph nodes.

Few studies have questioned if Apc loss or mutation directly affects T cell functions at the molecular level. We recently unveiled a direct involvement of Apc in T cell biology and the molecular mechanism responsible for the altered inflammatory control in Apc mutant mice intestine. As mentioned above, Apc loss impairs microtubule organization at the immunological synapse and centrosome reorientation toward the cell contact area in human CD4 T cells (Aguera-Gonzalez et al., 2017). Moreover, we observed that the NFAT transcription factor forms microclusters along microtubules. Therefore, Apc-dependent alteration of the microtubule network impairs NFAT nuclear translocation and its transcriptional activity. Intestinal Tregs from Apc mutant mice appear particularly affected, since they undergo altered differentiation and produce lower amount of the anti-inflammatory cytokine IL-10 (Aguera-Gonzalez et al., 2017), suggesting a dysregulation of the intestinal microenvironment at precancerous stages.

Recently, we showed that Apc is involved in cytoskeleton remodeling at the immunological synapse of CTLs. Indeed, Apc depletion impairs both microtubule and actin cytoskeletons, and as a consequence, it alters the morphology and stability of cytotoxic synapses formed by *ex vivo* differentiated CTLs. Additionally, polarized targeting and dynamics of lytic granules, as well as their fusion at the plasma membrane, are affected, thus diminishing the efficiency of tumor target cell killing by Apc-defective CTLs (Juzans et al., 2020) (see **Figure 5**). This phenotype shares some similarities with the one of CTLs from Wiscott-Aldrich Syndrome patients, who carry mutations in the gene encoding WASP and present actin cytoskeleton defects (De Meester et al., 2010; Houmadi et al., 2018) reducing their tumor cell killing ability.

Collectively, these data highlight how functional defects of the polarity regulator Apc may have a dual impact on familial adenomatous polyposis and colorectal cancer development, first, by altering the intestinal epithelial homeostasis, and second, by impairing T cell surveillance functions, further favoring the development of precancerous lesions and tumor growth.

CONCLUDING REMARKS

As we reviewed here, the fine interplay between actin and microtubule cytoskeleton and intracellular vesicle traffic is crucial for T cell functions, from migration to TCR signaling, immunological synapse formation, T cell activation, and effector functions. The detailed molecular mechanism of this crosstalk is not fully understood. An array of molecules linking cytoskeletal structures and their regulatory molecules, together with those linking plasma membrane-anchored proteins with the cytoskeleton, is key for this regulation, and their specific action needs further investigation. Likewise, novel cellular features needing cytoskeleton interplay are currently being unveiled. For instance, the role of mechanical forces in T cell physiology is becoming a field of active investigation, and the role of cytoskeletal crosstalk needs its further integration in these processes. *In vivo*, T cells continuously move in a crowded environment from which they may receive mechanical cues. In this sense, intermediate filaments, a third important component of the cell cytoskeleton, appear to play a key role in other cells in ensuring mechanical cell stability, as well as mechano-transduction from the cell surface to the nucleus. Intermediate filament dynamics, function, and interplay with various cell components are still poorly investigated in T cells and will be an interesting field of investigation. Interesting, polarity regulators as Apc ensure the interplay between the three cytoskeletal structures.

AUTHOR CONTRIBUTIONS

MM and MJ created the figures. AA and VDB edited the figures. All authors contributed equally in writing and editing the manuscript and read and approved the submitted version.

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REVIEW
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Modulation of TCR Signaling by Tyrosine Phosphatases: From Autoimmunity to Immunotherapy

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Early TCR signaling is dependent on rapid phosphorylation and dephosphorylation of multiple signaling and adaptor proteins, leading to T cell activation. This process is tightly regulated by an intricate web of interactions between kinases and phosphatases. A number of tyrosine phosphatases have been shown to modulate T cell responses and thus alter T cell fate by negatively regulating early TCR signaling. Mutations in some of these enzymes are associated with enhanced predisposition to autoimmunity in humans, and mouse models deficient in orthologous genes often show T cell hyper-activation. Therefore, phosphatases are emerging as potential targets in situations where it is desirable to enhance T cell responses, such as immune responses to tumors. In this review, we summarize the current knowledge about tyrosine phosphatases that regulate early TCR signaling and discuss their involvement in autoimmunity and their potential as targets for tumor immunotherapy.

Keywords: phosphatase, T cell, TCR signaling, autoimmunity, immunotherapy

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INTRODUCTION

Effective T cell responses require naïve T cell activation, proliferation and differentiation into effector and memory cells. Naïve T cells are activated when their T cell receptors (TCR) interact with a specific antigen presented by the major histocompatibility complex (MHC) on an antigen presenting cell (APC). In this process the extracellular engagement sensed by the TCR must be transmitted to the inside of the cell, whereupon signaling must propagate rapidly and alter gene expression to induce a lasting cellular response. After the response has been triggered, signaling must be turned off. Therefore, TCR signal propagation must be fast and reversible. These qualities are provided by post-translational protein modifications (reviewed in Deribe et al., 2010) that alter the properties of a protein by reversible addition of a chemical group such as a phosphate (phosphorylation) or another protein such as ubiquitin (ubiquitination) to one or more amino acids. Tyrosine phosphorylation is one of the main, although by no means only, post-translational modification driving early TCR signaling.

TYR PHOSPHATASES

Tyrosine phosphorylation controls a wide range of cellular processes in eukaryotic cells and is regulated by the opposing dynamic activities of tyrosine kinases and phosphatases. In fact, there is a similar number of both groups of enzymes in the human genome: 84 genes encode for catalytically active tyrosine kinases (Robinson et al., 2000; Manning et al., 2002) and 74 for phosphatases

known to dephosphorylate Tyrosine residues (hereafter Tyr phosphatases), all of which have mouse orthologs (Alonso and Pulido, 2016). This review will focus on Tyr phosphatases, which belong to the protein tyrosine phosphatase (PTP) superfamily, also known as the PTPome (Alonso et al., 2004; Alonso and Pulido, 2016).

Tyr phosphatases share a catalytic mechanism, in which the catalytic residue performs a nucleophilic attack on the phosphate group of the substrate, leading to the formation of an intermediate that is subsequently hydrolyzed (Tonks, 2006). The catalytic residue is generally cysteine, with a few exceptions such as the STS phosphatases, in which aspartic acid performs the nucleophilic attack (Alonso and Pulido, 2016). Tyr phosphatases are very diverse in terms of structural domains and motifs, which contributes to their heterogeneous subcellular localization. A subgroup of receptor Tyr phosphatases have a transmembrane domain that places them on the plasma membrane, where they can control cellular responses to extracellular stimuli. Some cytoplasmic phosphatases have an SH2 domain that allows them to bind Tyr-phosphorylated proteins, which are often transmembrane receptors and adaptors. This provides a rapid and reversible mechanism to direct phosphatases to the inner face of the plasma membrane, where they can regulate membrane proximal signaling in a dynamic manner. Phosphatases with a FERM domain interact with actin and localize at the interface between the plasma membrane and the cortical cytoskeleton. Phosphatases with a nuclear localization and/or a nuclear export signal are restricted to the nucleus or to the cytoplasm, or shuttle between both compartments. This diversity is relevant since it gives the phosphatase family the potential to regulate any cellular process in any subcellular region.

Tyr Phosphatases in T Cells

The essential role of tyrosine phosphatases in regulation of T cell activation was highlighted by early experiments in which pervanadate, a potent inhibitor of tyrosine phosphatases, was administered to T cells *in vitro* (Heffetz et al., 1990). Treatment of T cells with pervanadate resulted in rapid activation of the cells, including induction of proximal TCR signaling and production of IL-2, despite the absence of TCR engagement (Secrist et al., 1993). This finding shows that, taken as a whole, phosphatases dominate over kinases to maintain T cells in a resting state in the absence of antigenic stimulation. However, the picture is much more nuanced, as multiple phosphatases are involved, with potentially overlapping roles, to regulate both T cell homeostasis and responses. In addition, some phosphatases are required to initiate TCR signaling, such as CD45, while others amplify it, such as low molecular weight phosphotyrosine protein phosphatase, LMPTP. Clinical observations also point to an important role of Tyr phosphatases in T cell signaling and immunity. It has been demonstrated that perturbations in the expression or function of some Tyr phosphatases can lead to immunodeficiency on the one hand, when the altered phosphatase, for example CD45, is required for TCR signaling (Kung et al., 2000; Tchilian et al., 2001), or on the other hand, autoimmunity, when the altered phosphatase is a negative regulator of TCR signaling, for example, protein tyrosine phosphatase (PTP)N22 (Todd et al., 2007; Bottini and Peterson, 2014). These observations underscore how phosphatases are key in maintaining a delicate balance between immune responses that provide protection from infectious agents, while maintaining self-tolerance that prevents autoimmune disorders.

Of the 74 Tyr phosphatases in the genome, 37 were detected in a recent proteomic study of murine mature CD4 and CD8 T cells (Howden et al., 2019; **Figure 1**). Of note, this study found that the abundance of several phosphatases was modulated during differentiation of murine CD8 and CD4 T cells and/or T cell activation. Such regulated expression is consistent with previous data on human CD4 T cells (Castro-Sanchez et al., 2017), and highlights that both the number of phosphatases and the protein abundance of each expressed phosphatase shapes the T cell phenotype and the manner in which a T cell responds to antigen. Alteration of protein abundance, however, takes at least minutes if not hours or days to achieve, while early TCR signaling occurs within seconds. In this temporal scale, spatial regulation of proteins is the most efficient mechanism to control local protein concentrations. Early TCR signaling takes place in the context of the immunological synapse, a highly organized, dynamic contact between a T cell and an APC (reviewed in Dustin, 2014). To regulate TCR proximal signaling events, phosphatases must polarize to the area of the interaction, and position in close proximity to their substrates. The substrates are often transmembrane proteins, such as the ζ-chain, or cytoplasmic proteins localized at the inner face of the plasma membrane, such as the SRC-family kinase LCK. How do cytoplasmic phosphatases reach these substrates? Which adaptors or scaffolding proteins aid in the localization of phosphatases that themselves may lack specific localization domains or motifs? These questions have been frequently overlooked but answering them would greatly improve our understanding of the often nuanced manner by which Tyr phosphatases regulate T cell activation in health and disease.

To date, 15 Tyr phosphatases have been reported to regulate molecules involved in early TCR signaling (**Table 1**). In this review we will discuss their role in controlling proximal TCR signaling, their implication in autoimmunity and their potential as targets in immunotherapy.

Regulation of Early Tcr Signaling by Tyr Phosphatases

Signaling downstream of the TCR occurs through a network of rapid phosphorylation events on tyrosine residues of several effector and adaptor proteins (reviewed in Courtney et al., 2018). The TCR lacks intrinsic enzymatic activity, hence it relies on the SRC-family kinases LCK and FYN to initiate signaling. LCK phosphorylates CD3 and ζ-chains on their immunoreceptor tyrosine-based activation motifs (ITAMs) (Straus and Weiss, 1992; van Oers et al., 1996). These serve as docking sites for the recruitment of the 70 KDa ζ-chain associated protein kinase, ZAP70, to the TCR, where it is phosphorylated and activated by

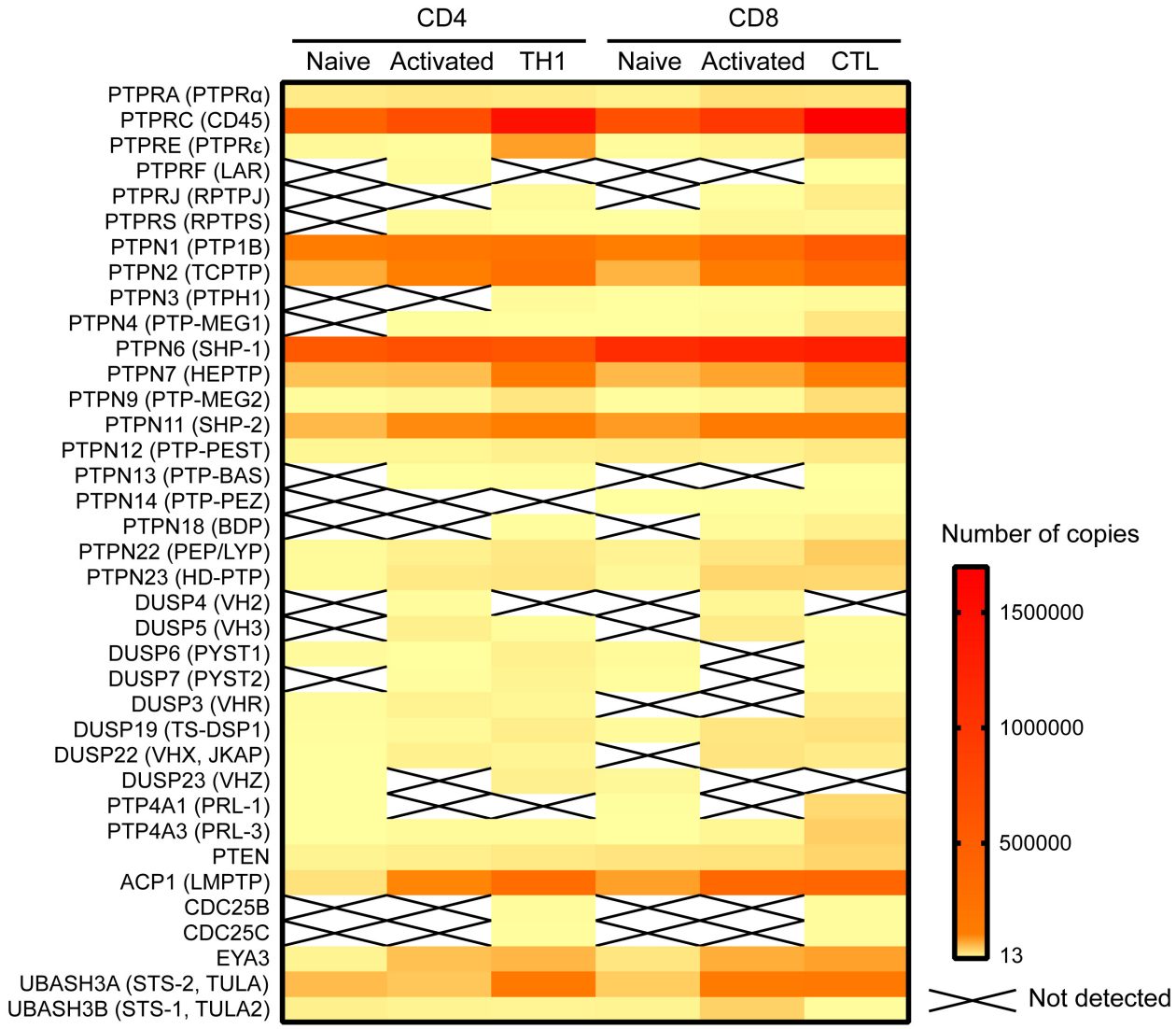

FIGURE 1 | Expression of Tyr phosphatases in primary murine CD4 and CD8 cells. Data on the number of copies of Tyr phosphatases was extracted from the proteomic study by Howden et al. (2019) and visualized in a heatmap using Prism software.

LCK (Chan et al., 1992; van Oers et al., 1996). Active ZAP70 phosphorylates, amongst other substrates, the scaffold protein linker for activation of T cells (LAT), which leads to the formation of a molecular complex that induces further distal signaling, resulting in T cell activation and effector function (Sommers et al., 2004). By regulating these proximal TCR signaling events, Tyr phosphatases determine activation thresholds and signal intensity and duration.

Regulation of SRC Family Kinases to Set the Activation Threshold and Maintain Peripheral Tolerance

Survival and functionality of naïve T cells in the periphery requires continuous tonic signals from self-peptide loaded MHC molecules (van Oers et al., 1993; Stefanova et al., 2002). However, this tonic signaling must not trigger cell activation, otherwise autoimmune pathology may develop. A precise threshold of T cell activation must therefore be set to ensure that naïve T cells are not activated by self-antigens but are able to respond to foreign antigens. Precisely how this equilibrium is maintained by subtle interactions between multiple signaling molecules is incompletely understood. A key initial trigger that has been well described is the regulation of the activity of the SRC-family kinases LCK and FYN (Seddon and Zamoyska, 2002) by phosphor/dephosphorylation of key residues (**Box 1**).

CD45

The highly expressed receptor-type tyrosine-protein phosphatase C (commonly known as CD45, encoded by the gene *PTPRC*) keeps LCK in a poised activation state in naïve T cells by dephosphorylating LCK^{Y505} (Stone et al., 1997;

TABLE 1 | Tyr phosphatases reported to regulate early TCR signaling.

Gene	Protein	Substrate in early TCR signaling	Localization features or domains
PTPRA	RPTPα	LCK, FYN	TM Receptor phosphatase
PTPRC	CD45	LCK, FYN, ζ-chain	TM Receptor phosphatase
PTPRE	RPTPε	LCK	TM Receptor phosphatase
PTPRH	SAP-1	LCK	TM Receptor phosphatase
PTPRJ	CD148	LCK	TM Receptor phosphatase
PTPN2	TCPTP	LCK, FYN	Nuclear and ER localization signals
PTPN3	PTPH1	ζ-chain	FERM domain
PTPN4	PTP-MEG1	ζ-chain	FERM domain
PTPN6	SHP1	LCK, ζ-chain, ZAP70	SH2 domains
PTPN11	SHP2	ZAP70, CD28,	SH2 domains
PTPN22	LYP	LCK, ζ-chain, ZAP70	Polyproline regions
DUSP22	VHX	LCK	Myristoylation signal
ACP1	LMPTP	ZAP70	None defined
UBASH3A	STS-2, TULA	ZAP70	UBA, SH3
UBASH3B	STS-1, TULA2	ZAP70	UBA, SH3

TM, Transmembrane; ER, Endoplasmic reticulum; FERM, protein 4.1, ezrin, radixin, moesin; SH2, Src Homology 2; UBA, Ubiquitin-Associated; SH3, Src Homology 3.

BOX 1 | Regulatory mechanism of key kinases involved in early TCR signaling transduction.

Src family kinases LCK and FYN. LCK activity is regulated by phosphorylation of two key residues, Y505 and Y394 (Yamaguchi and Hendrickson, 1996; Boggon and Eck, 2004). Phosphorylation of Y505 in the LCK C-terminal domain by the kinase CSK prompts an inhibited, *closed* conformation. Dephosphorylation of this inhibitory residue raises a *primed* conformation (Bergman et al., 1992), which allows trans-autophosphorylation on Y394 in the activation loop, leading to the fully active *open* conformation. A fourth conformation with both Y394 and Y505 phosphorylated has been found in T cells, and *in vitro* data suggests that this conformation is also active (Nika et al., 2010). FYN is regulated in a very similar way as LCK (Salmond et al., 2009). Phosphorylation of the inhibitory Y528 by CSK inactivates it, while dephosphorylation of this residue allows autophosphorylation on the activating residue Y417, resulting in full activation. Upon TCR stimulation, active LCK can also phosphorylate FYN^{Y417}, activating it (Filipp et al., 2008).

ZAP70. The activation of ZAP70 is regulated by localization and phosphorylation (reviewed in Au-Yeung et al., 2018; **Figure 4**). Binding of the SH2 domains of ZAP70 to pTyr in ITAMs of the ζ chains induces a conformational change in ZAP70 that aligns the SH2 domains, leading to increased affinity for the phosphorylated ITAMs, and exposes its activation loop, while also localizing ZAP70 in the proximity of LCK. Then, LCK phosphorylates Y315 and Y319 on the activation loop, and phosphorylation of Y493 on the kinase domain either by LCK or by autophosphorylation leads to full activation of ZAP70. Phosphorylation of Y292 on the activation loop and of Y492 on the kinase domain of ZAP70 dampen kinase activity, although the mechanism is not fully understood.

Seavitt et al., 1999; **Figure 2A**). This maintains basal levels of ζ-chain phosphorylation and provides tonic signaling needed for survival of naïve T cells (reviewed in Zamoyska et al., 2003). At the same time, and to prevent naïve T cell activation in the absence of antigen stimulation, CD45 also dephosphorylates LCK^{pY394}, inactivating it (D'Oro et al., 1996). The latter dephosphorylation, however, requires the high CD45 expression levels displayed by mature T cells. In fact, experiments manipulating CD45 expression have shown that T cells with very low amounts of CD45 had impaired T cell responses, because LCK is not sufficiently activated by dephosphorylation of pY505 (McNeill et al., 2007; Zikherman et al., 2010). Intermediate amounts of CD45 cause T cell hyperactivation, since CD45 abundance is enough to activate LCK through pY505 dephosphorylation, but not to limit its activation through pY394 dephosphorylation. Only the high physiologic CD45 abundance ensures sufficient primed LCK protein to trigger a T cell response while preventing T cell hyperactivation in the absence of antigen. This model provides a rationale for the consistent relative protein copy number found in several different primary T cell subsets, between LCK, CD45 and C-terminal Src kinase (CSK), the kinase responsible for Lck^{Y505} phosphorylation (**Figure 2B**). In both CD4 and CD8 T cells, a ratio of at least two CD45 molecules are found per LCK molecule to control LCK activity. In contrast, one molecule of CSK per LCK molecule seems to be sufficient to regulate LCK^{Y505} phosphorylation. Once antigen is encountered, there is evidence that segregation of CD45 from ligated TCRs in the immunological synapse is required to allow persistent phosphorylation of the ζ-chain that triggers TCR signaling (Leupin et al., 2000; Davis and van der Merwe, 2006; Varma et al., 2006; Cordoba et al., 2013; Chang et al., 2016). In fact, CD45-mediated tonic dephosphorylation of the ζ-chains in resting T cells helps prevent activation in the absence of antigen (**Figure 2A**; Courtney et al., 2019).

RPTPε

CD45 is one transmembrane receptor Tyr phosphatase with a well characterized role in regulation of SRC-family kinases in mature T cells. Further investigation into the function of other receptor Tyr phosphatases is likely to reveal new players in this regulation. Some experimental evidence has been reported for receptor-type tyrosine-protein phosphatase ε (RPTPε), receptor-type tyrosine-protein phosphatase H (RPTPH) and receptor-type tyrosine-protein phosphatase J (RPTPJ) so far. RPTPε (encoded by the gene *PTPRE*) has been proposed as a positive regulator of LCK activity, based on the observation that cells with low

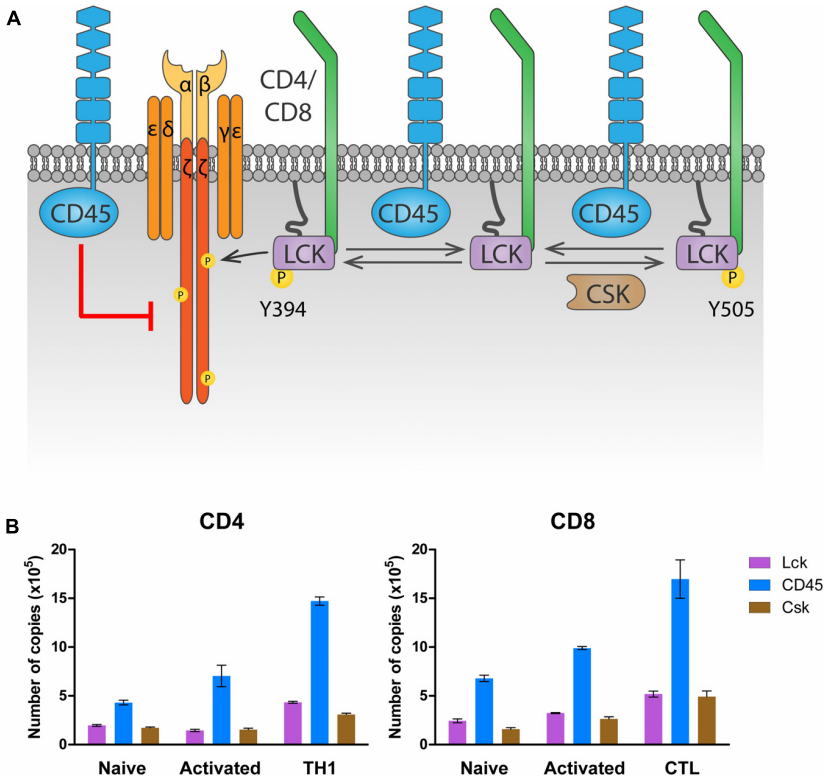

FIGURE 2 | Regulation of LCK by CD45 and CSK. **(A)** Regulation of tonic signaling. In absence of antigen stimulation, basal LCK activity ensures tonic ζ chain phosphorylation and signaling needed for naïve T cell survival. Phosphorylation of LCK on Y505 by CSK prompts an inhibited conformation (right). CD45 dephosphorylates Y505 to raise a *primed* conformation (center), which allows trans-autophosphorylation on Y394, leading to the fully active *open* conformation that phosphorylates the ζ chain (left). To avoid excessive LCK activation, CD45 dephosphorylates LCK on Y394. At the same time, CD45 dephosphorylates the ζ chain, inhibiting downstream signaling in the absence of antigen stimulation. **(B)** Number of LCK, CD45 and CSK molecules in CD4 and CD8 T cells. Data was extracted from the proteomic study by Howden et al. (2019) and visualized using Prism software.

levels of RPTPε (induced by incubation of T cells with a hepatitis C virus-derived small RNA) showed reduced phosphorylation on LCK^{Y394} and downstream molecules upon TCR stimulation, which resulted in impaired T cell activation (Bhattarai et al., 2015; Bhattarai et al., 2017). Analysis of the phenotype of *PTPRE* knockout cells would provide important further validation of its role in the regulation of LCK activity.

RPTPH

RPTPH (also known as SAP-1, encoded by the gene *PTPRH*) interacts with LCK both *in vitro* and *in vivo*, and overexpression of this phosphatase resulted in decreased phosphorylation of LCK^{pY394} and impaired T cell activation (Ito et al., 2003), suggesting that LCK is a direct substrate. RPTPH, however, was not detected in primary murine T cells (Howden et al., 2019) or Jurkat cells (Ito et al., 2003), hence a physiological role for this phosphatase in T cell regulation is unlikely.

RPTPJ

RPTPJ (also known as CD148, encoded by the gene *PTPRJ*), when overexpressed in Jurkat cells, bound to LCK and dephosphorylated both pY394 and pY505 residues, which resulted in a net inhibitory effect on LCK activity (Stepanek et al., 2011). Of note, RPTPJ was not detected in murine naïve T cells, while human naïve T cells express a significant amount (Stepanek et al., 2011; Castro-Sanchez et al., 2017). RPTPJ expression is induced and upregulated in murine and human effector T cells, respectively (**Figure 1**; Castro-Sanchez et al., 2017; Howden et al., 2019), so it may play a role in the regulation of T cell effector responses rather than in naïve T cell activation. RPTPJ knockout mice had no obvious phenotype with regard to T cell development, but T cell activation and recall responses in lineage specific knockouts have not yet been addressed for RPTPJ (Zhu et al., 2008).

SHP-1

Four cytoplasmic Tyr phosphatases are known to contribute to antigen discrimination and tolerance through dephosphorylation of Src family kinases on their activatory residues: Src homology 2-containing phosphatase 1 (SHP-1) (Stefanova et al., 2003), dual specificity protein phosphatase 22 (DUSP22) (Li et al., 2014), protein tyrosine phosphatase non-receptor type 2 (PTPN2) (Wiede et al., 2011) and protein tyrosine phosphatase non-receptor type 22 (PTPN22) (Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999; Wu et al., 2006). Of them, only DUSP22 (also known as JKAP or VHX) is permanently

located at the inner face of the plasma membrane, due to co-translational and irreversible myristoylation on its N-terminal Glycine (Schwertassek et al., 2010). In contrast, SHP-1, PTPN2 and PTPN22 must be recruited to the immunological synapse in an inducible way, which allows for spatial regulation of their activities.

SHP-1 (encoded by the gene *PTPN6*) is recruited to the immunological synapse by its SH2 domains, which only bind Tyr phosphorylated proteins, such as the chains of the TCR complex. In fact, SHP-1 was reported to be recruited to the TCR upon stimulation with a TCR antagonist (Stefanova et al., 2003), where it dephosphorylated LCK on pY394 to inhibit the response to the antagonist. In contrast, binding of an agonist rapidly activated ERK, which blocked interaction of SHP-1 with LCK by phosphorylating LCK on serine 59, allowing downstream signaling. These data suggest that SHP-1 may be important for T cells to discriminate between TCR agonists and antagonists. However, T cell specific deletion of SHP-1 resulted in a mild phenotype in terms of T cell activation and showed that SHP-1 is also involved in T cell differentiation and AKT signaling (Fowler et al., 2010; Johnson et al., 2013; Mercadante and Lorenz, 2017). Studies using knockdown strategies have also shown that SHP-1 induces T cell adhesion and mediates IL-10 signaling in T cells (Taylor et al., 2007; Azoulay-Alfaguter et al., 2017). The involvement of SHP-1 in so many diverse functions provides a rationale for its high expression in primary T cells. In fact, SHP-1 is the most abundant Tyr phosphatase in naïve T cells and is only outpaced by CD45 increased expression following T cell stimulation (**Figure 1**). Its putative involvement in diverse signaling pathways might be an issue when considering SHP-1 as a target in immunotherapy (see section "Concluding Remarks").

PTPN2

The spatial regulation of PTPN2 and PTPN22 remains poorly understood, despite their physiologic relevance. PTPN2 (also known as TCPTP) is important for establishing an appropriate T cell activation threshold that ensures tolerance (Wiede et al., 2011). It was suggested that PTPN2 regulates TCR signaling by dephosphorylation of SFKs, since a PTPN2 substrate-trapping mutant overexpressed in COS-1 cells bound LCK and FYN (Wiede et al., 2011). Whether this interaction takes place in a physiologic setting and how PTPN2 would reach these substrates in T cells remain unclear. The two described PTPN2 splicing variants, p45 and p48, localize to the nucleus (due to the presence of a nuclear localization signal) and to the endoplasmic reticulum (which requires the 19 C-terminal residues of the protein), respectively (Lorenzen et al., 1995). Small amounts of PTPN2 might reach the inner face of the plasma membrane and be stabilized there by its basic C-terminal residues, and additional mechanisms might translocate it to the immune synapse in an inducible manner. The use of fractionation techniques and microscopy would help clarify PTPN2 localization and how it regulates T cell activation thresholds.

PTPN22

PTPN22 (also known as PEP in mice or LYP in humans) is also important for antigen discrimination, since cells that lack PTPN22 show increased T cell activation particularly in response to low affinity agonists (Salmond et al., 2014). PTPN22 interacts with CSK (Cloutier and Veillette, 1996), and this interaction is relevant for PTPN22 function, as shown by the fact that a human *PTPN22*^{C1858T} variant, encoding an amino acid R620W substitution which impairs its interaction with CSK (Bottini et al., 2004), is associated with increased risk of autoimmunity (Bottini et al., 2004; Totaro et al., 2011; de Lima et al., 2017; Tizaoui et al., 2019). However, whether PTPN22 inhibits TCR signaling more efficiently when interacting with or when dissociated from CSK remains unclear (**Figure 3**). In support of the latter, PTPN22 was shown to dissociate from cytosolic CSK and translocate to lipid rafts upon TCR stimulation, where it can access its substrates and inhibit TCR signaling (Vang et al., 2012).However, a mechanism for PTPN22 recruitment to and stabilization at the plasma membrane is missing. Another model, supported by a study using super-resolution imaging, suggests that interaction with CSK is induced upon integrin stimulation, and this interaction is important for driving PTPN22 to the plasma membrane and for downregulation of integrin signaling (Burn et al., 2016). Inducible interaction of PTPN22 and CSK upon TCR stimulation has also been reported (de la Puerta et al., 2013), but how the PTPN22-CSK complex would be recruited to and stabilized at the plasma membrane to reach its substrates is unclear. CSK reaches the plasma membrane because, via its SH2 domain, it binds phosphorylated Tyr on membrane adaptor proteins including phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) 1 (Davidson et al., 2003). However, the pool of CSK binding to PAG differs from the pool of CSK binding to PTPN22 (Davidson et al., 2016). Therefore, another mechanism would be needed to localize the PTPN22-CSK complex on the plasma membrane. The polyproline regions on PTPN22 allow interaction of this phosphatase with other proteins, hence other potential interaction partners could direct PTPN22 to the plasma membrane. Apart from CSK, the proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1) is the only PTPN22 interaction partner identified so far (Voisinne et al., 2019). PSTPIP1 has been suggested to inhibit TCR signaling and localizes at the plasma membrane through its F-BAR and SH3 domains, interacting both with the cytoskeleton and with CD2 (Marcos et al., 2014). Further study of the interaction between PSTPIP1 and PTPN22 might help understanding the spatial regulation of PTPN22 in T cells, which is crucial to understand how the R620W polymorphism drives autoimmunity (further discussed in section "Tyr Phosphatases in T Cells").

Tyrosine Phosphatases Induce Amplification and Branching of Early TCR Signaling

Once TCR signaling is initiated by activation of SRC family kinases, it rapidly amplifies and branches to orchestrate the T cell response. Some of this branching is amplified by FYN, which induces amplification and diversification of TCR signaling by contributing to activation of the MAPK pathway (Lovatt et al., 2006) and by triggering cytoskeletal rearrangements downstream of the TCR (Chapman and Houtman, 2014). This is promoted

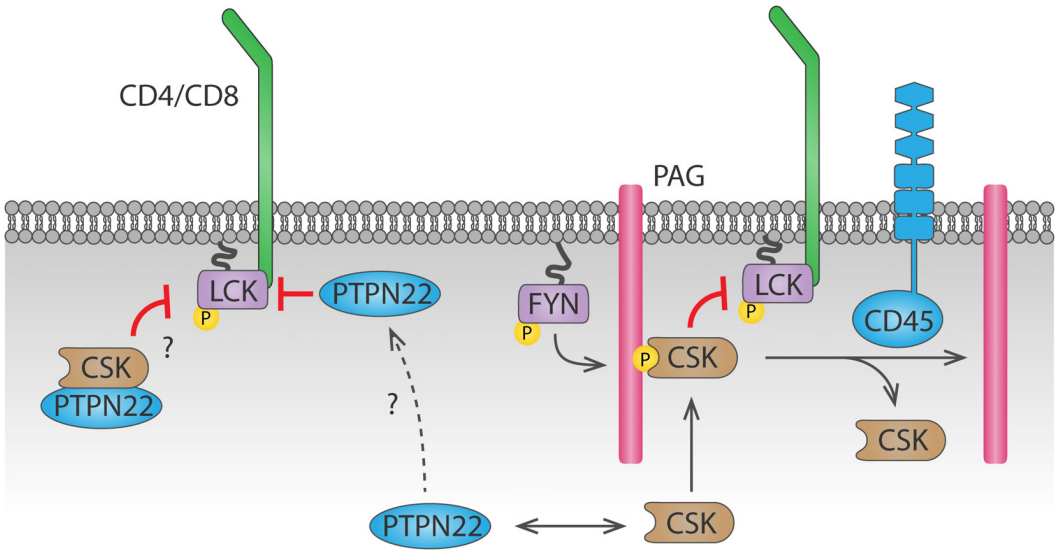

FIGURE 3 | Dynamics of PTPN22 and CSK-mediated LCK regulation. CSK is recruited to the plasma membrane by binding to phosphorylated PAG through its SH2 domain. CSK can then phosphorylate LCK and inhibit it. Recruitment of CSK is inhibited by CD45-mediated dephosphorylation of PAG. Whether PTPN22 inhibits LCK activity when bound to CSK or when dissociated from it remains unclear. The mechanism by which PTPN22 is recruited to the plasma membrane to dephosphorylate LCK is currently unknown.

by CD45 and receptor-type tyrosine-protein phosphatase α (RPTPα, encoded by the gene *PTPRA*), which activates FYN by dephosphorylating it on Y528 (Shiroo et al., 1992; Maksumova et al., 2007). In addition, dephosphorylation of Tyr on the adaptor protein PAG by a Tyr phosphatase, probably CD45, also sustains LCK and FYN activity (Davidson et al., 2003), since docking sites for CSK are lost upon PAG dephosphorylation.

LMPTP

LMPTP, encoded by the gene *ACP1,* positively regulates signaling downstream of the TCR by dephosphorylation of ZAP70 on the inhibitory Y292 (Bottini et al., 2002). This dephosphorylation prevents binding of the ubiquitin ligase c-CBL to ZAP70 and in consequence reduces ZAP70 degradation and prolongs TCR signaling. Although microscopy has shown that LMPTP localizes at the plasma membrane in lymphocytes (Gjorloff-Wingren et al., 2000), the mechanism of such localization remains unknown, since there is no obvious localization motif in its sequence, and no interaction partners have been identified. LMPTP is phosphorylated by SRC-family kinases on Tyr 131 and 132, and this increases its catalytic activity, generating a positive feedback loop for TCR signaling amplification (Tailor et al., 1997; Bucciantini et al., 1999).

Tyrosine Phosphatases Drive Negative Feedback Loops and Signal Termination

Once downstream effectors of TCR signaling have been activated and the cellular response has been triggered, signaling must be terminated. Several Tyr phosphatases contribute to this process by dephosphorylation of SRC-family kinases, the ζ-chain and ZAP70.

SHP-1 has been proposed to contribute to signal termination by inhibition of LCK, since it is recruited to the TCR between 20 and 40 min after TCR stimulation with antigenic peptides (Stefanova et al., 2003). Recently, it was proposed that the thousand-and-one amino acid kinase 3 (TAOK3) is involved in the crosstalk between LCK and SHP-1 (Ormonde et al., 2018). Using the Jurkat cell line and anti-CD3 stimulation, the authors concluded that TAOK3 promotes TCR signaling by blocking LCK interaction with SHP-1. However, the only T cell phenotype of *TAOK3*$^{-/-}$ mice reported so far was a reduction in CD8 T cell number (Hammad et al., 2017). A deeper analysis of T cell responses in these mice would help understand the relevance of TAOK3/SHP-1 crosstalk for T cell activation.

PTPN22 has been shown to dephosphorylate the ζ-chain both *in vitro* and in pervanadate-treated Jurkat cells (Wu et al., 2006). PTPN22 has also been suggested to dephosphorylate ZAP70 (**Figure 4B**). When a substrate-trapping PTPN22 mutant was expressed in Jurkat cells, ZAP70 was found among the bound proteins, and PTPN22 was shown to dephosphorylate ZAP70^{pY319} *in vitro* (Wu et al., 2006). Consistent with this observation, treatment of Jurkat cells with a PTPN22 inhibitor resulted in increased ZAP70 phosphorylation upon TCR stimulation (Vang et al., 2012). Evidence of direct dephosphorylation of these substrates in primary T cell is, however, not yet available.

The highly homologous non receptor phosphatases PTPN3 and PTPN4 (PTPH1 and PTP-MEG1, respectively) are both able to bind to and dephosphorylate the ζ-chain *in vitro* (Sozio et al., 2004; Young et al., 2008), and overexpression of either enzyme in Jurkat cells downmodulated T cell activation, although PTPN4 to a lesser extent (Han et al., 2000). However, none of the single knockout or the double *PTPN3*$^{-/-}$*PTPN4*$^{-/-}$ mice

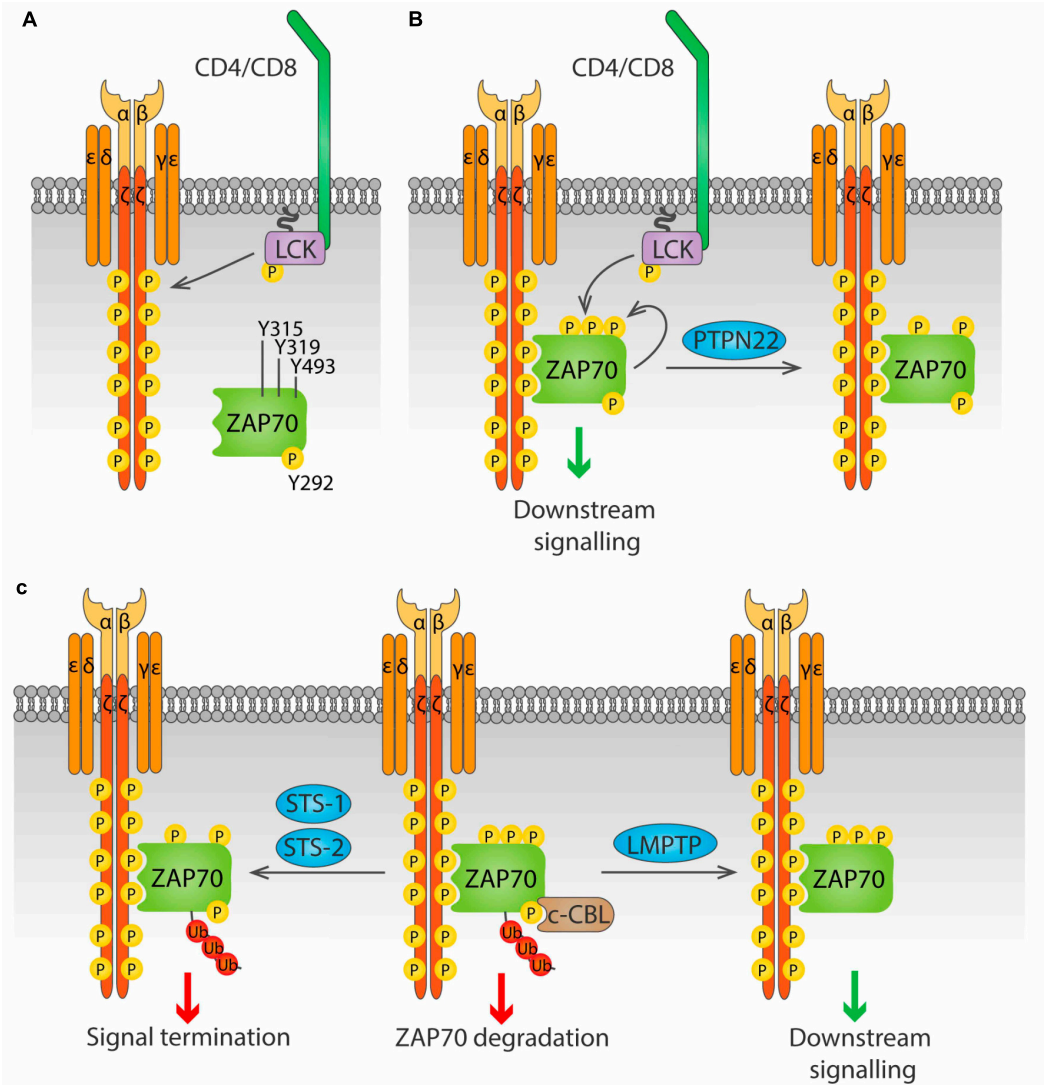

FIGURE 4 | Regulation of ZAP70 by Tyr phosphatases. **(A)** ZAP70 is regulated by the phosphorylation status of three key residue, Y315, Y319 and Y493. Phosphorylation of the ζ chains by LCK upon antigen stimulation provides docking sites for ZAP70. **(B)** ZAP70 binds to phosphorylated ζ chains through its tandem SH2 domains. Binding to ζ chains induces a conformational change in ZAP70 that exposes Y315 and Y319, that can then be phosphorylated by LCK. Phosphorylation of Y493 either by LCK or by autophosphorylation leads to full activation of ZAP70. PTPN22 is able to dephosphorylate Y319, inhibiting ZAP70. **(C)** Phosphorylation on Y292 allows binding of the ubiquitin ligase c-Cbl to ZAP70, and subsequent addition of poly-ubiquitin that leads to ZAP70 degradation (center). This process is avoided by the Tyr phosphatase LMPTP, which dephosphorylates Y292 and blocks c-Cbl binding, prolonging ZAP70 signaling (right). The phosphatases Sts-1 and Sts-2 can bind ubiquitinated ZAP70 and dephosphorylate Y319, terminating ZAP70 signaling (right).

showed abnormalities in T cell activation or development (Bauler et al., 2008), suggesting that loss of these two phosphatases can either be compensated or lack relevance *in vivo*.

The two highly similar phosphatases STS-1 (also known as TULA-2, encoded by the *UBASH3B* gene) and STS-2 (also known as TULA, encoded by the *UBASH3A* gene) negatively regulate T cell activation through dephosphorylation of ZAP70^{pY319} (Carpino et al., 2004; Luis and Carpino, 2014) (**Figure 4C**). These phosphatases only bind to and dephosphorylate ubiquitinated ZAP70, providing a link between ubiquitination and phosphorylation-mediated regulation of early TCR signaling (Yang et al., 2015; Hu et al., 2016). Whether STS-1 and STS-2 are functionally redundant or have unique roles in T cell regulation remains largely unknown. Cells from STS-1$^{-/-}$STS-2$^{-/-}$ mice show increased T cell proliferation and cytokine production upon *in vitro* TCR stimulation compared to WT cells. In contrast, responses of T cells lacking only one STS member are only modestly increased, suggesting that these proteins are functionally redundant (San Luis et al., 2011). However, *in vivo* studies point to differential, although partially overlapping, roles of STS-1 and STS-2. Survival from systemic *Candida albicans* infection was significantly enhanced not only in STS-1$^{-/-}$STS-2$^{-/-}$ mice, but also in each single knockout mouse (Naseem et al., 2015). Similarly, lack of either phosphatase

exacerbates pathology in a model of inflammatory bowel disease (IBD) (Newman et al., 2014). Nevertheless, in the latter study, only the double knockout mice showed enhanced cytokine production in the colon, and only double knockout CD4 T cells showed greater colitogenic capacity than wild type CD4 T cells when both were injected in T cell deficient, STS sufficient mice. The different outcomes are likely due to T cell-extrinsic effects of STS deficiency in the full knockout model used. Study of mice that lack STS-1, STS-2 or both specifically in the T cell compartment would help shed light into the specific functions of these proteins in T cell biology.

Tyrosine Phosphatases Mediate Inhibitory Receptor Signaling

Several inhibitory receptors control T cell activation by inhibiting early TCR signaling (reviewed in Fuertes Marraco et al., 2015). This control is important to avoid T cell hyperactivation and damage derived from chronic antigen exposure. Inhibitory receptors lack intrinsic enzymatic activity but have cytoplasmic tails with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) or an immunoreceptor tyrosine-based switch motif (ITSM) that are phosphorylated upon ligation and TCR signaling. The phosphorylated domains can serve as docking sites for the Tyr phosphatases with SH2 domains such as SHP-1 and SHP-2. This binding not only localizes SHP-1 and SHP-2 close to phosphorylated substrates, but also promotes a conformational change that leads to activation of the phosphatases (Hof et al., 1998; Wang et al., 2011). Below, we discuss the role of SHP-1 and SHP-2 in inhibition of early TCR signaling, and consequently T cell activation, downstream of several inhibitory receptors.

The role of SHP-1 and SHP-2 in signaling through the inhibitory receptor programmed cell death protein 1 (PD-1) is perhaps the most extensively studied, although it has been controversial. Initially, SHP-2, but not SHP-1, was shown to bind PD-1 upon PD-1 ligation, subsequently downregulating T cell activation through dephosphorylation of the ζ-chain and ZAP70 (Sheppard et al., 2004; Yokosuka et al., 2012). However, the finding that *SHP-2*$^{-/-}$ mice show intact PD-1-mediated signaling and cell exhaustion (Rota et al., 2018) suggested that another phosphatase was recruited to PD-1, SHP-1 being the likely candidate. This controversy was recently resolved by Celis-Gutierrez and colleagues (Celis-Gutierrez et al., 2019). Using mass spectrometry, they defined the PD-1 interactome during PD-L1 ligation and antigen stimulation. They showed that in wild-type cells, SHP-2 is the main PD-1 interactor, binding 50 times more PD-1 molecules than SHP-1, despite the latter being approximately six times more abundant than SHP-2. In *SHP-2*$^{-/-}$ cells, however, SHP-1 replaced SHP-2 and mediated PD-1 signaling. Consistently, only the double knockout *SHP-2*$^{-/-}$*SHP-1*$^{-/-}$ showed impaired PD-1-mediated T cell inhibition. This finding suggests that concomitant inhibition of both SHP-1 and SHP-2 would be needed to efficiently block PD-1 intracellular signaling in an immunotherapy setting. In the same study, the interactome of B and T lymphocyte attenuator (BTLA), another inhibitory receptor, was analyzed upon treatment of T cells with pervanadate (Celis-Gutierrez et al., 2019). Results showed that, consistent with a previous report (Watanabe et al., 2003), both SHP-1 and SHP-2 bind BTLA. However, contrary to PD-1, BTLA preferentially binds SHP-1 rather than SHP-2. This difference has implications for downstream inhibitory signaling. PD-1, recruiting mainly SHP-2, preferentially inhibits phosphorylation of CD28 over the ζ-chain, while BTLA, recruiting both SHP-1 and SHP-2, inhibits the phosphorylation of both CD28 and ζ-chain (Hui et al., 2017; Xu et al., 2020).

The role of SHP-1 and SHP-2 in cytotoxic T-lymphocyte antigen 4 (CTLA-4) signaling is poorly understood, with conflicting results being reported by different groups in the last 25 years. SHP-2 was initially shown to bind to CTLA-4 in T cells, although this binding would likely be indirect (Marengere et al., 1996; Schneider and Rudd, 2000). Supporting the need for an intermediate protein between CTLA-4 and SHP-2, another study did not find CTLA-4/SHP-2 association *in vitro* (Guntermann and Alexander, 2002; Yokosuka et al., 2010). Conflicting results are likely due to the different methodologies (immunoprecipitation *vs.* microscopy), cells (cell lines vs. primary murine cells), and conditions (*in vitro* proteins vs. cells; with endogenous *vs.* overexpressed proteins) used. Of note, CTLA-4 can exert inhibitory functions in a cell extrinsic manner and by signaling-independent mechanisms such as competition with CD28 for CD80/CD86 and transendocytosis of these ligands upon engagement (Walker and Sansom, 2015). Hence, the contribution of phosphatase-mediated signaling to CTLA-4 inhibition remains unclear. The study of the endogenous CTLA-4 interactome in primary T cells during antigen stimulation may help to identify whether there are cell intrinsic effects of CTLA-4 signaling and would be beneficial for applications in immunotherapy.

SHP-1 has been linked to signaling through two other inhibitory receptors, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and leucocyte-associated immunoglobulin receptor-1 (LAIR-1). Inhibition of T cell effector functions by CEACAM1 requires recruitment of SHP-1 (Nagaishi et al., 2006). During TCR stimulation, CEACAM1 ITIMs are phosphorylated by LCK, and serve as docking sites for SHP-1, which then dephosphorylates ZAP-70 and ζ-chain (Chen et al., 2008). On the other hand, SHP-1 constitutively interacts with LAIR-1 (Sathish et al., 2001a), a negative regulator of T cell activation highly expressed in naïve T cells (Maasho et al., 2005; Jansen et al., 2007). Although the relevance of this interaction for LAIR-1-mediated T cell inhibition has not been explored, it might be one of the mechanisms by which SHP-1 establishes T cell activation thresholds (Johnson et al., 1999; Sathish et al., 2001b).

Lastly, SHP-2, but not SHP-1, is recruited to platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) (Newman et al., 2001), and ligation of PECAM-1 with agonist peptides during antigen presentation leads to SHP-2-dependent dephosphorylation of ZAP-70 and inhibition of T cell activation (Clement et al., 2015).

Altogether, regulation of T cell responses by inhibitory receptors strongly relies on SHP-1 and SHP-2, which makes these phosphatases attractive targets to enhance T cell responses.

Despite a considerable improvement in the last years, more studies will be needed to clearly understand which functions are exclusive to SHP-1 or SHP-2, and in which situations loss of one of them can be compensated by the other. Strategies targeting both phosphatases are tempting, however their high expression and their regulatory role in important T cell functions such as cytokine signaling and adhesion will make it necessary to evaluate disruption of SHP-1/2 function for potential secondary effects.

TYROSINE PHOSPHATASES IN AUTOIMMUNITY

Most T cell responses to pathogens are appropriately regulated, however approximately 4-5% of the population of developed countries suffers from an autoimmune disease (Hayter and Cook, 2012; Roberts and Erdei, 2020), the onset of which is generally considered to result from a failure of tolerance. In this context, it is striking that polymorphisms in genes encoding phosphatases are among the most frequently associated with autoimmune disease (Burton et al., 2007; Todd et al., 2007). Here, we will review the current evidence and understanding of several autoimmune diseases associated with PTPs and their aberrant expression (**Table 2**), and discuss what these diseases might tell us about the function of those PTPs.

Rheumatological Diseases

The rheumatological diseases are the archetype of autoimmune disease. This group of diseases is characterized by inflammation, predominantly affecting the joints, such as in rheumatoid arthritis (RA), but also connective tissues, such as in systemic sclerosis, and sometimes involving other specific organs, such as the skin, eye, mouth and serosae, as seen in systemic lupus erythematosus (SLE).

PTPN22 is the most extensively studied phosphatase in relation to autoimmune disease, and polymorphisms in the *PTPN22* gene are heavily associated with rheumatological diseases. The *PTPN22*^{C1858T} missense mutation (rs2476601), which leads to the R620W substitution, is the strongest non-HLA genetic association for autoimmune disease (Stanford and Bottini, 2014). In 2004, a significant association was first reported between the R620W variant and both RA (Begovich et al., 2004) and SLE (Kyogoku et al., 2004). These findings have subsequently been replicated numerous times, and *PTPN22*^{C1858T} has additionally been shown to be a risk factor for development of other rheumatological disease including ANCA-positive vasculitis (specifically microscopic polyangiitis, granulomatosis with polyangiitis, and giant cell arteritis, but not eosinophilic granulomatosis with polyangiitis) (Carr et al., 2009; Jennette et al., 2013; Serrano et al., 2013; Cao et al., 2015) and systemic sclerosis (Gourh et al., 2006). Northern European Caucasians are the most common carriers of this mutation, with a minor allele frequency of >10%, while individuals of Middle Eastern, Asian and African decent are more rarely affected (<1%) (Zheng et al., 2012). It is possible that this reflects a protective effect of the SNP against an infectious threat such as tuberculosis (Boechat et al., 2013). Despite this geographical variation, carriage of the *PTPN22*^{C1858T} SNP within populations with a lower minor allele frequency still appears to act as a susceptibility allele for RA (Mastana et al., 2007; Sfar et al., 2009; Ates et al., 2011).

The R620W mutation is not simply associated with RA, but has been shown to alter the pathogenesis and phenotype of the disease in patients with RA. Both homo- and heterozygosity for the *PTPN22*^{C1858T} allele are strongly associated with rheumatoid factor (RF)-positive disease (the presence of circulating antibodies), while RF-negative disease shows no association (Begovich et al., 2004; Kokkonen et al., 2007). Furthermore, the C1858T variant is strongly associated with the additional presence of anti-cyclic citrullinated (anti-CCP) antibodies (Johansson et al., 2006; Kokkonen et al., 2007), earlier disease onset (Johansson et al., 2006; Karlson et al., 2008), quicker progression of radiological joint destruction (Lie et al., 2007), and erosive disease (Raslan et al., 2016). Interestingly, the presence of the *PTPN22*^{C1858T} SNP has no effect on the efficacy of anti-TNFα drug treatments used in RA (Potter et al., 2008), and studies examining its effect on efficacy of methotrexate have similarly shown mixed results without a convincing effect (Fedele et al., 2013; Majorczyk et al., 2020).

The effect of the *PTPN22*^{C1858T} SNP is not confined to T cells, but also involves B cells and myeloid cells, although detailed description of their involvement is outside the scope of this review. In T cells, an early study demonstrated that T cells from human donors heterozygous for the R620W variant secreted significantly less IL-2 in response to TCR stimulation (Vang et al., 2005). Several subsequent studies demonstrated reduced calcium mobilization and CD25 expression in response to TCR stimulation in C1858T homozygous human CD4 T cells (Rieck et al., 2007), resulting in reduced T cell proliferation (Aarnisalo et al., 2008) and IL-2 production (Aarnisalo et al., 2008; Chuang et al., 2009). T cells from healthy human homozygotes without clinically apparent autoimmune disease demonstrated reduced ζ-chain phosphorylation in response to TCR stimulation, due to increased phosphatase activity (Vang et al., 2013).

At a cellular level, the outcome of these alterations in signaling appears to be a shift towards a pro-inflammatory state lacking autoimmunity-protective mechanisms. Patients with SLE carrying the *PTPN22*^{C1858T} risk allele show a skewing towards high serum IFNα and low TNFα compared with patients without the SNP (Kariuki et al., 2008), a profile that has been implicated as a risk factor for SLE (Niewold et al., 2007). Furthermore, circulating levels of anti-inflammatory cytokines such as IL-10 have been shown to be reduced in individuals with RA carrying the *PTPN22*^{C1858T} SNP (Ghorban et al., 2019). Reduced IL-10 mRNA expression was also demonstrated in heterozygous patients with ANCA-positive vasculitis, due to high basal PTPN22 phosphatase activity conferring decreased phosphorylation of ERK; this correlated clinically with a higher rate of relapsing disease (Cao et al., 2012). In T cells from healthy human donors homozygous for *PTPN22*^{C1858T}, CD4 T cells produced significantly more IFNγ compared to those from individuals without the mutant allele, and significantly less IL-17, suggesting a skew in CD4 T cell differentiation away from Th17 towards Th1 (Vang et al., 2013). Additionally, $CD4^{+}Foxp3^{+}$ regulatory T cells (Treg) appear to be altered in the presence of the SNP. In chimeric mice reconstituted

TABLE 2 | Tyr phosphatases associated with autoimmune diseases.

PTPase	SNP	Disease	Effect of SNP on disease	References
PTPN22	rs2476601 (C1858T)	RA	↑ susceptibility	Begovich et al., 2004; Mastana et al., 2007; Sfar et al., 2009; Ates et al., 2011
			↑ RF-positive and anti-CCP	Begovich et al., 2004; Johansson et al., 2006; Kokkonen et al., 2007
			Earlier disease onset and quicker progression	Johansson et al., 2006; Karlson et al., 2008; Potter et al., 2008; Raslan et al., 2016; Majorczyk et al., 2020
			Methotrexate/anti-TNFa efficacy unaffected	Fedele et al., 2013
			↓ serum anti-inflammatory cytokines	Ghorban et al., 2019
		JIA	↑ susceptibility	Kaalla et al., 2013
		SLE	↑ susceptibility	Kyogoku et al., 2004
			↑ risk of complications	Reddy et al., 2005; Moez and Soliman, 2012; Ostanek et al., 2014
			High IFNa, low TNFα in serum	Kariuki et al., 2008
		ANCA+vasculitis	↑ susceptibility	Carr et al., 2009; Jennette et al., 2013; Serrano et al., 2013; Cao et al., 2015
			Higher rate of relapsing disease	Cao et al., 2012
		Systemic sclerosis	↑ susceptibility	Gourh et al., 2006
		Psoriatic arthritis	↑ susceptibility	Bowes et al., 2015
		IBD	↓ susceptibility to CD, but not UC	Martín et al., 2005; Diaz-Gallo et al., 2011
		T1DM	↑ susceptibility	Bottini et al., 2004
			↑ additional diabetes-related autoantibodies	Hermann et al., 2006
			↑ total and naïve Tregs	Valta et al., 2020
	rs33996649 (G788A)	RA	Protective	Rodríguez-Rodríguez et al., 2011; López-Cano et al., 2017; Bae and Lee, 2018
		SLE	Protective	Orrú et al., 2009; López-Cano et al., 2017; Bae and Lee, 2018
		IBD	↓ susceptibility to UC; no effect on CD risk	Bae and Lee, 2018
	rs2488457	RA, JIA	↑ susceptibility in Chinese populations	Feng et al., 2010; Huang et al., 2012; Fan et al., 2015
	(C-1123G)	IBD	↑ UC disease severity in Chinese	Chen et al., 2013
PTPN2	rs2542151	RA	↑ susceptibility	Burton et al., 2007
			↑ risk of erosive joint damage	Ciccacci et al., 2016
		IBD	↑ susceptibility to CD and UC	Burton et al., 2007
			↑ risk in smokers	Parkes et al., 2007; Weersma et al., 2009; van der Heide et al., 2010; Glas et al., 2012; Zhang et al., 2013
		T1DM	↑ susceptibility	Burton et al., 2007
			Earlier disease onset	Cooper et al., 2008; Espino-Paisan et al., 2011
	rs7234029	RA	Poorer response to adalimumab (anti-TNFa)	Conigliaro et al., 2017
		JIA	↑ susceptibility	Thompson et al., 2010
		IBD	↑ susceptibility to CD	Burton et al., 2007
			Earlier onset of CD; increased strictures	Parkes et al., 2007; Weersma et al., 2009; Glas et al., 2012; Zhang et al., 2013
	rs1893217	IBD	↑ susceptibility to CD and UC	Burton et al., 2007; Parkes et al., 2007; Weersma et al., 2009; Anderson et al., 2011; Glas et al., 2012; Zhang et al., 2013
CD45	rs17612648 C77G	MS	↑ susceptibility	Jacobsen et al., 2000
			No association in other studies	Barcellos et al., 2001; Ballerini et al., 2002; Gomez-Lira et al., 2003; Nicholas et al., 2003; Cocco et al., 2004; Szvetko et al., 2009
	C59A	MS	↑ susceptibility	Jacobsen et al., 2002
		Psoriasis	Overexpression correlates with severity	Zhang et al., 2014
SHP-1		RA	Alterations in SHP-1 mediated signaling	Li et al., 2013
		MS	↓ SHP-1 mRNA and protein in PBMCs	Christophi et al., 2008
		Psoriasis	↓ SHP-1 à ↑ sensitivity to inflammation	Eriksen et al., 2005, 2010
SHP-2		SLE	↑ SHP-2 in PBMCs from patients	Wang J. et al., 2016
		IBD	Increased susceptibility to CD	Burton et al., 2007
		T1DM	Increased susceptibility	Burton et al., 2007
LMPTP		IBD	Protective effect in females	Gloria-Bottini et al., 2007
		T1DM	Protective effect in females	Gloria-Bottini et al., 2007

RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; JIA, juvenile idiopathic arthritis; SLE, systemic lupus erythematosus; ANCA, anti-neutrophil cytoplasmic antibodies; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; T1DM, Type 1 diabetes mellitus; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cell.

1:1 with WT and PTPN22 R619W (the murine equivalent of R620W) bone marrow, more Tregs carrying the R619W mutation developed, indicating that PTPN22 exerts a cell intrinsic bias towards development of this lineage (Knipper et al., 2020). In *PTPN22*^{C1858T} carriers with type 1 diabetes, higher frequencies of total and naïve Tregs have been seen, suggesting that in humans also PTPN22 exerts an effect on circulating numbers of these cells (Valta et al., 2020). Furthermore, Tregs from C1858T homozygous human donors were not able to suppress the secretion of IFNγ by conventional CD4 T cells, suggesting the balance between regulatory and effector/memory cells is disrupted in such individuals (Vang et al., 2013).

The *PTPN22*^{C1858T} variant is also associated with juvenile idiopathic arthritic (JIA), and notably this association has been demonstrated by meta-analysis to be strongest with the RF-positive polyarticular JIA subtype, which is most similar to RA (Kaalla et al., 2013). Furthermore, susceptibility to ANCA (anti-neutrophil cytoplasmic antibody)-positive vasculitis is increased in the presence of the R620W allele, and specifically to involvement of lung, skin, ear/nose/throat, and peripheral neuropathy (Cao et al., 2015). Another rheumatological disease associated with the *PTPN22*^{C1858T} SNP is SLE, in which homozygosity poses a much higher risk (OR 4.37, vs. 1.37 for heterozygotes) (Kyogoku et al., 2004). Similarly to RA, the presence of the SNP not only confers increased susceptibility to SLE, but may also alter its clinical course: carriage of *PTPN22*^{C1858T} is associated with increased risk of renal complications of SLE (Reddy et al., 2005; Moez and Soliman, 2012), as well as secondary antiphospholipid syndrome (Ostanek et al., 2014). Higher titers of anti-cardiolipin and lupus anticoagulant antibodies were also found in SLE patients carrying *PTPN22*^{C1858T} (Ostanek et al., 2014). These associations illustrate the fact that C1858T is predominantly linked to autoimmune diseases characterized by the presence of circulating auto-antibodies (Begovich et al., 2004; Padyukov et al., 2011; Zheng et al., 2012), and suggests that pathogenic B cells play a role in R620W-associated disease. Although the role of PTPN22 in B cell receptor signaling is less well defined, human B cell activation is inhibited by the C1858T polymorphism, suggesting that impaired elimination of autoreactive B cells may be a factor (Menard et al., 2011; Metzler et al., 2017). Given the evidence of T cell influence in *PTPN22*^{C1858T} associated diseases, it is likely that follicular helper T cells (Tfh), which are essential for B cell responses in the germinal centers, are relevant. This seems to be the case in mice at least, where knockout of *Ptpn22* led to increased Tfh proliferation and accumulation in the germinal centers, as well as enhanced IL-21 production (Maine et al., 2014), while in non-obese diabetic (NOD) mice expressing the R619W variant there were increased Tfh and germinal center B cell numbers, associated with increased anti-islet auto-antibodies (Schmiel et al., 2018).

In addition to C1858T, other *PTPN22* polymorphisms have been identified, although none are as frequent nor as widely studied. The G788A missense mutation (rs33996649) causes a substitution of arginine to glutamine at position 263 (R263Q), located in the catalytic domain. This results in a change in conformation at the active site, manifesting as reduced phosphatase activity (Orrú et al., 2009). Despite conferring loss-of-function, G788A has been shown to be protective against RA (Rodríguez-Rodríguez et al., 2011; López-Cano et al., 2017; Bae and Lee, 2018) and SLE (Orrú et al., 2009; López-Cano et al., 2017; Bae and Lee, 2018). The *PTPN22*$^{C-1123G}$ SNP has also been linked to a higher risk of RA and JIA, but only affecting Chinese individuals (Feng et al., 2010; Huang et al., 2012; Fan et al., 2015); in Caucasian populations it was not demonstrated to increase risk of RA independently of C1858T, with which it is often co-expressed (Dieudé et al., 2008).

A further ubiquitously expressed phosphatase, PTPN2, has also been linked to RA (Burton et al., 2007) and JIA (Thompson et al., 2010), as well as other autoimmune diseases to be discussed in more detail later in this section. Similarly to PTPN22, SNPs in the *PTPN2* gene have been shown to confer specific disease phenotypes and/or response to therapies. For example, the rs2542151 SNP is associated with higher risk of erosive joint damage in RA patients (Ciccacci et al., 2016). Furthermore, the rs7234029 SNP has been linked to poorer response to treatment of RA with adalimumab (Conigliaro et al., 2017), an anti-TNFα monoclonal antibody.

Further T cell PTPs that have been implicated in RA and other rheumatological diseases include SHP-1 and SHP-2. Administration of the SHP-1 agonist regorafenib to mice with inflammatory arthritis significantly decreased incidence and severity of joint inflammation via increased phosphatase activity and decreased IFNγ secretion by splenic T cells (Markovics et al., 2020). However, the effects of SHP-1 dysregulation are not limited to T cells, due to its widespread expression in all hematopoietic cells as well as epithelial cells (Lorenz, 2009). In rheumatoid arthritis, inflammation associated with alterations in SHP-1-mediated signaling are mediated through T cells, B cells and macrophages (Li et al., 2013), while deletion of SHP-1 in B cells in mice causes an SLE-like disease (Pao et al., 2007). With regards to SHP-2, SHP-2 activity is higher in PBMCs from patients with SLE than from healthy individuals, and SHP-2 inhibition has been shown to significantly reduce T cell proliferation and production of IFNγ and IL-17 (Wang J. et al., 2016). Analogously, lupus prone mice treated with a SHP-2 inhibitor exhibited less severe disease (Wang J. et al., 2016).

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is an umbrella term for ulcerative colitis (UC) and Crohn's disease (CD), which are characterized by chronic inflammation in the gastrointestinal tract, leading to symptoms of abdominal pain, diarrhea, rectal bleeding, weight loss and fatigue. An acute severe flare may lead to complications such as toxic megacolon or bowel perforation, while long term inflammation can cause severe ulceration, abscesses and bowel strictures.

Polymorphisms in the *PTPN2* gene have been heavily linked with several autoimmune diseases including IBD (Glas et al., 2012; Zhang et al., 2013). There are several SNPs that have been identified by genome wide association studies (GWAS) as being associated with IBD: rs2542151 (located 5.5 kb upstream from the PTPN2 gene), rs7234029, and rs1893217 (Glas et al., 2012; Zhang et al., 2013). All three SNPs are associated with CD (Burton et al., 2007; Parkes et al., 2007; Weersma et al., 2009;

Glas et al., 2012; Zhang et al., 2013), while rs2542151 (Anderson et al., 2011; Glas et al., 2012; Zhang et al., 2013), and rs1893217 (Anderson et al., 2011) are also associated with UC. As well as conferring susceptibility to IBD, the presence of the rs7234029 correlates with a stricturing disease phenotype and earlier onset of CD (Glas et al., 2012). Interestingly, a recent meta-analysis of 13 studies showed differences between ethnicities, with rs2542151 increasing risk of both CD and UC in Caucasian but not in Asian study populations (Zhang et al., 2013). Furthermore, a study investigating the differences in genetic background between smoking and non-smoking Dutch-Belgian patients with Crohn's disease found that the rs2542151 *PTPN2* SNP only increased susceptibility in the smoking cohort, but not in the non-smoking or complete cohort (van der Heide et al., 2010).

IBD is characterized by loss of tolerance to intestinal commensal bacterial and self-antigens, due to dysregulated CD4 T cell differentiation, with enhanced differentiation of Th1 and Th17 cells, as demonstrated by elevated levels of IFNγ, IL-17, and IL-22 in the intestinal biopsies and serum of patients with IBD (Fujino et al., 2003; Maloy and Powrie, 2011). In mouse models of colitis, T cell-specific loss of PTPN2 leads to increased numbers of Th1 and Th17 cells in the colonic lamina propria, mesenteric lymph nodes and spleen, corresponding with earlier onset and increased severity of disease (Spalinger et al., 2015). Mirroring this, humans with IBD carrying the PTPN2 rs1893217 SNP have greater Th1- and Th17-associated gene expression in colonic biopsies (Spalinger et al., 2015). Furthermore, there is impaired induction of regulatory T cells (Treg) in PTPN2 deficient colitic mice compared to PTPN2 competent counterparts (Spalinger et al., 2015). A recent study using a *Ptpn2* haplo-insufficient auto-inflammatory mouse model demonstrated that reduced PTPN2 expression (as occurs in human carriers of *PTPN2* SNPs) led to increased disease severity, mediated through a Treg intrinsic mechanism in which PTPN2 dephosphorylation of STAT3 prevents pathogenic loss of FoxP3 after acquisition of RORγt by Tregs (Svensson et al., 2019). However, this mouse model expresses very little ZAP-70, so the outcome may differ from otherwise normally signaling cells lacking PTPN2. These results are also somewhat conflicting with previous studies suggesting that loss of PTPN2 enhanced Treg number and/or function (Wiede et al., 2011; Yi et al., 2014; Bothur et al., 2015) so the influence of PTPN2 on Treg differentiation may depend on the inflammatory environment present in the different autoimmune models. In CD8 T cells, PTPN2 deficiency induces enhanced thymic positive selection and accumulation of peripheral effector/memory T cells, leading to systemic autoinflammatory disease, which was reproducible in wild-type recipient mice following adoptive transfer of CD8 T cells (Wiede et al., 2011).

In addition to its interaction with LCK and FYN, PTPN2 is also known to negatively regulate JAK/STAT pathways (Simoncic et al., 2002; ten Hoeve et al., 2002). JAK/STATs mediate signaling through receptors for inflammatory cytokines such as IL-2 and IFNγ (Simoncic et al., 2002), as well as cytokines, such as IL-7, that direct T cell differentiation and homeostasis (Pike et al., 2017). PTPN2 may also regulate the T cell repertoire by controlling thymocyte lineage commitment and TCR specification through both LCK and STAT5 dephosphorylation (Wiede et al., 2017a). Thus, PTPN2 downregulates T cell activation and differentiation/development through two independent mechanisms. However, the postulated effect of PTPN2 on JAK/STAT signaling has been challenged by the finding that a *PTPN2* risk allele (rs1893217) correlated with reduced PTPN2 expression and reduced (rather than increased, as might be expected) phosphorylated STAT5 in response to IL-2 and IL-15 (Long et al., 2011), highlighting its probable complex action in multiple cell lineages.

It is important to note that, like PTPN22, the action of PTPN2 is not confined to the T cell compartment. This is demonstrated by the differences in disease phenotypes between mice that are completely deficient in PTPN2 and those with conditional deletion in T cells alone. In the former, autoimmune disease is more severe and occurs at a much earlier stage of life (You-Ten et al., 1997; Heinonen et al., 2004; Wiede et al., 2017b), confirming that PTPN2 plays an essential role in other cell types of both the innate and adaptive immune system to prevent autoimmunity. Moreover, PTPN2 is also expressed in tissues out with the hematopoietic system, and it is likely that its role in autoimmune disease is mediated through these as well. For example, PTPN2 is also expressed in intestinal epithelial cells, where it modulates cytokine secretion in response to TNFα and regulates epithelial permeability (Scharl et al., 2009, 2011).

Polymorphisms in the *PTPN22* gene are also associated with IBD, although the different SNPs differ in their effect. Interestingly, the classical C1858T SNP does not have any effect on risk of UC (Martín et al., 2005), while the rarer SNPs G788A and C-1123G do: the former reduces the risk of UC (Bae and Lee, 2018), while in Chinese populations the latter increases UC disease severity (Chen et al., 2013). Conversely, PTPN22 C1858T reduces the risk of CD, while G788A has no effect on CD risk (Diaz-Gallo et al., 2011; Bae and Lee, 2018).

Type 1 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder characterized by absence of pancreatic insulin secretion (type 1) or lack of peripheral response to insulin (type 2), leading to elevated blood glucose levels and, if untreated, macro- and microvascular complications such as ischemic heart disease, stroke, peripheral neuropathy, nephropathy, and retinopathy. Type 1 diabetes (T1DM) is an autoimmune disease caused by antibody-mediated destruction of insulin producing beta cells in pancreatic islets of Langerhans that usually manifests during childhood or adolescence and persists lifelong.

Increased risk of T1DM has been linked to SNPs in both of the phosphatases already discussed, PTPN22 (Bottini et al., 2004) and PTPN2 (Burton et al., 2007; Cooper et al., 2008; Espino-Paisan et al., 2011). In children with risk-associated HLA genotypes, carriage of the PTPN22 R620W SNP is associated with earlier onset of clinical T1DM, reflected in earlier appearance of islet auto-antibodies, as well as a higher likelihood of developing additional diabetes-associated auto-antibodies such as glutamic acid decarboxylase autoantibodies and islet antigen-2 autoantibodies (Hermann et al., 2006). Similarly, *PTPN2* polymorphisms are associated with earlier onset of disease

(Espino-Paisan et al., 2011). This is backed up by mouse models, in which adoptive transfer of PTPN2-deficient CD8 T cells resulted in beta cell destruction and development of autoimmune diabetes, and this was exacerbated by co-transfer of PTPN2-deficient CD4 T cells (Wiede et al., 2014). Recently, novel mutations in coding regions of *PTPN2* were identified as susceptibility factors for development of childhood-onset T1DM in a Japanese population (Okuno et al., 2018), but these findings are yet to be replicated more widely.

Again, it is noteworthy that expression of PTPN22 and PTPN2 is not confined to T cells: PTPN22 expression is restricted to all hematopoietic cells, while PTPN2 is expressed more ubiquitously. Thus, the effects of their relevant SNPs on predisposition to autoimmune diseases are not mediated solely through T cells. For example, PTPN2 regulates cytokine-induced pancreatic β cell apoptosis (Moore et al., 2009), β cell insulin secretion (Xi et al., 2015), and insulin receptor signaling in muscle and liver (Galic et al., 2003), all of which contribute to T1DM pathogenesis. To attempt to determine the effect of PTPN2 deficiency in T cells specifically, Wiede et al. recently utilized a NOD mouse model (in which autoimmune diabetes occurs spontaneously) in which PTPN2 was lacking only in T cells. Their results demonstrated that T cell specific deficiency of PTPN2 led to increased incidence and earlier onset of autoimmune diabetes (Wiede et al., 2019). This was associated with pancreatic islet infiltration by CD8 and Th1 cells, as well as expansion of Tfh and B cells in the spleens, inguinal lymph nodes, and pancreatic draining lymph nodes, reinforcing the role for auto-antibodies in the disease pathogenesis (Wiede et al., 2019).

Mutations in the *PTPN11* gene (encoding SHP-2) are also associated with increased risk of T1DM (Burton et al., 2007), while an *ACP-1* (encoding LMPTP) polymorphism reduces risk. The latter association is subtler and appears to influence Th1/Th2 orientation depending on gender. The presence of the ACP1*A allele, which leads to low LMPTP activity, increases female susceptibility to allergic disorders (Th2-mediated), while reducing female susceptibility to T1DM and Crohn's (Th1-mediated) compared to males (Gloria-Bottini et al., 2007). However, the mechanism behind this may lie outside of T cells: in diabetes, at least, LMPTP appears to be a key promoter of insulin resistance through its dephosphorylation of the insulin receptor in the liver (Stanford et al., 2017).

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) caused by autoimmune neuronal demyelination leading to signal conduction block or slowing. The symptoms can be variable due to the potential for the disease to affect any part of the CNS; patients may experience some recovery between episodes (relapsing-remitting MS, the most common form) or there may be no remission phase (primary and secondary progressive MS). In the majority of cases, the disease is progressive, with accumulation of neurological deficits over time, and it is one of the leading causes of disability in the developed world. In contrast to the previously discussed antibody-mediated autoimmune diseases, MS is classically driven by CNS-infiltrating T lymphocytes causing destruction of the myelin sheath and the oligodendrocytes that produce it, in response to myelin antigens. Correspondingly, the T cell tyrosine phosphatases implicated in this disease are distinct from those discussed in the previous sections. Indeed, *PTPN22*^{C1858T} shows no correlation with MS risk. The notion of MS being a purely T cell driven disease has been challenged somewhat recently by the success of anti-CD20 monoclonal antibody treatments for MS (Bar-Or et al., 2008; Hauser et al., 2009, 2017), revealing an important role for B cells in the pathogenesis. However, it is thought that these pathogenic B cells play more of a role in antigen presentation and T cell activation rather than antibody production (Jelcic et al., 2018), and the autoreactive T cell remains the central player in MS.

Mutations in the *PTPRC* gene, encoding CD45, are associated with MS. Different highly conserved isoforms of CD45 may be expressed due to alternative splicing of exons 4, 5 and 6, giving rise to CD45RA, RB, and RC, respectively (Trowbridge and Thomas, 1994; Pulido et al., 1988). Different isoforms are expressed at distinct stages of T cell development (for example CD45RB on naïve cells; CD45RO on activated and memory cells) (Clement, 1992), and they differ in their ability to modulate TCR signaling. This has been suggested to be related to their relative size, which influences their ability to form homodimers (Xu and Weiss, 2002), as well as the speed and efficiency with which CD45 may be excluded from the TCR-pMHC complex in the immunological synapse to reduce local phosphatase activity, enhancing phosphorylation and TCR signaling (Leupin et al., 2000; Davis and van der Merwe, 2006; Cordoba et al., 2013; Carbone et al., 2017). A C77G point mutation, which prevents silencing of exon 4 splicing, leading to overexpression of the CD45RA isoform in T cells (Thude et al., 1995; Lynch and Weiss, 2001), has been described at greater frequency in patients with MS compared to healthy controls (Jacobsen et al., 2000). The alteration in isoform expression has been suggested to lead to reduced dimerization and autoinhibition of CD45, thereby enhancing CD45 phosphatase activity. T cells from heterozygous healthy human donors and patients with MS demonstrated increased proliferation and IL-2 production in response to TCR ligation (Do et al., 2006). A similarly enhanced proliferation was seen in response to stimulation with IL-2 (Windhagen et al., 2007). In addition, Tregs from C77G carriers showed impaired responsiveness to TCR/CD28 stimulation and reduced ability to suppress conventional CD4 T cells (Pokoyski et al., 2015). However, the association between the C77G SNP and MS has only been corroborated by some subsequent studies (Ballerini et al., 2002) but not others (Barcellos et al., 2001; Gomez-Lira et al., 2003; Nicholas et al., 2003; Cocco et al., 2004; Szvetko et al., 2009), although this disparity may be because of the case-control design of most primary studies and low allelic frequency in most populations (Tchilian and Beverley, 2006). It has furthermore been argued that any potential role played by CD45 in MS may actually relate to its function in oligodendrocyte development and myelination in the CNS (Nakahara et al., 2005). A further human CD45 polymorphism, C59A, alters alternative splicing, leading to expression of CD45RA on memory T cells and monocytes, and has been linked to MS in one MS multiplex family (Jacobsen et al., 2002).

In mice, a single point mutation in the CD45 wedge motif, glutamate 613 to arginine (E613R), prevents the formation of CD45 dimers, and negative regulation of CD45 is lost, leading to development of lymphoproliferative disease and severe autoimmune lupus-like nephritis (Majeti et al., 2000). Thymocytes from these mice exhibit enhanced TCR-induced MAPK activation and calcium flux, undergo positive selection more readily, and have higher numbers of peripheral T cells. These mice are more sensitive to experimental autoimmune encephalomyelitis (EAE) (Hermiston et al., 2009), a Th1 cell driven inflammatory demyelinating disorder of the central nervous system (CNS) frequently used as a mouse model of MS.

Alterations in SHP-1 signaling are also associated with MS, as well as other autoimmune diseases. So-called "motheaten" mice have a recessive *Ptpn6* frameshift mutation that leads to an absence of SHP-1 protein (Green and Shultz, 1975; Shultz et al., 1993; Tsui et al., 1993), and exhibit severe skin inflammation, as well as interstitial pneumonitis and a range of hematological abnormalities, including hyperproliferative T cells (Minton, 2013). PBMCs from patients with MS have reduced levels of SHP-1 mRNA and protein (Christophi et al., 2008), due to increased DNA methylation of the *SHP-1* promoter (Kumagai et al., 2012). This acquired deficiency of SHP-1 is thought to lead to T cell induced inflammation through a reduction in dephosphorylation of targets such as STAT1, STAT6, NFκB and consequent increase in STAT-responsive inflammatory genes (Feng et al., 2002; Christophi et al., 2009). Furthermore, treatment of PBMCs from MS patients with IFNβ (a current treatment for MS) induces SHP-1 activity with corresponding reduced inflammatory gene expression, and the therapeutic effect of IFNβ is also dependent on SHP-1 (Christophi et al., 2009). This is backed up by EAE mouse models, in which heterozygous deletion of SHP-1 led to increased IFNγ production and increased expansion of MBP (myelin basic protein, the predominant auto-antigen) specific T cells in response to lower antigen concentrations, and these mice developed a more severe EAE phenotype (Deng et al., 2002). However, acquired deficiency of SHP-1 is not likely to be a direct cause of MS, rather it confers susceptibility to auto-inflammatory demyelination if other conditions are met, as has been demonstrated in mice (Croker et al., 2008). In addition, while T cells play a significant role in the pathogenesis of MS, the effects of SHP-1 deficiency in other cells types such as myeloid cells and oligodendrocytes is also expected to be important (Gruber et al., 2015).

SHP-2 may also participate in T cell driven pathology in MS, as treatment of mice with a SHP-2 inhibitor enabled resistance to induction of EAE following inoculation with myelin oligodendrocyte glycoprotein35-55 (MOG) protein, via prevention of infiltration of CD8 T cells into the CNS (Luo et al., 2014). These observations are yet to be borne out in human studies, where the picture is likely to be more complicated.

Psoriasis

Auto-reactive T cells also play a central role in psoriasis, a chronic relapsing inflammatory skin disease characterized by epidermal hyperplasia and desquamation. Specifically, epidermal CD8 T cells that respond to skin epitopes mediate the initiation phase of the disease (Johnston et al., 2004; Lande et al., 2014; Arakawa et al., 2015), and subsequent amplification of skin inflammation is driven by a predominantly Th17 response (Lowes et al., 2013; Girolomoni et al., 2017). The central importance of the Th17 axis has been highlighted by recent success of anti-IL-17 monoclonal antibodies in the treatment of psoriasis (Mease et al., 2014; McInnes et al., 2015).

Aberrations in the same phosphatases as those linked to MS are also associated with psoriasis. T cells from patients with psoriasis are more sensitive to IFNα-induced stimulation, leading to increased STAT signaling and pro-inflammatory IFNγ production (Eriksen et al., 2005). This has been shown to be mediated through reduced expression of SHP-1 in psoriatic T cells, and was reversible by the forced expression of SHP-1 in T cells from the skin of psoriasis patients (Eriksen et al., 2010). In contrast to MS, in psoriasis the reduction in SHP-1 is due to demethylation of the promotor 2 of the gene (Ruchusatsawat et al., 2006).

CD45 has been shown to be significantly overexpressed in the bone marrow hematopoietic stem cells and PBMCs of patients with psoriasis, compared to those from healthy controls (Zhang et al., 2014). This higher level of CD45 expression correlated with disease severity index (Zhang et al., 2014), suggesting that this could be used as a biomarker for severity.

Interestingly, while the PTPN22 R620W polymorphism does not associate with skin psoriasis, it does increase the risk of psoriatic arthritis (Bowes et al., 2015), suggesting that the two diseases have diverging pathogeneses, and PTPN22 may in some way alter the balance or phenotype of CD8 and/or Th17 cells, particularly when the known action of PTPN22 on CD4 T cell differentiation is taken into consideration (Vang et al., 2013).

Other Autoimmune Diseases Associated With T Cell PTPs

There are several other autoimmune diseases that have been linked to PTP mutations or altered expression. *PTPN22*^{C1858T} is the predominant association, and has been linked to Grave's disease (Velaga et al., 2004; Heward et al., 2007), vitiligo (Cantón et al., 2005), myasthenia gravis (MG) (Vandiedonck et al., 2006; Chuang et al., 2009), Addison's disease (Skinningsrud et al., 2008), and alopecia areata (Lei et al., 2019). Grave's disease (autoimmune-mediated hyperthyroidism) is also associated with polymorphisms in the *PTPN2* gene (Todd et al., 2007).

The tyrosine phosphatases discussed here are those most studied with respect to autoimmune disease, but the list is not exhaustive. Although several human PTP SNPs have been linked to autoimmunity through GWAS, there is still much work to be done in order to deepen our understanding of the immunopathogenic mechanisms. It is striking that diseases that are strongly auto-antibody mediated, such as most rheumatological diseases, are affected by alterations in PTPN22 and PTPN2, whereas T cell driven diseases such as MS and psoriasis lean more heavily towards changes in other PTPs such as CD45 and SHP-1. This may suggest that the different PTPs influence different types of immune response, or be due to the relative influence of each PTP on different populations of T cells,

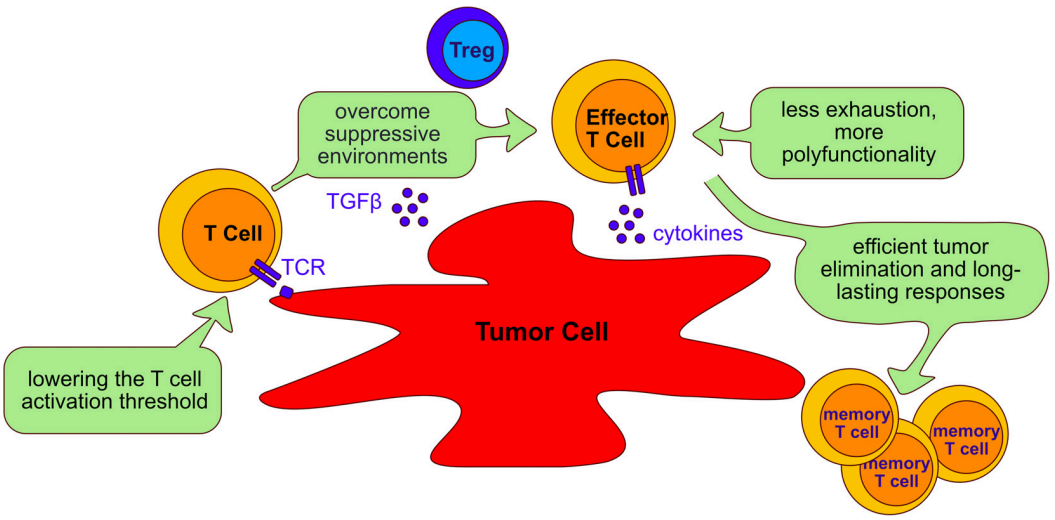

FIGURE 5 | Aims of adoptive cell transfer therapy for treatment of cancer. Adoptive cell transfer strategies aim to: (I) lower the T cell activation threshold to allow response to tumor antigens; (II) overcome the suppressive environment generated by tumor cells and immunosuppressive cells such as Tregs; (III) reduce T cell exhaustion and increasing polyfunctionality of T cells; (IV) enable long lasting responses that allow persistent tumor elimination. The beneficial role of targeting the phosphatases PTPN2, PTPN22, SHP-1 and SHP-2 to improve adoptive cell transfer therapy is discussed in the text (Section "Tyrosine phosphatases as targets in immunotherapy").

for example increased Th1 and Tfh responses, compared with enhanced CD8 and Th17 functions. Autoimmune diseases are polygenic, and it is likely that an individual PTP mutation confers only modest relative risk of developing disease; rather disease occurs in the context of complex genetic and environmental pre-disposing factors. Deciphering the relative contributions to disease of individual PTPs and interrogating them as potential therapeutic targets should be a focus for future work.

TYROSINE PHOSPHATASES AS TARGETS IN IMMUNOTHERAPY

Immunotherapy is the use of the immune system to fight cancer. There are different kinds of immunotherapy, for instance, monoclonal antibodies that target inhibitory molecules like PD-1 and CTLA-4 are called checkpoint inhibitors. Additionally, tumor antigens can be used to target cancer cells. Adoptive T cell therapy (ACT) is a novel modality of immunotherapy using either tumor infiltrating lymphocytes (TILs) from the patient or engineered T cells with a TCR or a chimeric antigen receptor (CAR) that recognizes tumor antigens. Both options can induce complete and durable regression of tumors (Johnson et al., 2009; Rosenberg et al., 2011; Robbins et al., 2015). Despite the successful treatment of a proportion of cancer patients with ACT, the majority of patients do not yet benefit from the therapy, especially when treating solid tumors. The challenges faced by adoptively transferred T cells in eliminating tumors is illustrated in **Figure 5** and below we discuss studies that have targeted four phosphatases, PTPN2, PTPN22, SHP-1 and SHP-2 as a strategy of overcoming these hurdles and improving ACT in several cancer models (**Table 3**).

Regulating T Cell Activation Thresholds to Improve Adoptive Cell Transfer

Limitations of ACT using TILs or engineered T cells are largely imposed by resistance mechanisms of cancer cells and their evasion from immune surveillance. Tumor antigens can be divided into tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) (Magalhaes et al., 2019; Janelle et al., 2020). TSAs refer to antigens and neoantigens that often newly arise from acquired genetic variants. As these are antigens that the adaptive immune system has not experienced previously, TSAs usually elicit vigorous immune responses that are specific to the cancer cells (Robbins et al., 2013; Lu et al., 2014) and are thought to elicit fewer on-target off-tumor effects in the patient because their expression is restricted to the tumor (Wang and Cao, 2020). TAAs on the other hand include embryonic/differentiation antigens and overexpressed self-antigens. TAAs are widely used as targets for immunotherapy because they often expressed across several cancer types (Janelle et al., 2020). However, it is presumed that overexpressed TAAs induce weaker T cell responses because T cells expressing TCRs with strong affinity to self-antigens would be eliminated by negative selection during development in the thymus (Aleksic et al., 2012). Since TAAs are frequently used as target antigens for ACT, enhancing the responses of T cells that express low-affinity TCRs would potentially improve immunotherapy of cancer (**Figure 5**). Targeting the T cell activation threshold in a cell intrinsic manner could expand the TCR repertoire available to ACT. PTPs that limit the threshold of TCR activation in response to low-affinity antigens are interesting potential targets in this regard.

PTPN22 is important for regulating TCR sensitivity to low-affinity agonists, and murine PTPN22-deficient OT-1 CD8 T cells were more permissive for the production of an effector response

TABLE 3 | Functional outcomes of Tyr phosphatase deletion in T cells.

PTPase	KO Mouse	Functional Outcome	References
PTPN2	pLck-Cre; PTPN2$^{fl/fl}$	↑ effector/memory T cells	Wiede et al., 2011
	Mx1-Cre; PTPN2$^{fl/fl}$		Wiede et al., 2017b
	pLck-Cre; PTPN2$^{fl/fl}$ OT-1	↑ response to low affinity ligands	Wiede et al., 2011
	PTPN2 sgRNA/Cas9 OT-1	↑ response B16-OVA	LaFleur et al., 2019
	Lck-Cre; PTPN2$^{fl/fl}$ HER2 CAR	↑ PD-1 and LAG-3 expression	Wiede et al., 2020
	CD4-Cre; PTPN2$^{fl/fl}$	↑ Th1 and Th17, ↓ Treg	Spalinger et al., 2015
PTPN22	PTPN22$^{-/-}$ Rag1$^{-/-}$ OT-1	↑ effector/memory T cells	Hasegawa et al., 2004; Brownlie et al., 2012
		↑ response to low affinity ligands & tumors	Salmond et al., 2014; Brownlie et al., 2017, 2019
		↑ polyfunctionality	Salmond et al., 2014
		↑ resistance to suppressive cytokines and Tregs	Brownlie et al., 2017
		↑ proliferation	Knipper et al., 2020
	PTPN22 sgRNA/Cas9	↑ response to tumors	Cubas et al., 2020
	PC3-Cre; PTPN2$^{fl/fl}$ OT-1	↑ Treg suppression & IL-10 secretion	Brownlie et al., 2012
	PTPN22$^{-/-}$	↑ Treg	Maine et al., 2012; Knipper et al., 2020
SHP-1	Lck-Cre; SHP-1$^{fl/fl}$	↑ CD8 T cells proliferation	Fowler et al., 2010; Stromnes et al., 2012
		↑ polyfunctionality	Stromnes et al., 2012
		↑ response to leukemia cells	Stromnes et al., 2012
		↑ resistance to Tregs	Mercadante and Lorenz, 2017
		↓ short-lived effector cell formation	Fowler et al., 2010
	shRNA KD OT-1	↑ polyfunctionality	Snook et al., 2020
	SHP-1 sgRNA/Cas9 CD19 CAR	↑ polyfunctionality	Ruella et al., 2020
		↑ response to tumors	Ruella et al., 2020
	Motheaten mutant	↑ IL-2 production in CD4 T cells, ↓ requirement for CD28 co-stimulation	Sathish et al., 2001b
		↑ Treg suppression	Iype et al., 2010
SHP-2	SHP-2 sgRNA/Cas9 CD19 CAR	↑ degranulation & IL-2 production	Ruella et al., 2020
	CD4-Cre; SHP-2$^{fl/fl}$	↓ colitis-associated colorectal cancers	Liu et al., 2017
		↑ Th1 differentiation & IFNγ production	Liu et al., 2017
		↑ response to colon cancers	Zhao et al., 2019

to a self-antigen (Salmond et al., 2014). Moreover, weak agonists stimulated substantially more *PTPN22*$^{-/-}$ OT-1 CD8 T cells to produce IFNγ, TNFα, and GM-CSF (Salmond et al., 2014). Similarly, knockout of PTPN2 in OT-1 CD8 T cells resulted in enhanced cell proliferation and this effect was more pronounced in cells that were stimulated with lower-affinity peptides (Wiede et al., 2011). Studies have shown that deletion of PTPN22 or PTPN2 in CD8 T cells improved tumor clearance in a number of mouse tumor models (Brownlie et al., 2017, 2019; LaFleur et al., 2019; Wiede et al., 2020). In particular, responses to weak affinity tumor antigens were enhanced by knockout of PTPN22 which suggests that this strategy might be beneficial in promoting T cell responses to weaker TAAs (Brownlie et al., 2017, 2019).

Several studies have assessed the influence of SHP-1 on modulating the ability of T cells expressing TCRs of different affinities to control tumor cells. Hebeisen et al. found that the effectiveness of TCR-engineered CD8 T cells to kill tumors was limited by two different mechanisms (Hebeisen et al., 2013). The first was characterized by preferential expression of the PD-1 inhibitory receptor within T cells expressing the highest supraphysiological affinity TCR, and T cells with this variant TCR benefited most from PD-L1 blockade. The second was associated with the progressive increase of SHP-1 expression in a TCR affinity-dependent manner. In contrast to PD-L1 blockade, inhibition of SHP-1 (and partially SHP-2) using the PTP inhibitor sodium stibogluconate (SSG) resulted in increased degranulation and cytotoxicity of engineered T cells for all TCR affinity variants. These results suggest that SHP-1 may play a dual role and restrict not only T cell signaling of lower affinity TCRs (Stefanova et al., 2003), but also of higher and supraphysiological affinities. This role seems to be independent of PD-1 signaling because only T cells with the highest affinity TCR variant benefited from PD-L1 blockade. Together these results indicated that targeting SHP-1 in T cells with engineered TCRs can augment their functional efficacy. However, another study demonstrated that although SHP-1 knockdown functionally enhanced low-affinity T cells, it showed limited therapeutic benefit for the treatment of B16 melanoma cells *in vivo* (Snook et al., 2020). A partial CRISPR-mediated knockout of SHP-1 in the SUP-T1 cell line resulted in increased phosphorylation of CD3 chains and of ERK1/2 in all NY-ESO1-TCR affinity variants used, with the exception of the lowest affinity variant (Presotto et al., 2017). TCR variants considered to have an optimal affinity for pMHC showed the greatest increase in pERK1/2 in SHP-1 knockout cells.

There appears to be an optimal window for TCR affinities and increasing TCR-pMHC affinities and binding half-lives above a natural level can lead to less functional T cells (Kalergis et al., 2001; McMahan et al., 2006; Thomas et al., 2011). It seems that

maximal biological activity occurs between a well-defined affinity window with K_D ranging from 5 to 1μM (Schmid et al., 2010; Irving et al., 2012), but this could differ between various TCRs. SHP-1 seems to restrict not only signaling of lower-affinity TCRs, but also of high-affinity TCRs (Hebeisen et al., 2013). Thus, deletion of SHP-1 in human T cells would need to be tested for each TCR and whether functional enhancement occurred would need to be monitored. Furthermore, SHP-1 deficient CD4 T cells produced more IL-2 and in these cells loss of SHP-1 obviated the requirement for CD28 co-stimulation (Sathish et al., 2001b). Engagement of co-stimulatory receptors such as CD28, LFA-1 and CD2 can significantly lower the threshold of responsiveness of the TCR (Viola and Lanzavecchia, 1996; Bachmann et al., 1997, 1999). Targeting SHP-1 in T cells using shRNAs or CRISPR might be a promising strategy to improve adoptive T cell transfer. Indeed, deletion of SHP-1 using shRNAs T cells demonstrated enhanced cytotoxicity *in vitro* (Stromnes et al., 2012).

Although several studies have shown that the phosphatases SHP-1 and SHP-2 have overlapping substrate specificities, other studies have indicated that they preferentially co-localize with the TCR and PD-1, respectively (Yokosuka et al., 2012; Presotto et al., 2017). Upon PD-1-ligand interaction, PD-1 and the TCR form microclusters which downregulate TCR downstream signaling by recruiting SHP-2 (Yokosuka et al., 2012). PD-1/PD-L1 and TCR complexes co-localize at the membrane and together exclude CD45 (Carbone et al., 2017). This might shift the balance in favor of PD-1 signaling and attenuate TCR signaling. It was suggested that PD-1 might increase the threshold that needs to be overcome by TCR stimulation to initiate signals (Celis-Gutierrez et al., 2019). Thus, SHP-2 deficiency might also result in enhancement of TCR activity by lowering the activation threshold. However, CRISPR-mediated knockout of SHP-2 in SUP-T1 cells did rather decrease ERK1/2 phosphorylation using TCRs with increasing affinities and had no impact on proximal TCR/CD3 signal initiation (Presotto et al., 2017). In summary, although knockout of some PTPs to lower the threshold for T cell activation by tumor antigens might be a viable way to improve T cell-mediated responses to cancer cells, there remain questions about whether such a strategy would be suitable for TCRs of all affinities or whether it would benefit only a subset of tumor specific T cells.

Targeting Phosphatases to Mitigate T Cell Exhaustion

T cell-mediated tumor responses are complex and very high-affinity CD8 T cell responses to tumor cells can lead to tolerization in the tumor microenvironment (Janicki et al., 2008; Zahm et al., 2017). Indeed, continual or prolonged exposure to the tumor antigen can induce functional exhaustion of the T cells (Schietinger et al., 2016) and tumor-infiltrating TCR-engineered T cells can progressively lose the ability to produce IFNγ and TNFα (Stromnes et al., 2015). Exhausted T cell responses have been reported in tumor settings as well as during chronic viral infections. Upregulation of inhibitory molecules (PD-1, CTLA-4, Tim-3, LAG-3, etc.) or functional dysregulation such as decreased cytotoxicity and reduced polyfunctional cytokine expression are characteristics of exhausted T cells. Polyfunctionality describes the ability of T cells to express two or more cytokines simultaneously and polyfunctional T cells are thought to be more efficient in fighting cancer cells (Ma et al., 2013). Thus, if PTPs could be targeted in order to make T cell less prone to exhaustion and more polyfunctional, it may improve T cell function in the tumor after ACT (**Figure 5**).

The influence of PTPN22 in T cell exhaustion has been studied most extensively in the context of chronic viral infections. Infection of PTPN22$^{-/-}$ and control mice with lymphocytic choriomeningitis virus (LCMV) clone 13 resulted in chronic infection of the host and PTPN22$^{-/-}$ mice controlled the viral infection more efficiently than control mice (Jofra et al., 2017). In this context, the presence of PTPN22 was able to promote CD8 T cell exhaustion; however, this was a consequence of T cell-extrinsic effects, namely, loss of PTPN22 from other hematopoietic cells. Another study found that in PTPN22$^{-/-}$ mice after chronic LCMV infection, PTPN22$^{-/-}$ CD8 T cells were less exhausted and more polyfunctional (Maine et al., 2016). This was dependent on CD4 T cell help because depletion of CD4 T cells in PTPN22$^{-/-}$ mice led to exhaustion of CD8 T cells. Interestingly, the increased prolration and inflammatory cytokine expression of PTPN22$^{-/-}$ CD4 T cells were also regulated by T cell-extrinsic effects. Other recent work has also suggested that loss of PTPN22 might be beneficial for anti-tumor responses, since PTPN22$^{-/-}$ mice showed increased tumor rejections in combination with anti-PD-L1 immunotherapy (Cubas et al., 2020). This effect was dependent on T cells and IFNα signaling, although the precise mechanism remains to be determined. In the same study, the frequency of the PTPN22 R620W SNP was determined in a cohort of patients with non-melanoma skin cancer and compared with healthy controls. Interestingly, the frequency of R620W was reduced in patients, suggesting a protective effect of this SNP, and homozygous patients showed improved overall survival after anti-PD-L1 therapy. The effect of the R620W SNP, however, might be cancer origin related, as another study showed that the frequency of R620W carriers was significantly increased in chronic lymphocytic leukemia (CLL) cases compared to healthy controls (Hebbring et al., 2013). Additionally, PTPN22 was overexpressed in CLL patients and PTPN22 overexpression inhibited antigen-induced apoptosis of CLL cells (Negro et al., 2012). Thus, the outcome of checkpoint inhibition therapy may depend on the PTPN22 allele expressed and the particular cancer under study.

In a comparable study, adoptive transfer of PTPN2$^{-/-}$ or wildtype CD8 T cells into mice chronically infected with LCMV showed that the PTPN2$^{-/-}$ CD8 T cells proliferated more and expressed higher percentages of granzyme B^{+} cells (LaFleur et al., 2019). Interestingly, TIM-3^{+} cell frequencies were also enhanced in the PTPN2$^{-/-}$ CD8 T cell population. Killing assays using TIM-3^{+} PTPN2$^{-/-}$ or control CD8 T cells isolated from LCMV infected mice, showed increased killing of target cells by the TIM-3^{+} PTPN2$^{-/-}$ T cells. Consistent with this, PTPN2 deficient OT-1 CD8 T cells were superior in controlling the growth of B16-OVA melanoma cells and these T cells expressed higher frequencies of granzyme B^{+} cells. Similar results were obtained for MC38 colon adenocarcinoma cells. Another study found

that PTPN2 deficient T cells were more efficient in restraining AT3-OVA mammary carcinoma cells, but the PTPN2$^{-/-}$ OT-1 CD8 T cells expressed lower frequencies of PD-1^{+} or LAG-3^{+} cells (Wiede et al., 2020). Strikingly, PTPN2 deletion also enhanced cytotoxicity and cytokine production of Her2-specific CAR T cells. These PTPN2$^{-/-}$ CAR T cells expressed more PD-1 and LAG-3 than control T cells, so would potentially be more susceptible to checkpoint inhibition. Thus, the question remains whether human PTPN2-deficient T cells expressing a tumor-specific CAR or TCR would similarly express increased exhaustion markers. It is possible that the higher affinity of the CAR compared to the TCR favors the generation of exhausted T cells, but this remains to be proven. Nevertheless, PTPN2 could be an interesting target for improving T cells for ACT.

CRISPR-mediated knockout of SHP-1 and SHP-2 has also been studied in CD19-specific human CAR (CAR19) T cells and resulted in higher degranulation and higher expression of IL-2 (Ruella et al., 2020). SHP-1 deficient CAR19 T cells also secreted more IFNγ, TNFα, and IL-2 and these T cells were more efficient in killing tumor cells *in vitro* and *in vivo*. From these results the authors concluded that SHP-1 and SHP-2 deficiency reduced T cell exhaustion.

In summary, targeting of several PTPs in T cells has been shown to improve T cell polyfunctionality and decrease the exhaustion of those T cells upon chronic antigen encounter. Such studies indicate that this approach might be a promising strategy either alone or in combination with checkpoint inhibitors, to improve T cell efficacy in protecting against cancers.

Overcoming the Suppressive Tumor Microenvironment by Targeting Phosphatases

A major challenge in therapy of solid tumors is the suppressive microenvironment that can dampen T cell responses (Wellenstein and de Visser, 2018). This microenvironment can recruit suppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells or produce suppressive cytokines, such as TGFβ and IL-10, that can inhibit T cell function (**Figure 5**). Additionally, the metabolism of cancer cells can limit oxygen and nutrients such as glucose, and can accumulate waste products such as lactate that inhibit T cells directly (Anderson et al., 2017). Cancer cells often express ligands for inhibitory receptors on T cells. For instance, expression of the PD-1 ligand, PD-L1, in several different solid tumors was associated with worse survival of cancer patients (Wang X. et al., 2016). The hostile tumor microenvironment can lead to dysfunction or exhaustion of T cells in the tumor, or can prohibit T cell infiltration into the tumor. PTPs that are involved in the regulation of sensitivity to suppressive factors in the microenvironment would be attractive targets to improve ACT. Remarkably, OT-1 T cells that lack PTPN22 were found to be more resistant to both TGFβ-mediated suppression and suppression by PTPN22-sufficient Treg cells (Brownlie et al., 2017). It was suggested that increased IL-2 production by murine PTPN22 deficient T cells helped them overcome the suppressive effects of TGFβ in the tumor microenvironment. This led to a better response of CD8 T cells to tumors and a more efficient elimination of tumor cells.

CD4 T cells can be beneficial for cancer therapy as they can enhance CD8 T cell-mediated elimination of cancer cells (Li et al., 2016). Interestingly, deletion of PTPN2 in CD4 T cells led to increased frequencies of Th1 and Th17 cells and the loss of Treg cells. When re-stimulated *in vitro,* PTPN2$^{-/-}$ CD4 T cells expressed more IFNγ and IL-17 (Spalinger et al., 2015). Given that pro-inflammatory responses are thought to be helpful in the suppressive tumor environment, deletion of PTPN2 in CD4 T cells could enhance CD8 T cell-mediated tumor elimination and might also inhibit the development of Tregs. Further studies are needed to analyze the benefits of PTPN2 loss in different T cell subpopulations.

In light of the above findings, one might consider the use of small molecule inhibitors that target phosphatases such as PTPN22 and PTPN2 as a viable therapeutic option to improve ACT. A recent study has shown that tumor clearance was improved in PTPN22$^{-/-}$ mice when combined with anti-PD1 therapy, in support of this strategy (Cubas et al., 2020). However, such an approach requires caution as PTPN22 is expressed in all hematopoietic cells and was found to be important for conventional dendritic cell homeostasis (Purvis et al., 2020). In mice PTPN22 deficiency led to increased frequencies of Tregs in the thymus and the periphery (Maine et al., 2012; Knipper et al., 2020). Moreover, PTPN22$^{-/-}$ Tregs were more suppressive and secreted more IL-10 than their wildtype counterparts (Brownlie et al., 2012) which might promote a more suppressive tumor environment. PTPN2 is also expressed ubiquitously and plays multiple roles in different cells (Mosinger et al., 1992; Spalinger et al., 2018) so that the use of small inhibitors targeting PTPN2 in cancer might present the risk of causing additional side-effects. In addition, PTPN2 is 72% identical to another Tyr phosphatase, PTP1B, within the catalytic domain (Romsicki et al., 2003), which might pose problems for the development of inhibitors specific for PTPN2.

Small molecule inhibitors that target the action of the phosphatases SHP-1 and/or SHP-2 are currently undergoing extensive clinical trials for efficacy in cancer treatment (**Table 4**). Interestingly, the absence of SHP-1 in CD8+ T cells allowed them to resist suppression by Treg activity in a T cell-intrinsic manner, which may be crucial to survival of those cells once they enter the tumor microenvironment (Mercadante and Lorenz, 2017). However, inhibition of SHP-1 in Tregs led to increased suppressive function and TCR-antigen presenting cell (APC) conjugate formation (Iype et al., 2010). This is an important aspect when using small molecule inhibitors because they could also act on Tregs, thereby enhancing suppression in the tumor microenvironment. A preclinical study with the SHP-1 inhibitor TPI-1 showed anti-tumor effects in established B16 melanomas (Kundu et al., 2010). However, several phase I studies with the PTP inhibitor SSG in combination with IFNα showed no clinical response (Naing et al., 2011; Yi et al., 2011). Being phase I studies, anti-tumor effects were measured, but were not the primary focus. Moreover, SSG is not specific for SHP-1 but also inhibits SHP-2 (Pathak and Yi, 2001). Off-target effects might be one explanation for the lack of clinical efficacy. Another explanation

TABLE 4 | Clinical trials of SHP-1 and SHP-2 phosphatase inhibitors.

Trial number	Compound	Target(s)	Disease	Status	References
NCT00629200	Sodium stibogluconate	SHP-1 and SHP-2	Various solid tumors	Phase I completed, no objective response, adverse events in up to 68% of patients	Naing et al., 2011
NCT00498979	Sodium stibogluconate	SHP-1 and SHP-2	Malignant melanoma	Phase I completed, no objective response, dose-limiting toxicities	Yi et al., 2011
NCT03443622	SC-43	SHP-1	Refractory solid tumors	Phase I completed	
NCT00311558	Sodium stibogluconate	SHP-1 and SHP-2		Phase I completed	Yi et al., 2011
NCT03114319	TNO155	SHP-2	Advanced solid tumors	Phase I recruiting	
NCT04000529	TNO155	SHP-2	Advanced solid tumors	Phase I recruiting	
NCT04330664	TNO155	SHP-2	KRAS G12C mutation cancers	Phase I recruiting	
NCT03565003	JAB-3068	SHP-2	Advanced solid tumors	Phase I/IIa recruiting	
NCT03518554	JAB-3068	SHP-2	Advanced solid tumors	Phase I recruiting	
NCT04045496	JAB-3312	SHP-2	Advanced solid tumors	Phase I recruiting	
NCT03634982	RMC-4630	SHP-2	Relapsed/Refractory solid tumors	Phase I recruiting	
NCT03989115	RMC-4630	SHP-2	Relapsed/Refractory solid tumors	Phase I recruiting	
NCT04252339	RLY-1971	SHP-2	Advanced or metastatic solid tumors	Phase I recruiting	

could be that SHP-1 expression is altered in many malignancies, and small molecule inhibitors might influence not only SHP-1 activity in hematopoietic cells but also in the tumor cells themselves. A better approach may be to specifically target SHP-1 in CD8 T cells. Indeed, it was shown that SHP-$1^{-/-}$ CD8 T cells proliferated better and had improved cytolytic activity *in vitro*, and ultimately showed improved clearance of leukemia cells in a preclinical adoptive T cell therapy mouse model (Stromnes et al., 2012). In this study, a higher percentage of SHP-$1^{-/-}$ CD8 T cells secreted IFNγ and TNFα, which might be one of the mechanisms by which SHP-$1^{-/-}$ CD8 T cells were able to eliminate tumor cells more efficiently. SHP-1-deficient CD8 T cells produced more IL-2 and formed more stable and long-lasting conjugates with APCs (Sathish et al., 2007). However, another study could not confirm better B16 melanoma elimination by SHP-1-deficient OT-1 CD8 T cells (Snook et al., 2020). These discrepancies may be a reflection of different sensitivity to elimination by ACT in the different tumor models used in these studies. However, the latter study found that combination of transfer of SHP-$1^{-/-}$ T cells and anti-PD-1 treatment improved control of tumor growth indicating that therapies that combine inhibitory molecules blockade and ACT with T cells lacking PTPs could be a beneficial strategy for cancer treatment. This approach would need further testing in preclinical studies.

SHP-2 deficiency in CD4 T cells was found to augment colitis and reduced the incidence of colitis-associated colorectal cancers (Liu et al., 2017). SHP-2 deficiency also resulted in increased Th1 differentiation and IFNγ production. A recent study using the allosteric SHP-2 inhibitor SHP099 showed reduced tumor growth in an anti-PD-1-resistant non-small cell lung cancer mouse model (Chen et al., 2020). They found a higher percentage of CD8 T cells in tumors treated with the inhibitor. In a xenograft melanoma tumor model, tumor growth was inhibited by the SHP-2 inhibitor 11a-1 (Zhang et al., 2016). Moreover, tumor growth of colon cancer cells was reduced after treatment with different SHP-2 inhibitors (Zhao et al., 2019). CD8 tumor infiltrating lymphocytes from mice treated with the SHP-2 inhibitor produced more IFNγ and granzyme B. When colon cancer cells were injected into mice lacking SHP-2 in T cells, the resulting tumors were significantly smaller in the knockout mice. There are several ongoing clinical trials using SHP-2 inhibitors for cancer treatment (**Table 4**) but no efficacy data are yet available. However, SHP-2 is expressed in macrophages and SHP-2 inhibitors can negatively regulate suppressive M2-type tumor-associated macrophages (Chen et al., 2020) which could positively influence the outcomes for cancer treatment. Interestingly, two recent mouse studies confirmed an advantage in controlling tumor cell growth when using SHP-2 inhibitors in combination with anti-PD-1/PD-L1 therapy (Zhao et al., 2019; Chen et al., 2020). These results again indicate that combination therapies targeting PTPs and inhibitory molecule blockade might be an effective cancer treatment. In summary, using PTPs to improve the function of T cells in the tumor microenvironment or to make the cells less prone to tumor-intrinsic inhibitory mechanisms could be a valuable tool to improve T cell-mediated killing of tumor cells.

Other Challenges for Improving Adoptive T Cell Therapy

Ex vivo manufacturing of T cells on a commercial scale remains a challenge. Problems include the variability of the starting material between patients and limited understanding of the parameters that are necessary to produce high-quality T cells in sufficient numbers for the transfer (Amini et al., 2020). There is some evidence from animal models suggesting that multiple doses of adoptively transferred T cells are superior to a single infusion, and therefore, expanding as many cells as possible would be an advantage. PTPs are involved in the regulation of T cell proliferation after TCR stimulation. For instance, the presence of murine PTPN2 attenuated T cell activation and proliferation *in vitro*, indicating that deletion of PTPN2 in T cells could lead to better proliferation and increased cell numbers which would be advantageous. Additionally, PTPN22 deficient murine

T cells that were adoptively transferred into immunodeficient lymphopenic hosts showed more proliferation (Knipper et al., 2020) and patients are frequently rendered lymphopenic before ACT to improve engraftment of the transferred cells.

It is still out for debate which T cell subpopulation is more efficient in eliminating tumor cells and additionally form long-lasting memory responses after adoptive transfer into the cancer patient. Some studies show that effector T cells are most efficient in eliminating tumor cells initially, but fail to persist *in vivo* or form memory responses (Warren et al., 2010). This might be due to their terminal differentiation stage and consequently rapid exhaustion as a result of the extensive *in vitro* expansion protocol. A search for the T cell subpopulation with the highest proliferative potential has led to the identification of the stem cell-like memory T cell (T_{SCM}) subpopulation (Gattinoni et al., 2009, 2011). These memory T cells have a phenotype similar to naïve T cells, but they co-express memory markers, for instance CD95 and IL-2Rβ (Gattinoni et al., 2011). T_{SCM} cells represent the least differentiated memory subpopulation and undergo extensive proliferation in response to the homeostatic cytokines IL-15 and IL-7. In a humanized mouse model T_{SCM} cells were more efficient in eliminating mesothelioma tumor cells.

PTPN22$^{-/-}$ mice (Hasegawa et al., 2004; Brownlie et al., 2012) and mice that lack PTPN2 in T cells (Wiede et al., 2011, 2017b) show increased expansion of the effector and memory T cell compartment. Conditional knockout of SHP-1 in CD8 T cells resulted in greater expansion of the cells after stimulation with low peptide concentrations (Fowler et al., 2010). Additionally, SHP-1 deficiency limited the formation of short-lived effector cells and did not influence the generation of long-lived memory cells. In contrast, deficiency of the phosphatase SHP-2 in CD8 T cells did not affect the formation of memory T cells (Miah et al., 2017). Therefore, targeting PTPs to increase the proportion of memory T cells, and especially T_{SCM} cells, in the T cell product or after adoptive transfer could further improve ACT. In the future, more detailed analysis of the memory populations and the differentiation state of the cells is necessary to determine the potential of targeting PTPs to improve in vitro generation of T cells for adoptive T cell therapy.

CONCLUDING REMARKS

It is clear that phosphatases are essential for regulating T cell responsiveness. However, with the exception of CD45, T cell restricted loss of the other PTPs discussed here tended to have rather subtle effects. Nevertheless, the experience of GWAS studies that have linked mutations in multiple PTP genes with increased susceptibility to a wide variety of autoimmune diseases, point to the importance of PTPs in maintaining immune cellular homeostasis. These genetic PTP variants may affect the behavior of multiple hematopoietic (and possibly non-hematopoietic) cell types, as most are widely expressed, which undoubtedly contributes to autoimmunity. However, they also have clear effects on T cell behavior and T cells are generally considered to be key drivers of autoimmunity. Important features of autoimmune T cell behavior are their resistance to cellular exhaustion in the face of persistent antigen, their persistence in hostile inflamed tissue sites, and their resistance to both immunosuppressive cytokines, including TGFβ, and to the action of Tregs. A wealth of experimental evidence now suggests that these undesirable features of autoimmune T cells can be reproduced in a cell intrinsic fashion by the selective removal of any one of several PTPs. On the flip side, ACT experiments with T cells lacking these PTPs have shown improved anti-tumor activity in a variety of mouse models. Taking these lessons learned from studying autoimmune T cells, therefore, has interesting potential for the improvement of human T cell adoptive cell therapy and we await confirmation that these lessons will indeed be beneficial in a clinical setting.

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PC-S, ART, and SP performed the bibliographical searches and wrote the manuscript. RZ conceived the review, supervised the writing, and revised the manuscript. All authors read and approved the final version of the manuscript.

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REVIEW
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Control of T-Cell Activation and Signaling by Amino-Acid Catabolizing Enzymes

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Amino acids are essential for protein synthesis, epigenetic modification through the methylation of histones, and the maintenance of a controlled balance of oxidoreduction via the production of glutathione and are precursors of certain neurotransmitters. T lymphocytes are particularly sensitive to fluctuations in amino acid levels. During evolution, the production of amino-acid catabolizing enzymes by mainly antigen-presenting cells has become a physiological mechanism to control T-cell activation and polarization. The action of these enzymes interferes with TCR and co-stimulation signaling, allowing tuning of the T-cell response. These capacities can be altered in certain pathological conditions, with relevant consequences for the development of disease.

Keywords: amino acids, amino acid transporters, amino acid catabolizing enzymes, TCR signaling, immunoregulation

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INTRODUCTION

The activation of antigen-specific T lymphocytes drives them from quiescence to rapid clonal expansion, accompanied by effector differentiation. These profound functional modifications are permitted by rapid changes in metabolic programming to fulfill the abrupt increase in the requirement of nutrients and energy. Thus, lymphocytes are particularly vulnerable to alterations of the metabolic microenvironment.

Various amino-acid catabolizing enzymes expressed by stromal and immune cells have been identified and shown to be important regulators of these processes by reducing the level of essential amino acids available to proliferating T cells and, in certain cases, by producing bioactive compounds that affect cell viability and/or proliferation. As a consequence, these enzymes contribute to the immunosuppressive state involved in the development of cancer, and defective induction of their expression is suspected to conversely trigger autoimmunity.

In this review, we discuss aspects related to the modification of TCR signaling and their consequences on T-cell activation, proliferation, and differentiation resulting from variations in the level of amino acids and the presence of catabolites of amino-acid catabolizing enzymes.

AMINO-ACID TRANSPORT

The substantial new requirements of activated lymphocytes are fulfilled by activation-induced mechanisms. In particular, their highly rapid duplication requires amino acids for protein synthesis. Naive human primary T cells express an almost undetectable amount of amino-acid transporters (Ren et al., 2017). Some of the major transporters belong to the SLC7 family, which is comprised of cationic amino-acid transporters (CATs) and the light subunits of large amino-acid transporters (LATs). CATs are N-glycosylated membrane proteins specialized in the transport of cationic amino acids, e.g., arginine, lysine, and histidine. The heterodimeric LATs show broader substrate specificity toward different types of amino acids (neutral, cationic, negatively charged, etc.). SLC7A5, also known as LAT1, interacts with the glycoprotein SLC3A2 (CD98) to form a heterodimeric transporter dedicated to essential amino acids (tryptophan, phenylalanine and leucine, and to a lesser extent, histidine and glutamine). LAT1 can also transport several aromatic amino acid-related compounds, such as L-DOPA (Uchino et al., 2002) and citrulline, an intermediate catabolite from which arginine can be synthesized (Werner et al., 2017).

Both types of transporters are expressed within 24 h of T-cell activation (Hayashi et al., 2013; Sinclair et al., 2013). The induction of LAT1 in primary human T cells stimulated *in vitro* is dependent on activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) signaling. When LAT1 expression is blocked, cytokine secretion by T cells is impaired, suggesting that LAT1 is required for their full activation (Hayashi et al., 2013). Silencing of human CAT-1 in primary T lymphocytes for 24 h reduces arginine transport by 64% relative to control cells, resulting in a significant reduction of proliferation, whereas IFNγ, IL-2, and IL-6 secretion are not affected (Werner et al., 2016).

Thus, T cells can modulate the uptake of amino acids, in particular essential amino acids, to accommodate changes in their microenvironment and metabolic requirements (**Figure 1**).

AMINO-ACID CATABOLIZING ENZYMES

Amino-acid degrading enzymes have been shown over the last 20 years to be central players in the control of T-cell proliferation and differentiation. This category of molecules is mostly produced by antigen-presenting cells (APC). APCs use amino-acid catabolizing enzymes to reduce the availability of essential and semi-essential amino acids for T-cell activation in negative feedback control mechanisms of the immune response. Indeed, during T cell-APC cross-talk, APC activation leads to slightly delayed induction of the synthesis of some of these enzymes (Braun et al., 2005; Marquet et al., 2010).

Although genetically unrelated in most cases, these enzymes all act by degrading an amino acid and, in some cases, producing bioactive catabolites (**Table 1**). They can be classified based on their amino-acid substrate. Indoleamine 2,3, dioxygenase (IDO)1, its isoform IDO2, and tryptophan 2,3-dioxygenase (TDO) degrade tryptophan, whereas the arginases (Arg), Arg1 and Arg2, and the nitric oxide synthases (NOS), including inducible NOS (iNOS) and endothelial NOS (eNOS), degrade arginine (neuronal NOS is not expressed in the immune system). Finally, Interleukin 4 induced gene 1 (IL4I1) mainly degrades phenylalanine. IL4I1 is also able to catabolize tryptophan and arginine, although its activity against these amino acids is much lower (at least five-fold) than that toward phenylalanine [(Boulland et al., 2007; Yue et al., 2015; Molinier-Frenkel et al., 2016) and personal data].

These enzymes can also be divided between those that limit availability of their substrate amino acid (IDO1, IDO2, TDO, Arg1, Arg2, and IL4I1) and those that liberate products that are inhibitory or proapoptotic for T cells. The IDOs and TDO produce kynurenines (Kyns), iNOS and eNOS produce nitric oxide (NO), and IL4I1 liberates two toxic compounds, hydrogen peroxide (H_2O_2) and ammonia (NH_3), while converting its amino acid substrate into its ketoacid form. In a recent study, IL4I1 activity toward tryptophan was shown to produce the ketoacid indole-3-pyruvate, which may function as a precursor that can enter the Kyn pathway (Sadik et al., 2020). The enzymatic activity of iNOS can change when co-expressed with arginase. Under such conditions, the consumption of arginine by Arg1 favors the production of superoxide by iNOS. The interaction of NO with anion superoxide ($O2^{\cdot -}$) leads to the production of peroxynitrite, an extremely reactive compound (Xia and Zweier, 1997).

In the immune system, cells of myeloid origin are the main producers of these enzymes, with certain species-related differences. The main example is Arg1, which is constitutively expressed by granulocytes in humans, whereas it is a hallmark of macrophages activated by Th2 cytokines (M2) in mice (Munder et al., 1999). Mitochondrial Arg2, iNOS and eNOS can also be expressed by T cells (Ibiza et al., 2006; Yang et al., 2013; Geiger et al., 2016). iNOS is also expressed by mouse plasma cells and γδ T cells (Saini et al., 2014; Douguet et al., 2016b). Similarly, certain lymphocyte subsets, such as follicular B cells, mucosal associated invariant T cells (MAIT), and Th17 cells express IL4I1 (Molinier-Frenkel et al., 2019) (**Figure 2**).

THE EFFECT OF AMINO-ACID CATABOLIZING ENZYMES ON T-CELL SIGNALING

Engagement of the TCR by cognate MHC-peptide complexes leads to intracellular signaling, involving a cascade of protein phosphorylation and calcium fluxes that culminates with nuclear translocation of the transcription factors NFκB, NFAT, and AP1 and rearrangement of the actin and tubulin cytoskeleton. Expression of an activation program is essential for T-cell survival, proliferation, and differentiation. Signals from costimulatory molecules, such as CD28 engagement by B7 proteins or IL-2 binding to its high affinity receptor, amplify TCR signaling and, in parallel, activate the mammalian target of rapamycin (mTOR)C1 pathway, which is often described as a rheostat of T-cell activity, as it is sensitive to numerous environmental cues in addition to co-stimulation. The mTOR kinase controls both the exit from the quiescent state and

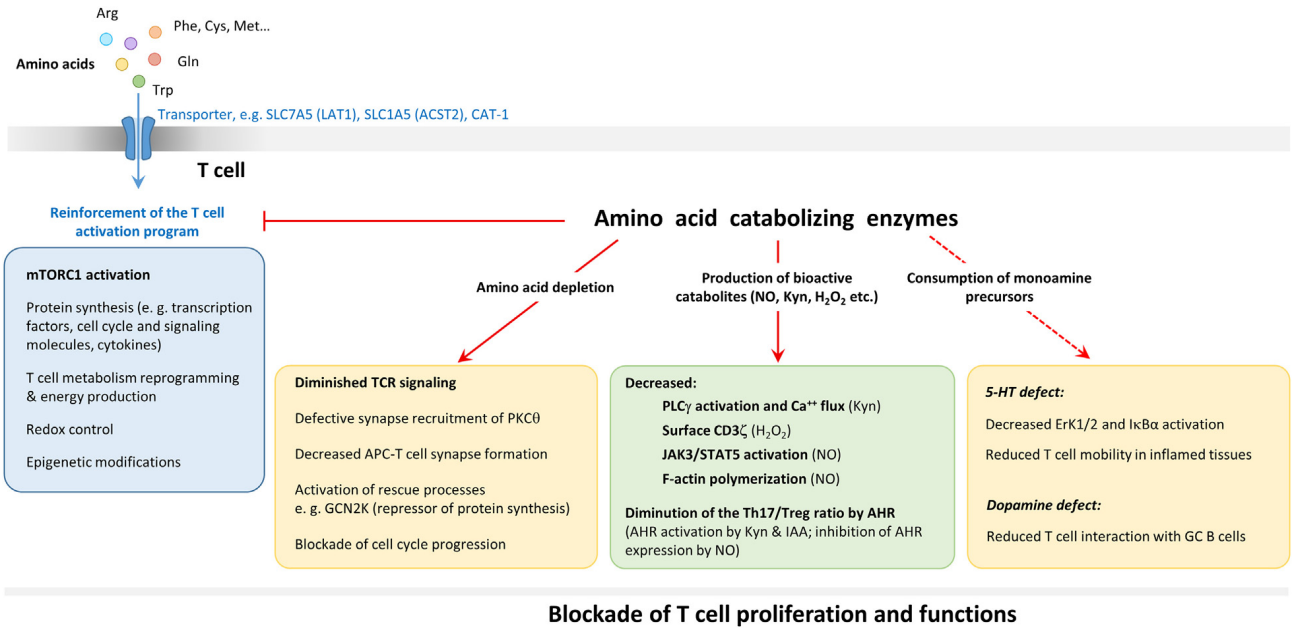
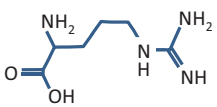
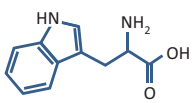
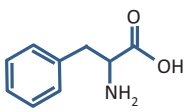


FIGURE 1 | Role of amino acids and amino-acid catabolizing enzymes in T-cell activation. Uptake of amino acids via cell surface transporters (CAT and the light subunits of LAT) is increased upon T cell activation. The intake of amino acid leads to the activation of the mammalian target of rapamycin complex 1 (mTORC1) pathway which controls protein synthesis and the reprogramming of T cell metabolism necessary for the full expression of the activation program. Amino acids are also required for protein synthesis, for the control of the redox balance (through glutathione tripeptide [GSH]) synthesis from cysteine and for epigenetic modifications of histones and ADN (through S-adenosylhomocysteine production from methionine). Amino acid catabolizing enzymes interfere with TCR signaling by starving T cells of amino acids and through the production of several bioactive metabolites (NO, kynurenine [Kyn], H_2O_2, etc.) acting at specific steps. Amino-acid catabolizing enzymes may also interfere with T-cell activation by degrading precursors of monoamines with costimulatory functions, such as serotonin (5-HT) and dopamine. Some of these effects are listed in the yellow and green boxes. For more detailed description of the action of amino-acids and their derivatives on TCR signaling, see **Figure 3**. The general effect of amino-acid catabolizing enzymes results in blockade of T-cell proliferation and function.

TABLE 1 | Characteristics of the amino acid-catabolizing enzymes expressed in the immune system.

Enzyme Acronyme	Nitric oxide synthase (inducible, iNOS; endothelial, eNOS)	Arginase (Arg1 and Arg2)	Indoleamine 2,3 dioxygenase (IDO1 and IDO2)	Tryptophan 2,3 dioxygenase (TDO)	Interleukin 4-induced gene 1 (IL4I1)
Main substrate		Arginine		Tryptophan	 Phenylalanine
Reaction	Arg → Citrulline + NO	Arg → Ornithine + Urea	Trp + O_2 → N-formyl-kynurenine	Trp + O_2 → N-formyl-kynurenine	Phe → H_2O_2 + NH_3 + Phenylpyruvate
Amino acid depletion	No	Yes	Yes	Yes	Yes
Expression in the immune system	Myeloid cells T cells	Myeloid cells (Arg1) T cells (Arg2)	Myeloid cells	Myeloid cells	Myeloid cells, B cells, Th17, MAIT
Expression in other tissues	Gastrointestinal tract, Lung, CNS (iNOS) Endothelium (eNOS)	Liver (Arg1) Prostate, thyroid (Arg2)	Placenta, lung (IDO1) Placenta, liver (IDO2)	Liver, pituitary gland	Testis, brain
Induction	IFNs (iNOS)	IL-4 and IL-10 in mouse macrophages (Arg1)	IFNs, CTLA4, TGFβ	Corticosteroids and glucagon	IFNs in myeloid cells (human); IL-4 in B cells (human, mouse)
Localization	Cytosol, granules (iNOS)	Cytosol, PMN* granules (Arg1) Mitochondria (Arg2)	Cytosol	Cytosol	Secreted

*Data on the expression in non-lymphoid tissues were obtained from The Human Protein Atlas. *PMN, polymorphonuclear cell.*

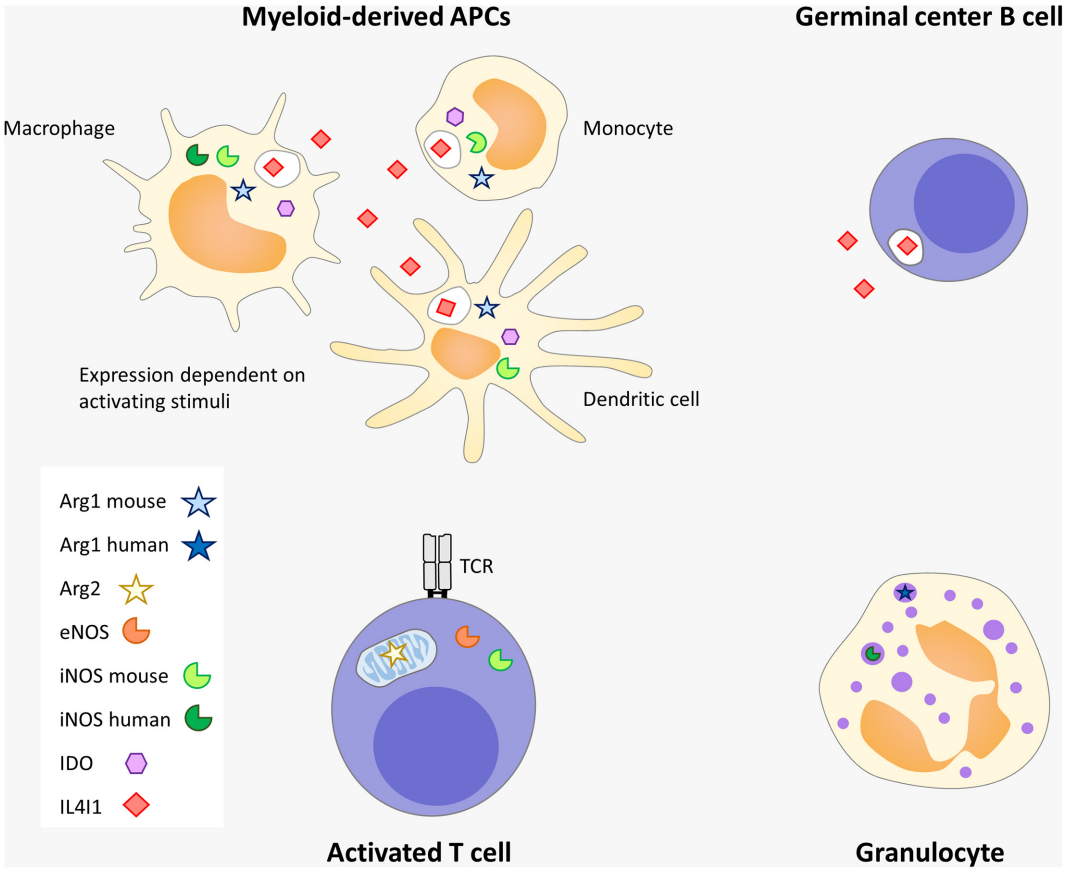


FIGURE 2 | Amino-acid catabolizing enzyme expression in immune cells. Myeloid-derived APC and granulocytes, including their poorly mature tolerogenic forms known as myeloid-derived suppressor cells (MDSC), are the strongest producers of immunosuppressive enzymes. IL4I1 is also produced by germinal center B cells (probably at the centrocyte stage) and by other subtypes of lymphocytes, such as Th17 and MAIT (not depicted). Arg2, iNOS and mitochondrial eNOS are expressed by T lymphocytes. Some differences exist between mouse and human. In humans, IDO, iNOS, and IL4I1 are induced in myeloid-derived APCs by inflammatory and Th1 signals whereas Arg1 is not expressed in this type of cells. In contrast, Arg1 is detected in human granulocytes, similar to iNOS, but in response to different stimuli. In the mouse, IL4I1 and Arg1 can be induced in macrophages by Th2 signals. IL4I1 is the only member of this group of enzymes which is secreted.

the outcome of T-cell activation and proliferation, including functional differentiation and acquisition of memory properties (Huang et al., 2020).

Certain amino-acid catabolizing enzymes interfere at various points of this signaling cascade (**Figure 3**). For example, IDO modulates activation of the exchange factor Vav1, which regulates actin polymerization downstream of the TCR by activating the small GTPase Rac1. Indeed, Li et al. (2009) showed a decrease in Vav1 expression and phosphorylation using co-culture systems of T cells together with IDO expressing cell lines. Consistent with this effect, the T cells showed defects in actin polymerization after activation, accompanied by a drop in p38 MAP kinase activation (Li et al., 2017). More recently, a diminution in the phosphorylation of the ζ chain of the CD3 complex was also observed (Eleftheriadis et al., 2016). Treatment with the IDO inhibitor 1-methyl tryptophan (1-MT) reversed these inhibitory effects. In mouse lymphocytes, the action of a derivative of Kyn, 3-hydroxyanthranilic acid, reduces PLCγ phosphorylation and calcium fluxes (Iken et al., 2012). The activity of IDO has also been implicated in the inhibition of protein kinase C (PKC)θ in experiments using D-1 MT and ectopic expression of IDO1 (Metz et al., 2012).

Decreased downregulation of the CD3 ζ chain has also been reported for IL4I1 partially due to H_2O_2 production (Boulland et al., 2007). We used an activation system involving TPH1 cells expressing or not IL4I1 as APCs and showed that IL4I1 inhibits several early signaling kinases downstream of the TCR, including ZAP-70, PLCγ, and ERK, diminishes calcium fluxes, and reduces the phosphorylation of the p65 subunit of NFκB. This in turn limits the acquisition of the activation markers CD69 and CD25. Unlike other amino-acid catabolizing enzymes, which are intracellular, IL4I1 is secreted by the APC at the interface with the T cell, leading to reduced synapse formation. Surprisingly, neither the products of the enzymatic reaction nor the absence of Phe is able to recapitulate the effect of IL4I1. In contrast, H_2O_2 administered either alone or with NH_4 and phenylpyruvate promotes activation of the TCR pathway (Aubatin et al., 2018). Indeed, oxidation by H_2O_2 inactivates tyrosine phosphatases involved in the inhibition of TCR signaling (Meng et al., 2002). However, it is important to note that H_2O_2 is a highly diffusible

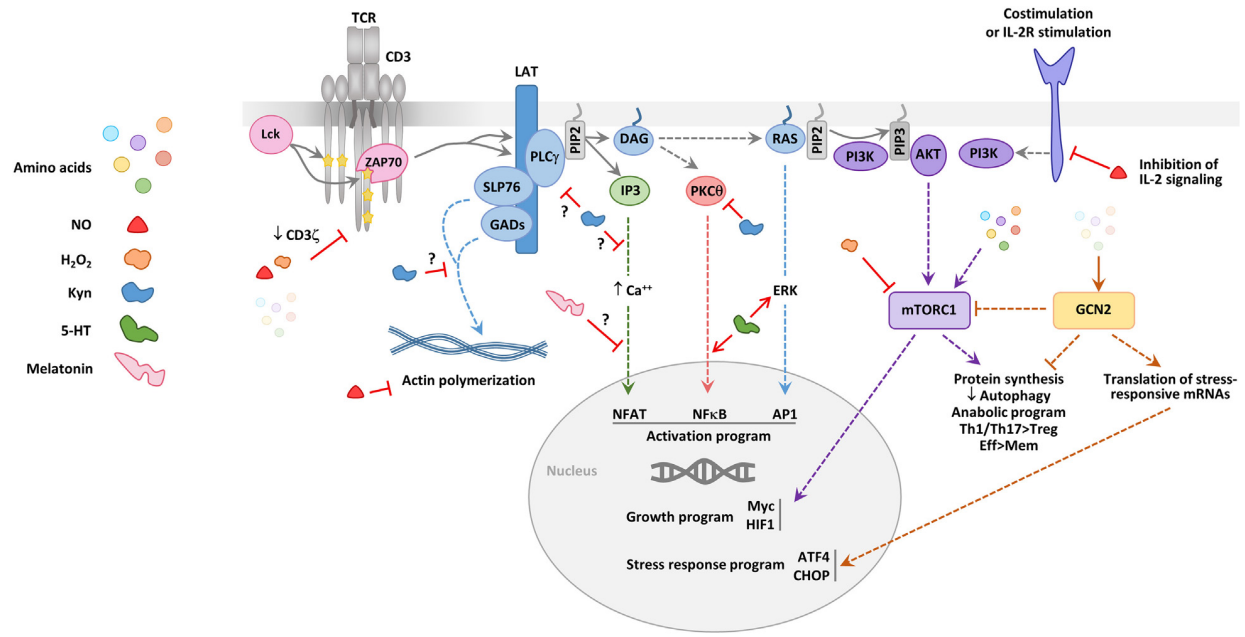


FIGURE 3 | Effect of amino acids and their derivatives on T cell signaling. A simplified scheme of the signaling events downstream of the TCR and costimulation or IL-2R signaling is provided. Early signaling (involving the successive recruitment and activation of the tyrosine kinases Lck and ZAP70) leads to the phosphorylation of the membrane-anchored linker for activation of T cells (LAT) adaptor, which represents a crucial signaling node. SLP76 and GADs are involved in pathways important for the reorganization of the actin cytoskeleton. The phospholipase C γ (PLCγ) degrades the lipid phosphatidylinositol biphosphate (PIP2) to produce diacyl-glycerol (DAG) and inositol triphosphate (IP3), two major signaling intermediates, which drive three distinct late signaling pathways, involving calcium mobilization, protein kinase Cθ (PKCθ) activation and RAS activation, respectively. These three signaling pathways lead to the activation and nuclear translocation of the transcription factors NFAT, NFκB, and AP1. PIP2 can also be degraded by the phosphatidylinositol 3 kinase (PI3K) to produce phosphatidylinositol triphosphate (PIP3) which recruits AKT. PI3K is activated downstream of TCR signaling effectors, including RAS, but also by costimulatory molecules, such as CD28 and the signaling chains of the IL-2 receptor. AKT drives one of the signaling pathways leading to the activation of the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 controls the initiation of protein synthesis and is central to the anabolic switch of activated T cells. High mTORC1 activity is linked to an increased effector (Eff) differentiation of $CD4^+$ and $CD8^+$ T cell and a decreased differentiation of Tregs and memory (Mem) T cells. Amino acids and some of the toxic metabolites produced by amino acid-catabolizing enzymes (NO, H_2O_2, and Kyn) can affect some of the early or late steps of the TCR signaling pathways. The effects mediated by amino-acid catabolizing enzyme production of these catabolites are depicted; in addition, some effects attributed to the monoamines 5-HT and melatonin are represented. NO, H_2O_2, and a decrease in the amino acid level lead to defects in early TCR signaling, in particular by diminishing CD3ζ expression. IDO activity, potentially through Kyn production, eNOS through NO production modify signaling pathways driving actin polymerization. High amino acids levels participate to activating the mTORC1 pathway, whereas low amino acid levels lead to the accumulation of empty tRNAs which are sensed by the stress kinase GCN2. GCN2 diminishes the general protein synthesis but favors the synthesis of a small set of proteins, such as activating transcription factor 4 (ATF4). ATF4 induces the transcription of genes involved in autophagy and response to cellular stress, including C/EBP Homologous Protein (CHOP). The kinases mTORC1 and GNC2 have opposite effects on the differentiation of Th1, Th17, and regulatory T cells.

molecule that variably affects T cells, depending on its local concentration, the duration of exposure, and the antioxidant systems of the T cell, which may be related to the T-cell subset and state of differentiation (Belikov et al., 2015). Finally, as IL4I1 binds to T lymphocytes, its action on TCR signaling may depend on its interaction with a surface receptor in addition to, or instead of, its enzymatic activity (Aubatin et al., 2018).

NO and peroxinitrite are powerful agents of protein nitration and nitrosylation which confers them important regulatory functions (García-Ortiz and Serrador, 2018). Macrophage-derived NO has long been known to limit T-cell activation by interfering with STAT5 phosphorylation (Bingisser et al., 1998). More recently, the expression of iNOS by eosinophils has also been linked to decreased TCR stimulation (Onyema et al., 2019). The co-culture of iNOS-expressing E1-polarized eosinophils with T cells expressing a GFP-coupled Nur77 protein, an early TCR-responsive molecule of which the expression directly correlates with the strength of the TCR signal, leads to decreased TCR activation after CD3/CD28 stimulation in an iNOS-dependent manner. Interestingly, in this study, the level of CD3ε and ζ chains decreased in T cells cultivated with WT eosinophils, but not iNOS-deficient eosinophils, and this correlated with the inhibition of T-cell proliferation by WT eosinophils. Similarly, iNOS has a detrimental effect on the organization of the immune synapse and the secretion of cytotoxic granules (Ferlito et al., 2006). However, NO production by eNOS in contact with the T-cell cytoskeleton is necessary for the correct organization of the immunological synapse and TCR signaling. Indeed, eNOS associates with actin upon TCR engagement to control the organization of the cytoskeleton and the resulting dynamics of signaling micro-clusters. Specifically, NO-mediated S-nitrosylation of F-actin residue Cys374 prevents actin binding to profilin 1, thus limiting actin polymerization. The resulting traction of the micro-clusters fosters the localization of PKC-θ

to the center of the immune synapse, thus facilitating its activation (García-Ortiz et al., 2017). Overall, these data suggest that different quantities, localization, and/or kinetics of NO production can have opposing effects on T-cell activation.

Arginine deficiency is well-known to block T cell proliferation (Rodriguez et al., 2017), whereas a sufficient level of arginine is necessary for the long-term survival and anti-tumor activity of T cells *in vivo*, independently of mTOR signaling. Impairment of early TCR signaling has been documented for Arg1. Depletion of arginine by macrophage-derived Arg1 or the growth of T cells in arginine-deprived medium leads to downregulation of the CD3 ζ chain (Rodriguez et al., 2003). This hallmark of T-cell dysfunction can also be observed in cancer patients in association with increased plasma activity of Arg1 released by myeloid-derived suppressor cells (MDSCs) (Rodriguez et al., 2009). Arginine-starved Jurkat T cells are still able to up-regulate IL-2 receptor chains and secrete IL-2 (Taheri et al., 2001), but are blocked at the G0–G1 transition of the cell cycle. This is due to decreased mRNA stability and a diminished translational rate of cyclin D3 and cyclin-dependent kinase 4 (Rodriguez et al., 2007). Cyclin D3 mRNA instability has been shown to result from a decrease in the level of the RNA-binding protein HuR (Rodriguez et al., 2010). These effects are all dependent on the general control non-derepressible 2 (GCN2) kinase (Rodriguez et al., 2007), an amino-acid sensor activated by uncharged tRNA molecules that inhibits eukaryotic initiation factor-2α (eIF2α) to repress protein synthesis. A pegylated form of Arg1 (PEG-Arg) has been used *in vitro* to limit the growth of cancer cells due to their dependence on arginine and is now being tested for its therapeutic effect in cancer (currently seven clinical trials[1]). However, PEG-Arg simultaneously limits arginine availability to T cells, thus blocking cell-cycle progression, despite the fact that it does not affect the acquisition of activation markers *in vitro* (Fletcher et al., 2015). *In vivo* administration of PEG-Arg induces the accumulation of granulocytic MDSCs via GCN2 activation. These MDSCs themselves show increased expression of Arg1 and are responsible for the inhibition of T-cell proliferation. Their accumulation is associated with enhanced tumor growth (Fletcher et al., 2015), suggesting that arginine starvation is a risky strategy for the treatment of cancer.

Similar to the situation for NOS, T lymphocytes themselves express the mitochondrial isoform of Arg (Arg2), showing a significant increase after activation. A recent analysis compared the proteome and metabolome of 72-h-activated and freshly isolated human naïve T cells. Arg2 transcription was higher in activated T cells, whereas among 429 differential metabolites, the levels of arginine, ornithine, and N-acetylornithine were lower, indicating that activation-induced Arg2 is metabolically active (Geiger et al., 2016). Murine T cells lacking Arg2 show faster and stronger activation marker dynamics, whereas their proliferative activity is not affected. *In vivo*, the lack of Arg2 allows the persistence of antitumor $CD8^+$ T cells and facilitates their differentiation into central memory T cells (Líndez et al., 2019). Arg2 is not expressed in peripheral blood regulatory T cells (Tregs), but its expression is induced by TCR stimulation and it is detected in Tregs from normal and inflamed skin. Arg2 expression by Tregs decreases mTOR signaling and enhances their suppressive activity (Lowe et al., 2019).

The T-cell inhibitory effect of arginine depletion is limited by the addition of citrulline, which can be endogenously converted into arginine (Bansal et al., 2004). T-cell activation induces the expression of the transporter LAT1 even under limiting arginine concentrations, allowing citrulline uptake by T cells. In a recent study, Werner et al. (2017) showed that arginine depletion induces both arginosuccinate synthase and arginosuccinate lyase, the two enzymes which allow the synthesis of arginine from citrulline, in T cells.

As previously mentioned for Tregs, certain effects of amino-acid catabolizing enzymes on T cells have been attributed to their inhibition of the mTOR pathway. Activation of naïve human T cells in the presence of IL4I1 limits the activation of the mTORC1 targets ribosomal S6 protein and p70S6K (Cousin et al., 2015). In HeLa cells, induction of IDO by interferon (IFN) γ depletes tryptophan and represses phosphorylation of p70S6K. The IDO1 inhibitor 1D-MT can reverse this inhibition, independently from GCN2 (Metz et al., 2012). In addition to its indirect effects on signaling pathways that are sensitive to amino-acid or kyn levels, IDO1 can directly interfere with intracellular signaling by recruiting the tyrosine phosphatases SHP1 and SHP2 through its immunoreceptor tyrosine-based inhibitory motifs (Pallotta et al., 2011). This function has been demonstrated in plamacytoid DCs (pDCs), in which IDO1 shifts from the cytosol to early endosomes to perform its signaling activity that is associated with amplification of a tolerogenic program (Iacono et al., 2020). Other amino-acid catabolizing enzymes may have properties independent from their catabolic activity, but this has not yet been explored.

Moreover, depending on the context, the simultaneous expression of these enzymes in the same cell or same microenvironment may modify their T-cell regulatory properties. This is known for the well-described co-expression of Arg1 and iNOS in cancer, which allows peroxinitrite formation, as stated above. IDO1 and Arg1 can also be expressed in the same tumor microenvironment. It has been demonstrated that TGFβ induces Arg1 expression in DCs, which is necessary for and followed by IDO1 expression. Polyamine production from the Arg1 catabolite ornithine favors Src kinase activation and the phosphorylation of IDO1, allowing its immunosuppressive signaling (Mondanelli et al., 2017). Stimuli produced by the anti-tumor response, such as IFNγ, are likely to induce contemporaneous expression of IDO1, IL4I1, and iNOS, with still undetermined consequences.

CONSEQUENCES OF AMINO-ACID CATABOLIZING ENZYME ACTIVITY ON T-CELL DIFFERENTIATION AND FUNCTION

Most amino-acid catabolizing enzymes, including IDO1 and IL4I1, decrease T-cell proliferation and modify the balance of effector versus regulatory T-cell differentiation (**Figure 4**).

[1] www.clinicaltrial.gov

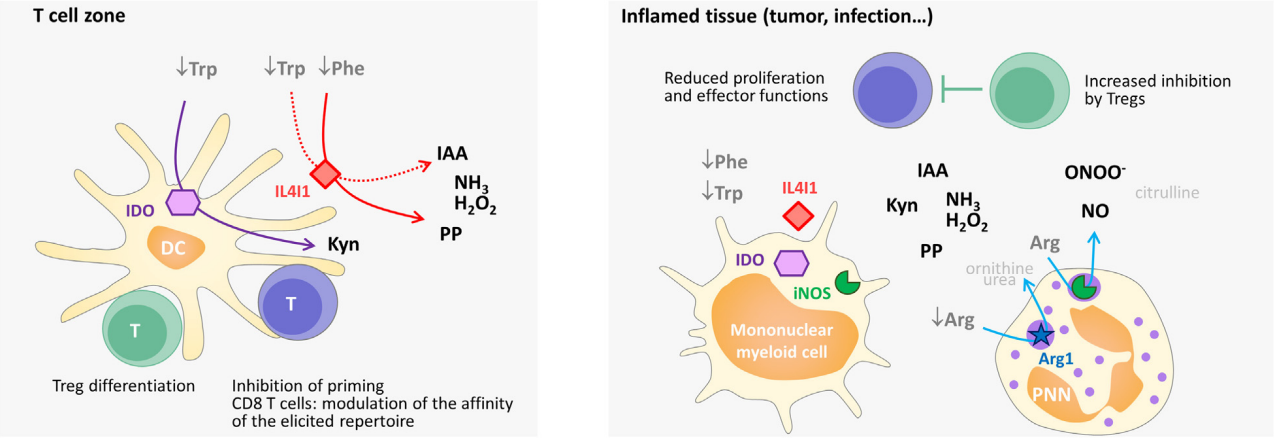


FIGURE 4 | Simplified scheme of the influence of immunosuppressive enzymes on T-cell priming, differentiation, and function in secondary lymphoid organs and in the periphery in humans. Mature dendritic cells in the T-cell zone (e.g., activated by IFNγ) can present antigens, as well as produce cytoplasmic IDO and secreted IL4I1. IDO degrades Trp and IL4I1 degrades Phe and, to a lesser extent, Trp. The level of these two essential amino acids declines in the T-cell microenvironment, whereas Kyn, phenylpyruvate (PP), IAA (indole-3 acetic acid), H_2O_2, and NH_3 accumulate. The combined effect limits the activation of naïve T cells or, in the case of CD4 T cells, favors their differentiation into regulatory T cells. By enhancing the activation threshold, IL4I1 can also restrain the repertoire of primed CD8 T cells to the high-affinity clones. In inflamed tissues, Arg-catabolizing enzymes can also be expressed, thus diminishing the concentration of available Arg (Arg1) and producing NO (iNOS) and peroxynitrite. Peroxynitrite ($ONOO^-$) results from the reaction of NO with O_2^-, which is produced by iNOS under conditions of low Arg levels. The combined effect of amino-acid starvation and the production of the various catabolites by Trp-, Phe-, and Arg-catabolizing enzymes diminishes the recruitment, proliferation and function of effector CD4 and CD8 T cells and increases the inhibitory function of regulatory T cells. Overall, this leads to lowering of the local T-cell response. The enzymatic reactions are indicated by arrows. Catabolic products that have no known specific impact on T-cell activation are shown in light gray. Some of these products are used for amino-acid regeneration (arginine from citrulline, proline from ornithine) or the production of polyamines (ornithine), which serve as building blocks for cell growth.

Plasmacytoid dendritic cells stimulated by CpG induce IDO activity, which stabilizes the suppressor phenotype of Tregs, while simultaneously blocking the IL-6 expression required for Th17 cell differentiation (Baban et al., 2009). During fungal infection of mice with *Paracoccidioides brasiliensis*, the absence of IDO1 is associated with an increased influx of Th17 cells to the infected lung and a concomitant reduction of the number of Th1 and Treg cells (de Araújo et al., 2017). Kyns, which are produced both by IDO and TDO, have been shown to bind to the aryl hydrocarbon receptor (AHR), a highly conserved ligand-activated transcription factor involved in controlling the balance of Treg versus Th17 differentiation (Mezrich et al., 2010; Opitz et al., 2011). Although certain AHR ligands promote the differentiation of Th17 cells, AHR activation by Kyns leads to Treg generation (Mezrich et al., 2010). In addition, tryptophan depletion can enhance the suppressive functions of Tregs by excluding PKCθ from the immune synapse, thus inhibiting its signaling activity (Zanin-Zhorov et al., 2010; Metz et al., 2012).

Differentiation of naïve $CD4^+$ T cells in the presence of IL4I1 also skews their polarization toward Tregs, whereas it does not substantially affect Th17 differentiation. This effect appears to involve diminution of mTORC1 signaling (Cousin et al., 2015). However, it has also been recently observed that IL4I1 degradation of tryptophan [a minor substrate in comparison to phenylalanine (Boulland et al., 2007)] produces indole derivatives that can activate the AHR pathway (Sadik et al., 2020; Zhang et al., 2020). Finally, IL4I1 modulates the priming of $CD8^+$ T cells. Indeed, the absence of IL4I1 lowered the activation threshold of cognate $CD8^+$ T cells in a mouse model of acute infection with the lymphocytic choriomeningitis virus, leading to extension of the responding repertoire to low-affinity clones and increased memory T-cell differentiation. Thus, IL4I1 may represent a mechanism to restrain T-cell activation to high-affinity $CD8^+$ T-cell clones (Puiffe et al., 2020).

Arg1 produced by MDSCs has also been suggested to play a role in Th17 differentiation. Indeed, RORγT and IL-17A expression decrease in T cells cultured with MDSCs treated with the Arg1 inhibitor Nor-NOHA (Wu et al., 2016). Consistent with this observation, mice with a conditional deletion of Arg1 in myeloid cells show decreased expression of IL-17A in the colorectum during experimentally induced colitis (Ma et al., 2020). High concentrations of NO provided by the NO donor NOC-18 can suppress the proliferation and function of polarized murine and human Th17 cells by inhibiting the expression of AHR (Niedbala et al., 2011). In accordance with this result, iNOS-deficient mice exhibit enhanced Th17 cell differentiation but no changes in Th1 or Th2 polarization (Yang et al., 2013). Conversely, the use of NOC-18 induces the proliferation and sustained survival of $CD4^+$ $CD25^-$ T cells, which acquire the expression of CD25 but not Foxp3 and present regulatory functions (Niedbala et al., 2007). In sharp contrast with these findings, physiological NO levels produced by the MDSCs of cancer patients or endogenously by $CD4^+$ T cells expressing iNOS can induce and stabilize the Th17 phenotype (Obermajer et al., 2013). Mouse γδ T cells also express iNOS, in particular following stimulation by inflammatory cytokines

(Douguet et al., 2018). The enzyme is essential for promoting optimal IL-2 production and proliferation of γδ T cells, but drives IL-17 production, which is associated with pro-tumor properties in a murine model of melanoma (Douguet et al., 2016a,b). These findings illustrate the dual role of NO on T cell activation at the level of T-cell differentiation, depending on its concentration.

OTHER AMINO ACIDS IMPORTANT FOR T-CELL SIGNALING AND ACTIVATION

Several other amino acids are involved in controlling T-cell function.

Recent metabolomics data have provided information on the importance of methionine uptake during T-cell activation. TCR engagement drives increased flow through the methionine cycle, which supplies the lymphocyte with methyl donors necessary for epigenetic modifications, as well as the first amino acid in protein synthesis (Martínez et al., 2017). Indeed, TCR stimulation upregulates and sustains both the transport of methionine and the expression of the enzymes involved in the production of S-adenosylhomocysteine from methionine. S-adenosylhomocysteine is necessary for histone methylation (Sinclair et al., 2019). Thus, although no specific enzyme that catabolizes methionine has been yet described, modifications of methionine availability should have important repercussions on the ability of T cells to respond to an antigenic challenge. Cancer cells have been recently shown to be metabolically dependent on methionine (Wang et al., 2019) and to avidly uptake this amino acid through the SLC43A2 transporter (Bian et al., 2020). Depletion of the tumor microenvironment of this amino acid by tumor cells may decrease its availability to infiltrating T lymphocytes. Consistent with this hypothesis, the absence of methionine decreases the $CD8^+$ T-cell immune response by dysregulating the transcription of essential genes due to deficient epigenetic reprogramming (Bian et al., 2020).

In the oxidizing environment of the extracellular space, cysteine exists primarily in its oxidized disulfide-bonded form cystine. Cysteine is an essential amino acid for T cells, as they are not equipped for its synthesis. Although cysteine and cystine are not required for early T-cell activation, their role in DNA and protein synthesis, proliferation, and cytokine secretion of antigen-stimulated T cells was shown long ago to be controlled by APCs through the extracellular release of cysteine (Angelini et al., 2002). Whereas naïve T cells cannot import cysteine or cystine, activated human T cells express transporters for both forms (Levring et al., 2012). Cysteine is the rate-limiting substrate for the synthesis of the glutathione tripeptide (GSH) which is required for T-cell proliferation and effector functions (Levring et al., 2015; Mak et al., 2017). Indeed, GSH protects signaling proteins from damage caused to cysteine and methionine residues by reactive oxygen species through its antioxidative activity. For example, GSH maintains the conformation of the membrane-anchored linker for activation of T cells (LAT) (Gringhuis et al., 2002) and supports mTOR and NFAT activation to drive the reprogramming of T-cell energy metabolism (Mak et al., 2017). Tumor-infiltrating MDSCs can limit T cell antitumor activity by consuming cystine and sequestering cysteine (Srivastava et al., 2010).

Glutamine is the most abundant free amino acid in the body. Glutaminolysis is a highly important source of biosynthetic precursors and energy in active T cells. T-cell activation strongly increases glutamine import and stimulates glutaminolysis. ERK and mTORC1 signaling are involved in promoting the expression of transporters and enzymes required for glutamine metabolism in T cells. As for cysteine or arginine, the absence of glutamine blocks T-cell proliferation but not the acquisition of early activation markers (Carr et al., 2010). The uptake of glutamine by its major transporter SLC1A5 (ACST2) is required for leucine import by the glutamine/leucine antiporter (see below) and mTORC1 activation (Nicklin et al., 2009), thereby promoting $CD4^+$ T-cell differentiation into Th1 and Th17 cells (Nakaya et al., 2014). The bacterial enzyme asparaginase, commonly used as an anticancer agent in lymphoblastic leukemia, catalyzes the deamination of asparagine and, to a lesser extent, glutamine, to aspartic acid and glutamic acid, respectively (Derman et al., 2020). The absence of asparagine affects T-cell activation and IL-2 production through inhibition of the mTORC1 pathway (Torres et al., 2016). Asparaginase kills tumor cells via combined asparagine and glutamine deprivation but its indications are limited by severe acute side effects and the induction of profound immunosuppression (Kim et al., 2015; Song et al., 2017).

Alanine is an amino acid that can be synthesized from pyruvate. Nevertheless, recent data have shown that lymphocytes depend on the import of extracellular alanine, which is vital for the transition from quiescence to activation of both naïve and memory T cells. Indeed, in the absence of extracellular alanine, early T-cell activation is delayed and the metabolic changes induced by activation are impaired (Ron-Harel et al., 2019).

Finally, leucine is the most common proteinogenic amino acid. The T-cell uptake of leucine involves the SLC7A5-SLC3A2 (LAT1–CD98) transporter, which imports branched amino acids while exporting glutamine (Fuchs and Bode, 2005). Along with arginine, leucine is a major activator of the mTORC1 complex, thus contributing to the costimulatory signal (Ananieva et al., 2016). The use of the leucine competitor N-acetyl-leucine-amide blocks T-cell activation, leading to anergy by limiting mTOR activation (Zheng et al., 2009). Consequently, leucine is involved in the differentiation of $CD4^+$ and $CD8^+$ T cells. For example, it has been shown that leucine addition reverses the ghrelin-induced inhibition of iTh17 cell differentiation through mTORC1 activation (Xu et al., 2015).

AMINO-ACID DERIVED COMPOUNDS

Certain neuroactive monoamines, such as dopamine, serotonin, and melatonin, are derived from enzymatic modifications of Trp, Tyr, or Phe. These monoamines are mainly known as neurotransmitters and signal through specific G-coupled receptors. More recent work demonstrates that they can also influence T-cell differentiation and function. Thus, amino-acid

catabolizing enzymes may also affect the T-cell response by decreasing the availability of these compounds.

Serotonin (hydroxytryptamine, 5-HT) is formed by the hydroxylation of Trp followed by decarboxylation. Certain immune-cell populations, including mast cells and T lymphocytes, can synthesize and release 5-HT, although 95% of the 5-HT in our body is produced by the nervous system of the gastrointestinal tract. The initial evidence that 5-HT has an influence on T cells was reported 35 years ago in rats (Steplewski and Vogel, 1985). 5-HT is an important neurotransmitter and its role in inflammation and immunity has been mainly studied in patients with psychiatric or neurodegenerative diseases. T cells produce 5-HT as an autocrine factor that acts through the 5-HT_3 receptor. Such production may facilitate T-cell infiltration in inflamed tissues by regulating T-cell responsiveness to chemokines (Magrini et al., 2011). *In vitro* addition of 5-HT to T-cell cultures induces rapid phosphorylation of ERK1/2 and IkBα through stimulation of the 5-HT_7 receptor (León-Ponte et al., 2007) and may also induce Ca^{++} release (Genius et al., 2015). 5-HT has been suggested to play a protective role in multiple sclerosis by attenuating the proliferation of and cytokine production by Th1 and Th17 cells and by favoring the expansion of $CD39^{+}$ $Foxp3^{+}$ T-regulatory lymphocytes, which secrete IL-10 (Sacramento et al., 2018).

The pineal gland synthesizes and releases melatonin (N-acetyl-5-methoxytryptamine) in response to decreased light. Melatonin is produced from Trp via 5-HT and principally acts as a regulator of circadian rhythms. As such, it may be involved in adjusting the immune system to circadian and seasonal fluctuations (Farez et al., 2016). However, as for 5-HT, the gastrointestinal tract is the largest producer of melatonin and several other extra-pineal sites contain melatonin-producing cells, including T cells. The biological effects of melatonin mainly depend on the activation of the specific G-coupled receptors MT1 and MT2, which are expressed by cells of the immune system (Farez et al., 2016). Melatonin has been suggested to participate in T-cell activation and protection from activation-induced cell death (Carrillo-Vico et al., 2005; Pedrosa et al., 2010). Melatonin also exhibits potent antioxidant properties, both direct and indirect, through the modulation of antioxidant gene transcription (Acuña-Castroviejo et al., 2014), which may interfere with T-cell activation. Melatonin is considered to be an anti-inflammatory agent (Tarocco et al., 2019) and is suspected to play a role in autoimmune diseases. The most important evidence was provided by a study of Farez et al., which showed a correlation between relapses of multiple sclerosis and decreased melatonin levels associated with increased exposure to sunlight (Farez et al., 2015). The effect of melatonin was attributed to MT1 stimulation and activation of the ERK1/2 kinases, leading to expression of the transcriptional repressor NFIL3, which blocks the differentiation of pathogenic Th17 cells. Concomitantly, melatonin favored the generation of protective Tr1 cells and their production of IL-10 via ROR-α activation of the *Il10* promoter.

Catecholamines, i.e., dopamine, noradrenaline, and adrenaline, are other neuroactive molecules that can influence the immune response. These molecules are derived from Phe via tyrosine, which is hydrolyzed to form the L-DOPA precursor. Lymphocytes can produce catecholamines, in particular dopamine (Bergquist et al., 1994). Catecholamines may participate in the fine-tuning of T-cell responses, but their effects have thus far not been extensively evaluated (Hodo et al., 2020). Five G-protein-coupled receptors (classified in the DR1-like and DR2-like families) mediate the effect of dopamine. TCR stimulation induces the expression of these receptors at the surface of human CD4 T cells (Kustrimovic et al., 2014). It has been suggested that dopamine diminishes T-cell activation via inhibition of Erk1/2 phosphorylation and reduced nuclear translocation of NFκB (Strell et al., 2009) or by limiting the expression of the upstream tyrosine kinases Lck and Fyn (Ghosh et al., 2003) and induces T-cell quiescence by up-regulating Krüppel-like factor-2 expression (Sarkar et al., 2006). However, varying doses of dopamine and stimulation of different dopamine receptors may determine divergent effects on T cells (Hodo et al., 2020). For example, *in vivo* data from mouse models deficient for DR3 (D2-like receptor) suggest that activation of this receptor favors Th1/Th17 but limits Th2 differentiation of naïve CD4 T cells (Contreras et al., 2016). Finally, one of the most exciting findings has been that dopamine secreted by follicular helper T cells facilitates the expression of the costimulatory molecule ICOS ligand (ICOSL) at the surface of germinal center B cells (Papa et al., 2017). This translates into an increase in the molecular dialogue between the two types of cells and the acceleration of B-cell exit from the germinal center (Papa et al., 2017). Interestingly, both Phe and L-DOPA are high-affinity substrates of IL4I1 [(Mason et al., 2004) and our unpublished data]. Thus, catabolism of their precursors by IL4I1 may reduce the availability of catecholamines, with a potential impact on the regulation of T-cell activation and function.

BACTERIAL-HOST INTERACTIONS IN THE PRODUCTION OF AMINO-ACID DERIVED METABOLITES

Several amino-acid catabolizing enzymes have a very ancient evolutionary origin, as they are detected in bacteria, in which they participate in maintaining the nutrient niche along with other metabolic enzymes. Their activity is essential for maintaining the equilibrium of the microflora and also influences the availability of amino acids and amino-acid derivatives to the host (Gao et al., 2018). Notably, a substantial amount of Trp absorbed from the diet is metabolized by gut microbes, which convert it into various compounds, including AHR-activating indole derivatives with T-cell inhibiting properties (Wojciech et al., 2020). As an illustration of the importance of such metabolism, the levels of AHR ligands produced by the gut microbiota have been recently shown to be reduced in patients with celiac disease (Lamas et al., 2020). Conversely, the activity of host amino-acid catabolizing enzymes can influence the availability of amino acids to the microbiota, with consequences on local inflammation, as shown by the role of host Arg1 on the composition of microbiota and bacterial production of protective polyamines

in a mouse model of inflammatory bowel disease (Baier et al., 2020). Thus, the microbiota participates in local immune homeostasis through its amino-acid catabolizing activity and alterations of such activity can lead to immunopathology. It is also probable that microbial amino-acid catabolizing enzymes have an impact on host immunity at non-mucosal sites, as the gastrointestinal tract requires amino acids for the production of immunoregulatory monoamines (melatonin, 5-HT). In certain instances, the activity of the bacterial enzymes may even surpass that of host amino-acid catabolizing enzymes. Indeed, it has been observed that the gut microbiota has a major influence on the level of circulating Trp, indole compounds, and serotonin (Wikoff et al., 2009; Clarke et al., 2013; O'Mahony et al., 2015).

CONCLUSION AND PERSPECTIVES

Aside from serving as the basic building blocks of proteins, amino acids can contribute to many critical processes in growing T cells, including energy metabolism, nucleotide synthesis, epigenetic remodeling, and redox control. T cells require prompt and massive intake of amino acids upon activation. They are thus equipped to sense amino-acid levels, directly and indirectly, via signaling molecules, some of which, like mTOR, control pathways downstream of TCR, costimulatory molecule, and cytokine receptor signaling. Their dependence on external amino-acid import makes T cells highly vulnerable to variations in their extracellular level. Several of the amino-acid catabolizing enzymes expressed in the proximal T-cell microenvironment play an important role in the control of T-cell activation, proliferation, and differentiation by regulating the level of essential and semi-essential amino acids. This effect can be coupled with the production of bioactive catabolites, which also regulate fundamental processes of activated T cells. These complimentary pathways to control T-cell functionality can become imbalanced in pathological situations, such as during cancer development, in which the expression of amino-acid catabolizing enzymes diminishes the quality and strength of the antitumor immune response.

Indoleamine 2,3, dioxygenase, Arg1 and iNOS have received much attention in the last 20 years. However, some aspects of their action have still not been completely elucidated. It is still not totally understood how they can affect the signaling of the T cell, while they are intracellular and produced by APCs. IL4I1 has been more recently identified as an immunosuppressive enzyme and its physiological role is still only partially characterized. As it is a secreted enzyme, its action may be mediated by mechanisms different from those of the intracellular enzymes. Given that several amino acids play a role in T cell activation, other unidentified amino-acid catabolizing enzymes may be involved in T-cell regulation. Finally, the interplay between different enzymes coexpressed by the same cell or in the same microenvironment has only been partially defined. It would be also worth investigating whether it is possible to reverse the effect of these enzymes on TCR signaling using the recently developed specific inhibitors.

Another set of questions remains concerning the action of amino acid catabolizing enzymes on the level of amino-acid derived monoamines that play a role in the neuro-immune axis. The expression of some of these enzymes at discrete sites of monoamine production may regulate specific functions. For example, IL4I1 is highly expressed by centrocytes, i.e., B cells that interact with follicular T helper cells during germinal center maturation of the B-cell response (Caron et al., 2009; Victora et al., 2010). In addition to inhibiting TCR signaling, this expression may interfere with dopamine production by the T cells and stop the dopamine-induced positive feedback loop that fosters B cell differentiation.

Whilst the role of amino acid catabolizing enzymes has been explored in the pathophysiology of various conditions, no major genetic alterations of these enzymes have been yet reported to be associated with human disease. However, further consideration should be given to patients affected by diseases in which a role of amino-acid catabolizing enzymes has been firmly demonstrated. Notably, in the context of cancer, treatments have been developed that target amino-acid metabolism of the tumor cells. These strategies can show considerable short-term efficacy. However, they carry a risk of facilitating relapse by dampening the antitumor T-cell response. This is especially important in the era of immunotherapy with immune checkpoint inhibitors and chimeric antigen receptor T cells (CAR-T). Indeed, Ninomiya et al. showed that CD19-targeted CAR-T lose their capacity to inhibit tumor cell growth in a xenograft lymphoma model when they express IDO (Ninomiya et al., 2015). Consistent with these results, IL4I1 expression in human melanoma has been recently associated with resistance to anti-PD-L1 (Sadik et al., 2020). Specific inhibitors of amino-acid catabolizing enzymes may thus enhance the efficacy of immune checkpoint inhibitors and CAR-T, whereas combining these new therapies with treatments targeting tumor metabolism may not be a valid strategy. Results from clinical trials should shed new light on these issues.

AUTHOR CONTRIBUTIONS

VM-F and FC conceived and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Direct Regulation of the T Cell Antigen Receptor's Activity by Cholesterol

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Biological membranes consist of hundreds of different lipids that together with the embedded transmembrane (TM) proteins organize themselves into small nanodomains. In addition to this function of lipids, TM regions of proteins bind to lipids in a very specific manner, but the function of these TM region-lipid interactions is mostly unknown. In this review, we focus on the role of plasma membrane cholesterol, which directly binds to the αβ T cell antigen receptor (TCR), and has at least two opposing functions in αβ TCR activation. On the one hand, cholesterol binding to the TM domain of the TCRβ subunit keeps the TCR in an inactive, non-signaling conformation by stabilizing this conformation. This assures that the αβ T cell remains quiescent in the absence of antigenic peptide-MHC (the TCR's ligand) and decreases the sensitivity of the T cell toward stimulation. On the other hand, cholesterol binding to TCRβ leads to an increased formation of TCR nanoclusters, increasing the avidity of the TCRs toward the antigen, thus increasing the sensitivity of the αβ T cell. In mouse models, pharmacological increase of the cholesterol concentration in T cells caused an increase in TCR clustering, and thereby enhanced anti-tumor responses. In contrast, the γδ TCR does not bind to cholesterol and might be regulated in a different manner. The goal of this review is to put these seemingly controversial findings on the impact of cholesterol on the αβ TCR into perspective.

Keywords: cholesterol, lipid, TCR, signaling, T cell, nanocluster, allostery

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INTRODUCTION

A eukaryotic plasma membrane is composed of a variety of lipids and sterols, such as cholesterol. The most common composition of the plasma membrane is 20–50% phosphatidylcholine, 20–25% sphingomyelin, 30–50% cholesterol, 10% phosphatidylserine and 25% phosphatidylethanolamine (van Meer et al., 2008; Marquardt et al., 2015). One important sterol is cholesterol (**Figure 1A**), that is synthesized by the cells themselves and can be taken up from the environment. It determines membrane fluidity and permeability (Heerklotz and Tsamaloukas, 2006; Subczynski et al., 2017). The tetracyclic structure of cholesterol is planar and rigid. As a result, increase in membrane cholesterol increases lipid packing and stiffness causing decreased fluidity of lipid bilayers.

Lipids are not randomly distributed within the membrane but are organized. Using model membranes lipid nanodomains called liquid-ordered (Lo) and liquid-disordered (Ld) domains can be distinguished (Veiga et al., 2001; Veatch et al., 2004). It has been argued that these nanodomains are also present in the plasma membrane of living cells, although in a less stable and smaller manner (Eggeling et al., 2009; Levental et al., 2011; Mueller et al., 2011). The Lo domains would correspond to the lipid rafts in cellular membranes and the Ld domains to the non-raft domains (Simons and Ikonen, 1997; Sharma et al., 2004). In cellular membranes the lipid rafts are enriched in sphingolipids and cholesterol and are most likely very small (10–40 nm) and short-lived (microseconds) and hence difficult to characterize. Important for the formation of these domains is the interaction between cholesterol and sphingomyelin that facilitates stable dimers (**Figure 1A**) (Demel et al., 1977; Veiga et al., 2001; Bjorkbom et al., 2011). In addition to the dimer, free cholesterol and free sphingomyelin also exist (Simons and Ikonen, 1997; Endapally et al., 2019). Rafts concentrate signaling molecules and thus are important for signaling (Simons and Ikonen, 1997). Non-raft domains are rich in unsaturated glycerophospholipids, mostly lack sphingolipids and contain less cholesterol. Lo domains are thicker than Ld domains, due to the loss of kinks in acyl chains (Subczynski et al., 2017). Another factor that contributes to nanodomain formation in cellular membranes is the lipid asymmetry between the outer and the inner leaflet. For example, phosphatidylserine is strongly enriched in the inner leaflet and sphingomyelin is mainly found in the outer leaflet (Fadeel and Xue, 2009). Another well-known asymmetry observed is of that of cholesterol where its affinity toward sphingomyelin leads to its enrichment in the outer layer (Wood et al., 2011), although due to its small hydrophilic group (**Figure 1A**) it possesses a very high flip-flop rate (Steck et al., 2002).

Transmembrane (TM) proteins are also not randomly distributed on the cell surface, but localize to certain lipid nanodomains. This is most likely dictated by the exact sequence of the TM region that interacts with the lipids, but also by interactions with other proteins. This localization impacts the function of these proteins, as it allows the vicinity to proteins with a similar lipid preference and guarantees a distance to proteins with a different lipid preference. For example, specific interaction of TM proteins with certain lipids has been demonstrated by structural biology for the bacteriorhodopsin-glycolipid S-TGA-1 (Essen et al., 1998), the cytochrome bc1 complex of the mitochondrial respiratory chain (Hunte, 2005), the metarhodopsin-cholesterol (Ruprecht et al., 2004), the β2-adrenergic receptor-cholesterol (Cherezov et al., 2007; Hanson et al., 2008) interactions or by functional assays for the epidermal growth factor receptor (EGFR)-ganglioside GM3 association (Coskun et al., 2011). These interactions might be the underlying reason for their preferential localization to certain lipid domains or not. In addition, these specific TM region-lipid interactions might directly influence the function of the TM protein. One well-studied example is the T cell antigen receptor (TCR)-cholesterol interaction (Schamel et al., 2017, 2019) and this is the focus of this review.

THE T CELL ANTIGEN RECEPTOR (TCR)

T cells are important for an adaptive immune response against pathogens and tumors and are involved in autoimmunity. In humans 95% of the T cells express an αβ TCR while 5% express a γδ TCR on their surface. The TCR expression is crucial for their development and activation. The αβ TCR (here denoted as TCR for simplicity) binds to pathogen-, tumor- or host-derived peptides presented on MHC molecules (pMHC) by the host's cells. This binding leads to the activation and proliferation of the T cells and downstream effector functions such as cytokine production, provision of help to B cells, regulation of the T cell response or killing of cells expressing the cognate pMHC.

The TCR is a trans-membrane protein complex composed of non-covalently bound TCRαβ, CD3γε, CD3δε, and $\zeta\zeta_2$ dimers (**Figure 1B**). All subunits are type I membrane proteins that contain either basic amino acid residues (arginine and lysine in TCRα; lysine in TCRβ) or acidic ones in their TM domains (aspartic acid in CD3ε, CD3δ, and ζ; glutamic acid in CD3γ) (Alarcon et al., 2003; Malissen, 2003). It is suggested that the potentially charged amino acids in the TM domains are involved in the interaction between the TCRαβ and CD3 (Call et al., 2002) as are also the ectodomains (Schamel et al., 2019).

With their variable extracellular regions TCRαβ bind to pMHC and the information of ligand binding is transduced through the membrane to the cytosolic tails of CD3 and ζ, that contain intracellular signaling motifs (**Figure 1B**). These motifs include the receptor-kinase (RK) motif that binds to the TCR's kinase Lck (Hartl et al., 2020), tyrosines in the context of the immunoreceptor tyrosine-based activation motifs (ITAMs) that can be phosphorylated (Reth, 1989; Weiss, 2010) and a proline-rich sequence (PRS) that can associate with the adaptor protein Nck (Gil et al., 2002). Further, basic rich sequences (BRSs) in CD3ε and ζ bind to negatively charged lipids of the inner leaflet of the plasma membrane in the resting TCR (Aivazian and Stern, 2000; Xu et al., 2008; Zhang et al., 2011). pMHC-binding leads to the exposure of these motifs with a consequent phosphorylation of the tyrosines by Lck. These phospho-tyrosines serve as docking sites for signaling proteins with SH2 domains (Acuto et al., 2008; Courtney et al., 2018). The latter then transduce the signal into the cells, causing activation of the T cell and subsequent effector functions.

THE αβ TCR BINDS TO CHOLESTEROL

Compared to techniques to study protein-protein interactions, the ones for identifying specific associations of lipids with the TM regions of proteins are scarce and have limitations. Thus, not much is known about lipid-protein interactions. Useful techniques include the following: (i) In living cells covalent cross-linking of lipid derivatives with a UV light inducible reactive groups to proteins as been successfully used (Thiele et al., 2000; Hulce et al., 2013). However, the lipids are not exactly the natural ones and thus some interactions might not be detected. (ii) Structural studies of membrane proteins, such as NMR or crystallization, might resolve lipids that either were co-purified with the protein or added during the analysis or crystallization

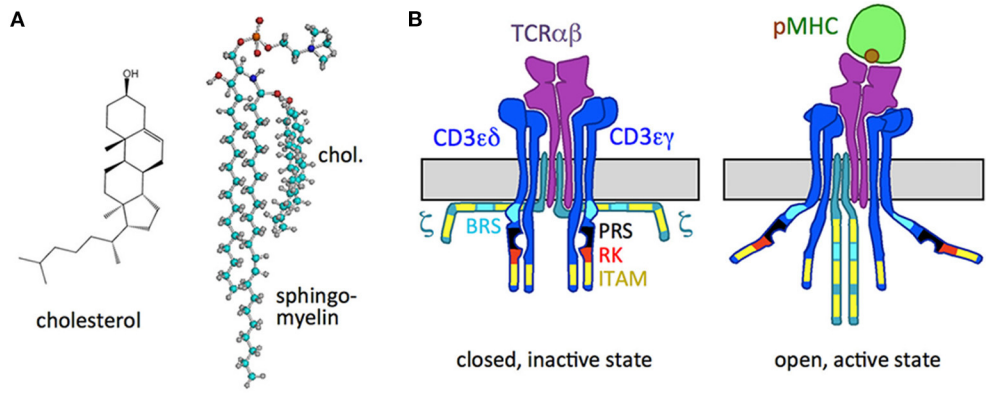

FIGURE 1 | Cholesterol and the TCR. **(A)** Structure of cholesterol and the cholesterol sphingomyelin pair. **(B)** Schematic of the resting, *inactive* TCR, in which the cytoplasmic signaling motifs of the CD3 and ζ subunits are not accessible (right), and of the *active* TCR with the pMHC ligand bound (left), in which the motifs are exposed. The ITAM, BRS, PRS, and RK motifs are indicated.

(Hunte, 2005). (iii) Although indirect, another approach is to modulate the lipid composition of the membrane, as e.g., is artificial liposomes, and then detect changes on the embedded membrane protein (Coskun et al., 2011). (iv) A complementary method is to use beads coupled to a lipid that are then used for pull-down assays to purify proteins that bind to the lipid (Beck-Garcia et al., 2013). However, this requires solubilisation of the membrane proteins by detergent that might be a source for artifacts. Due to these caveats it is recommended to use at least two complementary techniques. These experiments done with the TCR are described in the next paragraph.

Using a radioactive cross-linkable cholesterol derivative (Thiele et al., 2000), we could show that cholesterol specifically binds to the TCR in living cells, and it did not bind to other receptors tested (Molnar et al., 2012). This binding occurred to the TCRβ chain in the resting, i.e., non-ligand bound TCR (Molnar et al., 2012; Swamy et al., 2016). In a recent first high resolution structure of the complete TCR, bound cholesterol was not seen (Dong et al., 2019), most likely because digitonin was used to solubilize the TCR from the cell membrane, which is known to extract and remove cholesterol from the TCR (Schamel et al., 2005; Alarcon et al., 2006; Molnar et al., 2012). Interestingly, cholesterol sulfate, a naturally occurring derivative of cholesterol, competes with cholesterol in binding to the TCR (Wang et al., 2016).

The cholesterol-TCRβ interaction is dynamic, since only the non-ligand bound TCR associated with cholesterol and the ligand-bound TCR did not (Swamy et al., 2016). These binding characteristics were recapitulated using purified TCRs and cholesterol-coupled beads (Beck-Garcia et al., 2013; Swamy et al., 2016) as only the *resting* TCR bound to these beads. This demonstrated that the dynamic cholesterol binding is a property of the TCRβ TM region and is not a consequence of altered membrane properties caused by ligand engagement.

In addition to the cholesterol-TM region interaction and as mentioned above, the cytosolic tails of CD3ε and ζ might interact with the head groups of negatively charged lipids, such as phosphatidylserine, in the inner leaflet of the plasma membrane (Aivazian and Stern, 2000; Xu et al., 2008). Since this has already been reviewed by Wu et al. (2016), it will not be discussed.

THE γδ TCR DOES NOT BIND TO CHOLESTEROL

At first sight the γδ TCR looks similar to the αβ TCR. It also contains the CD3 and ζ subunits, but instead of TCRαβ it contains the highly related TCRγδ ligand-binding dimer. However, the TM region of TCRγ is partially different to the of TCRβ, and consequently using a radioactive cross-linkable cholesterol derivative (Thiele et al., 2000), we demonstrated that the γδ TCR does not bind to cholesterol (Swamy et al., 2016). Thus, the function of cholesterol on the activity of the TCR that we discuss in this review is limited to the αβ TCR and the γδ TCR must therefore be regulated by different mechanisms. A comparison of both TCRs was published recently (Morath and Schamel, 2020).

CHOLESTEROL REGULATES THE ALLOSTERIC SWITCH OF THE αβ TCR

As a prerequisite for allostery, the TCR exists in (at least) two different conformations that differ in their tertiary and/or quaternary structure (**Figure 1B**). Although most crystal structures of certain isolated domains of TCRαβ and CD3 did not provide information on these changes [Garboczi et al., 1996; Garcia et al., 1996; Rudolph et al., 2006; and the reason for that is discussed in a recent review Schamel et al. (2019)], a number of experiments have detected changes in the TCR structure upon ligand binding. These include NMR (Natarajan et al., 2017; Rangarajan et al., 2018), crystallography (Beddoe et al., 2009), and fluorescence-based or H/D exchange approaches (Beddoe et al., 2009; Hawse et al., 2012; Lee et al., 2015). In addition, biochemistry has provided evidence that the TCR

structure changes when pMHC (or stimulating antibodies) are bound. These include limited trypsin digest (Risueno et al., 2008), measuring the distance between two subunits (Lee et al., 2015), accessibility of an antibody epitope (Risueno et al., 2005), cholesterol-binding to TCRβ (Swamy et al., 2016), and exposure of the proline-rich sequence (PRS) (Gil et al., 2002; Minguet et al., 2007), the receptor-kinase (RK) motif (Hartl et al., 2020) and the tyrosines in the cytosolic tails of the CD3 and ζ subunits (Swamy et al., 2016).

The two conformations of the TCR are: (i) the *resting, inactive* conformation (TCR), in which the CD3ε RK motif cannot bind to the Lck and the cytoplasmic tyrosines are shielded and thus are not phosphorylated; and (ii) the *active* conformation, which is stabilized after pMHC or antibody binding, in which Lck binds to CD3ε and the exposed cytosolic tyrosines of CD3 and ζ are phosphorylated (Gil et al., 2002, 2005; Minguet et al., 2007; Lee et al., 2015; Swamy et al., 2016; Hartl et al., 2020). The switch to the *active* conformation is essential for TCR phosphorylation and T cell stimulation. This was confirmed using artificial ligands (Minguet et al., 2007) and TCR mutants that are trapped in the *resting* conformation (Martinez-Martin et al., 2009; Blanco et al., 2014; Dopfer et al., 2014).

Thus, the αβ TCR is allosterically regulated; binding of pMHC at one site (through the variable regions of TCRαβ) causes structural alterations and dynamic changes at other sites, e.g., in the CD3 subunits. As a side note, the γδ TCR does not show these changes and its activity is regulated in a different manner (Blanco et al., 2014; Dopfer et al., 2014; Juraske et al., 2018; Morath and Schamel, 2020).

The Monod-Wyman-Changeux model of allostery (Monod et al., 1965) proposes that the αβ TCR can switch spontaneously between the two states in the absence of ligand (**Figure 2**) (Schamel et al., 2017); experimental evidences support this notion (Mingueneau et al., 2008; de la Cruz et al., 2011; Swamy et al., 2016). The ligand binding can perturb the equilibrium between these two states. In fact, ligand only binds to the *active* state and thus shifts the equilibrium to the *active* state (Swamy et al., 2016); consequently the cytoplasmic motifs are exposed and the TCR becomes signaling active (**Figure 2**). So how does a T cell guarantee that in the absence of ligand not too many TCRs are in the *active* state? This is achieved by cholesterol, which binds only to the *resting* TCR, and hence shifts the equilibrium to *inactive* TCRs (**Figure 2**) (Swamy et al., 2016). Thus, the TCR has two opposing binding partners, one that leads to the accumulation of *inactive* TCRs (cholesterol) and the other one that promotes *active* TCRs (pMHC).

The spontaneous shift of the TCR between its conformations was seen when in the absence of pMHC the cholesterol concentration was lowered (by extraction with methyl-β-cyclodextrin or by oxidation to cholestenone), which caused accumulation of *active* TCRs and initiated spontaneous TCR signaling (Kabouridis et al., 2000; Rouquette-Jazdanian et al., 2006; Swamy et al., 2016). Although methyl-β-cyclodextrin is commonly used to extract or to deliver cholesterol to membranes, it has several undefined effects on the plasma membrane and cell viability. Apart from increasing membrane permeability, it also depolymerizes the actin cytoskeleton and thereby reduces cell stiffness (Mundhara et al., 2019). Hence, it is important to complement the results obtained by methyl-β-cyclodextrin with other methods. In our previous studies we employed cholesterol oxidase to reduce the amount of available membrane cholesterol and again observed an accumulation of TCRs in the *active* state (Swamy et al., 2016). Similarly, mutating the TCRβ TM region so that cholesterol can no longer bind led to a shift of the equilibrium toward the *active* state and low level of T cell stimulation (Petersen et al., 2004; Swamy et al., 2016). These reports show that the TCR TM regions are key regulators of the conformational states of the TCR and that changes at the TM regions are linked to changes at the cytosolic tails.

In conclusion, cholesterol is a natural negative allosteric regulator of the TCR that guarantees that in the absence of ligand most TCRs remain in the *resting* state.

In another model, the TCR acts as a mechanosensor, in which force that is applied via pMHC to the TCR changes the TCR's structure to a signaling active configuration (Kim et al., 2009; Schamel et al., 2019). Since cholesterol stiffens the membrane, its presence at the TCR might influence these changes.

CHOLESTEROL REGULATES αβ TCR NANOCLUSTERING

By using complementary techniques, several studies have suggested that on the surface of a resting T cell, the TCR exists in a monomeric and in a nanoclustered form (Schamel et al., 2005; Lillemeier et al., 2010; Kumar et al., 2011; Sherman et al., 2011; Schamel and Alarcon, 2013; Pageon et al., 2016; Martín-Leal et al., 2020). Other studies only found low amount of TCR nanoclusters and thus concluded that nanoclusters would not exist (James et al., 2011; Rossboth et al., 2018). Thus, the existence of TCR nanoclusters is still controversial, and technical limitations that contribute to this disagreement are discussed in several articles (Schamel and Alarcon, 2013; Platzer et al., 2020). For example, detergents might disrupt nanoclusters when being analyzed biochemically, in microscopy a low labeling efficiency might prevent the detection of nanoclusters or rapid blinking of a fluorophore attached to a TCR might lead to the detection of a nanocluster when in reality there is only one TCR present. Our own studies favor the existence of TCR nanoclusters. In fact, the amount and the size of the nanoclusters depend on the state of the cell (and this might be another confounding factor for detecting or not the nanoclusters). For example, a naïve T cell has less and smaller nanoclusters than an antigen-experienced T cell (Kumar et al., 2011; Schamel and Alarcon, 2013). Likewise, the cholesterol content of these cells increased from naïve to memory cells (Kaech et al., 2002; Kersh et al., 2003; Tani-ichi et al., 2005). These findings suggest that cholesterol is involved in the TCR nanoclustering (**Figure 3**) and three different approaches have shown that this is the case: (i) solubilisation of the TCR from T cell membranes with detergents that do not extract cholesterol preserved the TCR's nanoclustered form; in contrast, when cholesterol was extracted the nanoclusters disassemble to the monomeric TCRs (Schamel et al., 2005; Alarcon et al., 2006; Molnar et al., 2012). (ii) TCR nanoclusters disassembled when

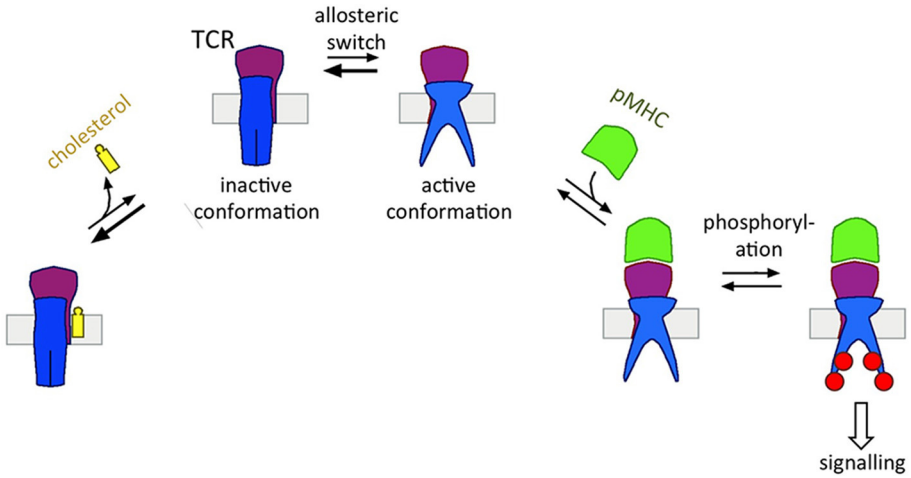

FIGURE 2 | Cholesterol's function of regulating the allosteric switch of the TCR. The TCR can switch spontaneously between the *inactive* and *active* state (allosteric switch). Cholesterol binds to the TCR**β** subunit only in the *inactive* TCR, thus shifting the equilibrium to the left side. The pMHC ligand binds to the TCR**αβ** subunits only in the *active* TCR, thus shifting the equilibrium to the right side. Only in the *active* state the TCR can be phosphorylated transmitting the signal of pMHC-binding downstream.

cholesterol was either extracted from the cells or when cholesterol levels were reduced pharmacologically as detected by immunogold electron microscopy or super-resolution fluorescence microscopy (Schamel et al., 2005; Alarcon et al., 2006; Molnar et al., 2012; Yang et al., 2016). (iii) Reconstituting the monomeric TCR in liposomes of defined lipid composition only allowed nanoclusters to form when cholesterol and sphingomyelin were present in the otherwise phosphatidylcholine-containing liposomes (Schamel et al., 2005; Alarcon et al., 2006; Molnar et al., 2012). This indicated that membrane proteins other than the TCR and lipids other than the ones mentioned are not required for TCR nanoclustering. Since sphingomyelin is mainly present in the outer leaflet of the plasma membrane, it is possible that cholesterol and sphingomyelin [maybe as a pair (Demel et al., 1977; Veiga et al., 2001; Bjorkbom et al., 2011)] bind to the N-terminal part of the TCRβ TM region. However, this remains to be tested. Concerning the mechanism of how cholesterol and sphingomyelin promote TCR nanoclustering, we suggested that cholesterol and sphingomyelin form a mini-raft-islet at the TCRβ TM region that is not favored to be in contact with the non-raft lipid domains that are around the TCR (Molnar et al., 2012; Beck-Garcia et al., 2015). Thus, these islets from several TCRs would come close to each other to shield each other from the non-raft domains, causing TCR nanoclustering.

By regulating TCR nanoclustering cholesterol defines the sensitivity of the TCR for activation through its ligand; a T cell with more and bigger nanoclusters is easier to activate than a cell with predominantly monomeric TCRs (Kumar et al., 2011). Indeed, it was shown that TCR nanoclusters possess a higher avidity toward multimeric pMHC than monomeric TCRs (Molnar et al., 2012). Further, TCRs within a nanocluster show positive cooperativity, so that if one TCR is stabilized in the *active* state by ligand-binding also the other TCRs in the nanocluster reside in the *active* state (Martinez-Martin et al., 2009; Schamel et al., 2017). TCRs in a given nanocluster that are stabilized in *inactive* state by cholesterol can spontaneously release cholesterol and thereby subsequently switch to the *active* conformation. Whether it is sufficient that one single TCR within a cluster binds to cholesterol to prevent the switch of all TCRs to the *active* conformation is not known. Since nanoclusters disassemble when cholesterol is extracted from the cells, the cooperativity of TCR within nanoclusters could be abrogated by cholesterol removal (Martin-Blanco et al., 2018). This result again showed that nanoclusters are required for the TCR cooperativity and that cholesterol is crucial for TCR nanocluster formation.

Studies show that naive T cells contain lower levels of cholesterol than activated T cells (Kersh et al., 2003; Tanichi et al., 2005). Indeed, upon activation of T cells cholesterol metabolism is reprogrammed to synthesize more cholesterol by upregulation of the Sterol Regulatory Element-Binding Protein-2 (SREBP-2) pathway and to transport less cholesterol out of the cell by downregulation of Liver X Receptor (LXR) target genes (Bensinger et al., 2008; Wu et al., 2016). Importantly, in antigen-experienced T cells, such as effector or memory T cells, the increased cholesterol levels contribute to enhanced TCR nanoclustering (Kumar et al., 2011). This might be a danger, since the nanoclusters lower the threshold for T cell activation due to increased avidity and cooperativity. Thus, a counter-regulation through cholesterol by keeping the TCRs in the *inactive* state might prevent spontaneous activation or activation by weak signals, in order to prevent autoimmune diseases. Indeed, elevated cholesterol levels in T cells have been linked to certain autoimmune diseases (see below).

Cholesterol sulfate, which is a low abundant derivative of cholesterol (Bergner and Shapiro, 1981) can bind to the TCR and disrupt the TCR-cholesterol interaction (Wang et al., 2016). This finding suggested that cholesterol sulfate binds to the same region as cholesterol. Interestingly, cholesterol sulfate disrupted

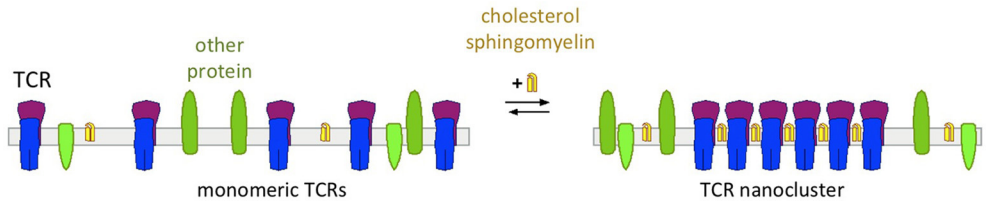

FIGURE 3 | Cholesterol's function of regulating nanoclustering of the TCR. With low levels of cholesterol and sphingomyelin TCRs are expressed as monomers on the cell surface (left)—as it is the case in naïve T cells. With increasing concentrations of cholesterol and sphingomyelin, these lipids bind to the TCR and cause TCR nanoclustering—as it is the case in activated and memory T cells.

TCR nanoclustering in liposomes and in T cells (Wang et al., 2016). The reduced TCR nanoclustering was paralleled by a reduced avidity of the T cells toward multivalent TCR ligands (Wang et al., 2016). Maybe the cholesterol-sphingomyelin pair is required for TCR nanoclustering (see above); and since cholesterol sulfate may not bind to sphingomyelin, it does not promote TCR nanoclustering.

Likewise, the lipid ceramide reduced TCR nanoclustering (Martín-Leal et al., 2020). This was observed in liposomes as well as in T cells that were treated with sphingomyelinase, which hydrolyses sphingomyelin to ceramide. This data suggest that the cholesterol-sphingomyelin pair drives TCR nanoclustering. Interestingly, signaling by the receptor CCR5 reduces ceramide levels in antigen-experienced T cells (Martín-Leal et al., 2020). In these cells, along with reduced ceramide levels, increased membrane cholesterol contributes to enhanced TCR nanocluster formation and increased sensitivity of these cells compared to naive T cells. In conclusion, T cells regulate their membrane lipid composition, in order to tune TCR nanoclustering and thus TCR signaling.

MODULATION OF CHOLESTEROL LEVELS TO TUNE αβ TCR FUNCTION IN THE TREATMENT OF DISEASES

As discussed, cholesterol modulates the activity of the TCR. Moreover, since dampening or increasing signaling by the TCR can be used to treat autoimmunity or cancer, respectively, it is not surprising that pharmacologically changing the cholesterol content of T cells has been used to ameliorate certain diseases.

Autoantibodies and the deposition of immune complexes are known to cause the autoimmune disease systemic lupus erythematosus (SLE). Overactive T cells contribute to the pathology by help provided to B cells and by the killing of host cells in a number of organs. Thus, a strong T cell activity contributes to SLE (Moulton and Tsokos, 2015). Importantly, T cells from SLE patients possess increased plasma membrane levels of cholesterol and glycosphingolipids (Jury et al., 2004; McDonald et al., 2014). These could lead to enhanced TCR nanoclustering and formation of signaling-promoting lipid rafts, consequently leading to increased T cell activation and effector functions as observed experimentally (McDonald et al., 2014). Extraction of cholesterol from the membrane of T cells from SLE patients using methyl-β-cyclodextrin indeed reversed the heightened signaling by the TCR (Krishnan et al., 2004). This may be a result of a disintegration of TCR nanoclusters and a partial disruption of lipid rafts. Reduced TCR signaling was also seen when the inhibitor N-butyldeoxynojirimycin was used, which normalized glycosphingolipid levels in T cells of SLE patients (McDonald et al., 2014). Similarly, inhibition of cholesterol biosynthesis in the SLE T cells by Atorvastatin reduced signaling and T cell activation (Jury et al., 2006). Statins are widely prescribed as drugs to reduce cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase, which is a key enzyme in the mevalonate pathway to synthesize cholesterol, but also to generate protein prenylations, such as farnesylation or geranylgeranylation. Thus, statins have multiple effects. Indeed, Simvastatin impairs T cell activation through inhibition of Ras prenylation (Ghittoni et al., 2005) and Lovastatin suppresses T cells proliferation due to reduced farnesol pyrophosphate levels (Chakrabarti and Engleman, 1991; Bietz et al., 2017). These anti-inflammatory effects of statins could be beneficial for autoimmune or inflammatory disorders but would worsen immune responses against cancer. In this review, we focus on the cholesterol-related effects.

In vivo extraction of cholesterol from plasma membrane of T cells using methyl-β-cyclodextrin in a mouse model of SLE delayed disease onset (Deng and Tsokos, 2008). This is line with reducing the T cells' activity by disruption of TCR nanoclusters and of lipid rafts. The latter mechanism was most likely involved, as clustering of lipid rafts in T cells by cholera toxin B promoted disease progression *in vivo* (Deng and Tsokos, 2008).

To treat cancer by increasing T cell activation has been proven to be a successful strategy (Iwai et al., 2002; Fritz and Lenardo, 2019). The enzyme acyl-CoA cholesterol acyltransferase 1 esterificates cholesterol and thus reduces cholesterol levels in T cells (Chang et al., 2006). Inhibition of this enzyme by Avasimibe led to elevated membrane cholesterol levels in $CD8^+$ T cells and enhanced signaling (Yang et al., 2016) most likely by increased TCR nanoclustering. Importantly, this led to enhanced T cell effector functions resulting in stronger killing of tumor cells in mouse melanoma and lung carcinoma models (Yang et al., 2016).

Additionally, combination of Avasimibe and anti PD1 treatments proved to be more potent than either monotherapies against cancer (Yang et al., 2016).

These preclinical findings show that the role of cholesterol in promoting TCR signaling (by inducing TCR nanoclustering and formation of lipid rafts) is dominant over its role in suppressing TCR signaling (by stabilizing the *inactive* TCR state).

CONCLUSION

Cholesterol specifically binds to the αβ TCR through its TCRβ subunit in the TCR's *inactive* conformation, thus supressing signaling. Cholesterol also promotes TCR nanoclustering and the formation of lipid rafts, both of which promote signaling. In a translational approach, this knowledge was recently used to pharmacologically enhance cholesterol levels in T cells, which potentiated the anti-tumor function of T cells in mouse models. This suggests that the cholesterol-induced nanoclustering and lipid raft formation are dominant in this setting and hence, cholesterol acted as a positive regulator of TCR signaling. What remains to be understood is, how the balance between positive and negative regulation through cholesterol interaction is regulated, in order to achieve fine-tuning of TCR activation and how this can be translated for the treatment of diseases that depends on the sensitivity of TCR activation.

Most likely, the TCR is an example protein for which its regulation by lipids is beginning to unfold. Most likely the influence of direct lipid-TM region interactions on the functioning of membrane proteins is much more widespread than currently thought.

AUTHOR CONTRIBUTIONS

SP-C, CD, and WS wrote the first draft of the manuscript and updated the last version. KR, AM, WW, CX, and BO completed and corrected the draft. All authors contributed to the article and approved the submitted version.

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Single-Molecule, Super-Resolution, and Functional Analysis of G Protein-Coupled Receptor Behavior Within the T Cell Immunological Synapse

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A central process in immunity is the activation of T cells through interaction of T cell receptors (TCRs) with agonistic peptide-major histocompatibility complexes (pMHC) on the surface of antigen presenting cells (APCs). TCR-pMHC binding triggers the formation of an extensive contact between the two cells termed the immunological synapse, which acts as a platform for integration of multiple signals determining cellular outcomes, including those from multiple co-stimulatory/inhibitory receptors. Contributors to this include a number of chemokine receptors, notably CXC-chemokine receptor 4 (CXCR4), and other members of the G protein-coupled receptor (GPCR) family. Although best characterized as mediators of ligand-dependent chemotaxis, some chemokine receptors are also recruited to the synapse and contribute to signaling in the absence of ligation. How these and other GPCRs integrate within the dynamic structure of the synapse is unknown, as is how their normally migratory Gαi-coupled signaling is terminated upon recruitment. Here, we report the spatiotemporal organization of several GPCRs, focusing on CXCR4, and the G protein Gαi2 within the synapse of primary human $CD4^+$ T cells on supported lipid bilayers, using standard- and super-resolution fluorescence microscopy. We find that CXCR4 undergoes orchestrated phases of reorganization, culminating in recruitment to the TCR-enriched center. This appears to be dependent on CXCR4 ubiquitination, and does not involve stable interactions with TCR microclusters, as viewed at the nanoscale. Disruption of this process by mutation impairs CXCR4 contributions to cellular activation. Gαi2 undergoes active exclusion from the synapse, partitioning from centrally-accumulated CXCR4. Using a CRISPR-Cas9 knockout screen, we identify several diverse GPCRs with contributions to T cell activation, most significantly the sphingosine-1-phosphate receptor S1PR1, and the oxysterol receptor GPR183.

These, and other GPCRs, undergo organization similar to CXCR4; including initial exclusion, centripetal transport, and lack of receptor-TCR interactions. These constitute the first observations of GPCR dynamics within the synapse, and give insights into how these receptors may contribute to T cell activation. The observation of broad GPCR contributions to T cell activation also opens the possibility that modulating GPCR expression in response to cell status or environment may directly regulate responsiveness to pMHC.

Keywords: lymphocyte, synapse, fluorescence, microscopy, receptor, tracking, signaling, screening

INTRODUCTION

The adaptive immune system depends on the activation of antigen-specific lymphocytes to deliver an appropriate and coordinated response to infection or cellular dysfunction. Central to this are T cells, which express clonally unique T cell receptors (TCRs) capable of recognizing a restricted range of antigen-derived peptides presented by major histocompatibility (MHC) molecules on antigen-presenting cells (APCs), such as B cells and dendritic cells (DCs). The recognition of cognate peptide-MHC (pMHC) by TCR leads to activation of the T cell and formation of a large interface with the APC; in either the form of a stable immunological synapse (synapse) or a motile kinapse (Dustin, 2007; Mayya et al., 2018). This involves spatial organization into distinct zones that correspond to transitions in an underlying filamentous actin (F-actin) network, described as supramolecular activation clusters (SMACs): the central (c)SMAC corresponds to sparse F-actin bundles that enable access for bidirectional vesicular budding and fusion; the actinomyosin- and talin-rich peripheral (p)SMAC stabilizes adhesion; and the dendritic F-actin-rich distal (d)SMAC is an important site for signal initiation (Freiberg et al., 2002; Sims et al., 2007; Fritzsche et al., 2017). It is important to point out that part of the cSMAC includes a synaptic cleft into which TCR-enriched extracellular vesicles, including synaptic ectosomes and exosomes, soluble secreted proteins, and multiprotein complexes are released (Stinchcombe et al., 2001; Mittelbrunn et al., 2011; Choudhuri et al., 2014; Saliba et al., 2019; Bálint et al., 2020)—a process that occurs through the ramified actin network (Fritzsche et al., 2017). Kinapses are related to synapses by symmetry breaking with the dSMAC converting into a leading lamellipodium and pSMAC into a talin-rich focal zone (Smith et al., 2005; Sims et al., 2007). A common feature of both synapse and kinapse is F-actin-dependent TCR microclusters/protrusions that integrate with the larger actin network to influence synapse/kinapse balance (Varma et al., 2006; Beemiller et al., 2012; Kumari et al., 2015, 2020; Cai et al., 2017). Immunoglobulin superfamily, tumor necrosis factor/receptor families, and integrin family receptors—e.g., TCR, CD28, CTLA-4, PD1, CD40L, HVEM, LFA-1—are well-mapped in the immunological synapse including the recently described CD2 corolla (Demetriou et al., 2020). However, it is also evident that proteins from other families have significant contributions in this context, including members of the G protein-coupled receptor (GPCR) family. GPCRs are the largest family (>800 members) of cell surface receptors in the human genome and activate intracellular heterotrimeric G proteins and arrestins in response to extracellular ligand-binding (Rosenbaum et al., 2009). TCR-derived signals act in part through G-proteins (Stanners et al., 1995; Ngai et al., 2008) and arrestins (Fernández-Arenas et al., 2014), and are sensitive to factors under GPCR control, e.g., cAMP (Ledbetter et al., 1986; Abrahamsen et al., 2004). Several GPCRs have important regulatory function during T cell-APC communication, including receptors for lysophosphatidic acid (Oda et al., 2013), adenosine (Linnemann et al., 2009), adrenaline (Fan and Wang, 2009), and dopamine (Papa et al., 2017); however, the most ubiquitous are members of the chemokine receptor family.

Classically, chemokine receptors coordinate migration of T cells and other leukocytes between blood, lymphoid organs, and inflamed tissue by directing cells along localized chemokine gradients. Orthogonal CCL21 and CXCL10 gradients promote synapse breaking, whereas orthogonal CXCL12 and CCL5 gradients are generally permissive of synapse formation (Bromley et al., 2000). Consistent with this, signals from the TCR and chemokine receptors may be reciprocally regulated (Peacock and Jirik, 1999; Dar and Knechtle, 2007) and chemokine-mediated signaling in T cells is at least partially dependent on components of the TCR signaling system, e.g., Lck (Inngjerdingen et al., 2002), ZAP70 (Kremer et al., 2003), and the TCR itself (Newton et al., 2009). CXCR4 and CCR5 (Molon et al., 2005; Contento et al., 2008), as well as CCR7 (Laufer et al., 2018) are recruited to the synapse to act as coreceptors that enhance TCR-derived signals, increase synapse lifetime, and augment cytokine mRNA stability (Kremer et al., 2017). Such recruitment appears to be driven by TCR triggering which might also synergise with chemokine-driven receptor activation. Furthermore, direct physical association with TCR might be required for recruitment of CXCR4 (Kumar et al., 2006; Trampont et al., 2010) and CCR7 (Laufer et al., 2018). In the case of CXCR4 such association appears to be dependent on phosphorylation of Ser-339 by G protein-coupled receptor kinase-2 (GRK2) that is in turn activated by TCR-activated tyrosine kinases (Dinkel et al., 2018). However, delivery of CXCR4 into the synapse is also reportedly driven by the actin-binding protein drebrin, which by bridging CXCR4 to actin leads to its accumulation in the actin-rich regions of the synapse (Pérez-Martínez et al., 2010). Actin-enrichment is restricted to the periphery of the synapse, away from the major accumulations of TCR at the center, and

hence would appear to be incompatible with simultaneous CXCR4 interaction with the TCR. Nonetheless, a C-terminally truncated form of CXCR4 associated with WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) syndrome does not exhibit correct recruitment to and stabilization of the synapse (Kallikourdis et al., 2013), confirming the importance of this domain for CXCR4 coreceptor function. The spatiotemporal organization of CXCR4 and other GPCRs within the synapse has not been extensively studied, and the extent to which GPCRs can influence TCR signaling in the absence of ligation is poorly understood.

Alongside these considerations is the question of to what degree GPCR effects on T cell activation are dependent on signaling through associated G proteins. This is perhaps best characterized for Gαs-coupled GPCRs, such as the adenosine or adrenergic receptors, which increase local cAMP concentration through activation of adenylate cyclase. Ligand-dependent activation of Gαs activates the inhibitory kinase Csk in a cAMP-dependent manner (Vang et al., 2001), thereby inhibiting TCR signaling through ZAP70 (Linnemann et al., 2009) and downstream activation of integrins (Dimitrov et al., 2019). The contribution of Gαi-coupled signaling, which inhibits adenylate cyclase, is less well-understood. Many T cell-expressed GPCRs couple preferentially to Gαi proteins, including all chemokine receptors, and this signaling pathway is the primary driver of chemotaxis (Legler and Thelen, 2018). Several studies have reported chemokine-dependent effects on T cell activation that are sensitive to inhibition by pertussis toxin (PTx), which inactivates Gαi proteins (e.g., Bromley and Dustin, 2002; Smith et al., 2013). Nonetheless, upon recruitment to the synapse, chemokine receptors have also been observed to shift preference from Gαi- to Gαq/11-coupled pathways (Molon et al., 2005), which drive cell adhesion rather than migration (Mellado et al., 2001). However, Gαq is believed to be inhibited by active GRK2 (Mariggiò et al., 2006), and so it is not clear how much GPCRs within the synapse could promote Gαq signaling even if they are able to physically couple. Interpretation of experiments involving inhibition by PTx are also complicated by the observation that PTx activates the TCR signaling pathway to drive desensitization of chemokine receptors (Schneider et al., 2009), thereby impacting receptor effects beyond just Gαi-coupled processes. Alongside G protein signaling, chemokine receptors are sensitive to tyrosine-phosphorylation at a DRY motif at the cytoplasmic end of transmembrane helix 3 (Mellado et al., 1998), which is highly conserved across almost all GPCRs. Such phosphorylation can be mediated by Src-family kinases (Hauser et al., 2016), generating docking sites for SH2-domain containing proteins in a manner similar to the TCR itself and many tyrosine-based co-receptors.

In this study we use fluorescence microscopy techniques to examine the spatiotemporal organization of GPCRs within the synapse and identify the underlying molecular determinants. We focus primarily on the chemokine receptor CXCR4 due to its relative significance in T cell activation, and existence of previously published insights into its gross distribution in the synapse (Molon et al., 2005; Pérez-Martínez et al., 2010). In order to simulate T cell-APC interactions in an imaging-permissive manner we use planar supported lipid bilayers (SLBs) loaded with anti-CD3 Fab' to mimic TCR-pMHC engagement, and the recombinant integrin ligand ICAM1 (intercellular adhesion molecule one) to drive adhesion through binding to LFA1. This approach has been widely used in combination with total internal fluorescence microscopy (TIRFM) to visualize only the events occurring at the synapse (Calvo and Izquierdo, 2018). Through both ensemble imaging and single-particle tracking, we observe initial segregation of CXCR4 to the dSMAC, followed by active recruitment to the center over time. This is not due to physical TCR-CXCR4 interactions and is not sensitive to CXCR4 engagement of chemokine, coupling to G protein, or C-terminal/DRY motif phosphorylation, but may be dependent on ubiquitination. We report concomitant exclusion of the G protein Gαi2 from the synapse, which may offer an explanation for the cessation of Gαi-mediated signaling by chemokine receptors upon T cell activation. Finally, we assess the sensitivity of T cell activation to knockout of 28 diverse GPCRs and identify significant contributions for several receptors. Investigation of a subset of these GPCRs did not reveal clear correlation between intra-synapse organization and costimultory potential, but did suggest commonalities in receptor dynamics that may be applicable to many GPCRs.

RESULTS

CXCR4 Undergoes Contact Time-Dependent Organization Within the T Cell Synapse

We began by quantifying the spatiotemporal organization of CXCR4 within the synapse to determine how it relates to the various SMACs and their distinct signaling environments. Primary human $CD4^+$ T cell blasts were transfected with mRNA encoding CXCR4 fused to a C-terminal HaloTag, then allowed to form contacts on SLBs presenting either ICAM1 alone at 200 molecules/μm^2 (non-activating) or ICAM1 and anti-CD3 (UCHT1) Fab', at 200 and 30 molecules/μm^2, respectively (activating). These were then imaged live at different time points using TIRFM, which visualizes only molecules within ∼100 nm of the SLB. Whereas, CXCR4 distribution exhibited no obvious organization in contact with the non-activating SLB, CXCR4 exhibited a clear exclusion from the center of the contact within minutes on activating SLB, and from both the cSMAC and pSMAC in the mature synapse (**Figures 1A–D**; **Supplementary Figure 1A**). This distribution shifted over the lifetime of the synapse, with gradual enrichment of CXCR4 within the cSMAC clearly evident after 30 min (**Figures 1A–D**). Three-dimensional confocal microscopy revealed large amounts of CXCR4 away from the planar bilayer interface that could be consistent with receptor endocytosis, but also with presence in extracellular vesicles that accumulate between the cell and the SLB (**Supplementary Figure 1B**). Delivery of intracellular CXCR4 toward the synapse could also contribute to this observation. Staining of endogenous CXCR4 with fluorophore-conjugated anti-hCXCR4 antibody following fixation at 10 and 30 min yielded comparable observations (**Figure 1E**), indicating

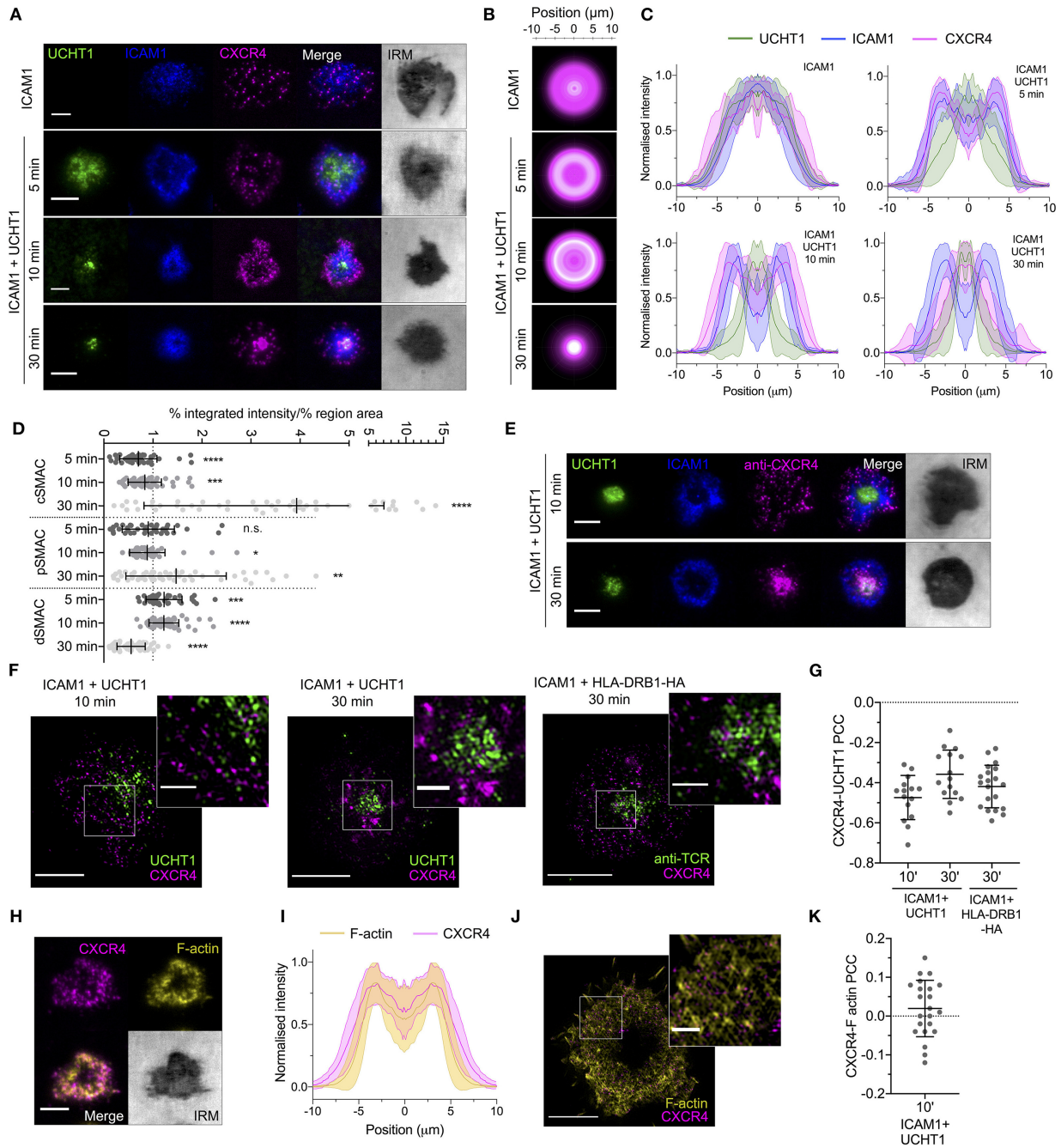

FIGURE 1 | CXCR4 distribution within the synapse. **(A)** Representative TIRFM examples of CXCR4-HaloTag-expressing CD4$^+$ T cell blasts interacting with non-activating (ICAM1) or activating (ICAM1 + UCHT1) SLB for 5–30 min. IRM, interference reflection microscopy. **(B)** Radial averages of CXCR4-HaloTag intensity from >30 cells for each indicated condition. **(C)** Cross-sectional normalized intensity profile of radial averages of all labeled proteins for each indicated condition. Plots are mean normalized intensity at each position ± std dev. **(D)** Relative enrichment of CXCR4-HaloTag intensity in cSMAC, pSMAC, and dSMAC regions of the synapse on activating SLB. Values are expressed as the percentage of total intensity within a region divided by the percentage of the total IRM-defined area that constitutes that region. A value of one indicates no relative enrichment or depletion from a region; >one indicates relative enrichment; <one relative depletion. Significance is shown relative to a value of one assessed with a one-sample, two-tailed *t*-test. Each point represents an individual cell; bars are mean ± std dev. **(E)** TIRF microscopy examples of anti-CXCR4-stained CD4$^+$ T cell blasts interacting with activating SLB for 10 or 30 min. **(F)** TIRF-SIM examples of CXCR4-HaloTag-expressing CD4$^+$ T cell blasts on SLBs presenting ICAM1 + UCHT1 Fab for 10 or 30 min (left & middle), or HA-restricted CD4$^+$ T cell clone 40 on SLB presenting ICAM1 + HLA-DRB1-HA for 30 min. Inserts correspond to white boxes. **(G)** Pearson's correlation coefficient (PCC) values for CXCR4-HaloTag vs. UCHT1 in TIRF-SIM-imaged cells. **(H)** TIRF microscopy example of F-actin and CXCR4-HaloTag in a CD4+ T cell blast on activating SLB. **(I)** Normalized intensity profile for radially averaged F-actin and CXCR4-HaloTag signals in >30 cells. **(J)** TIRF-SIM example of F-actin and CXCR4-HaloTag on activating SLB for 10 min. **(K)** PCC values for CXCR4-HaloTag vs. F-actin in TIRF-SIM-imaged cells. All scale bars are 5 μm except for zoomed inserts (1 μm). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s., not significant. All pooled data represent a minimum of $n = 3$ independent donors.

that at least some of the centrally accumulated CXCR4 remains at the cell surface or in extracellular vesicles. Late CXCR4 accumulation at the cSMAC was also evident in cells pre-stained with anti-hCXCR4 antibody before synapse formation (**Supplementary Figure 1C**), supporting the notion that CXCR4 in this region has been directly recruited from the plasma membrane.

Given the previous indications of a physical association between CXCR4 and the TCR (Kumar et al., 2006; Trampont et al., 2010), and that recruitment to the cSMAC would be an expected outcome of this, we examined the nanoscale organization of CXCR4 relative to TCR microclusters using TIRFM with structured illumination microscopy (SIM), which provides an effective isotropic resolution of ∼100 nm. This was performed in fixed cells to avoid movement of molecules during image acquisition. CXCR4 exhibited marked segregation from TCR-enriched regions of the synapse both 10 and 30 min after exposure to the SLB (**Figures 1F,G**). To determine if recruitment of CD4 to TCR-pMHC complexes impacts possible CXCR4-TCR interactions, we repeated these experiments with a high-affinity T cell clone specific to peptide corresponding to influenza H3 haemagglutinin residues 338–355 bound to HLA-DRB1*09:01 (as used in Saliba et al., 2019), which was used to replace UCHT1 Fab' on the SLB at 30 molecules/μm^2. CXCR4 in these cells underwent comparable organization to those activated with UCHT1 (**Supplementary Figure 1D**), and was similarly segregated from the TCR at the nanoscale (**Figures 1F,G**). These data, along with the different timing of TCR and CXCR4 accumulation in the cSMAC, argue against the formation of extensive stable CXCR4-TCR interactions.

Given the initial distal segregation of CXCR4, we examined whether this distribution correlated with well-described peripheral F-actin structures (Dustin and Cooper, 2000). In the mature, early (10 min) synapse, CXCR4 distribution correlated closely with that of F-actin stained with phalloidin (**Figures 1H,I**), in line with previous observations of CXCR4-actin connections in activated T cells (Pérez-Martínez et al., 2010). This organization was lost following acute inhibition of Src kinases or disruption of actin polymerisation (**Supplementary Figure 1E**), supporting the notion that CXCR4 redistribution depends on correct F-actin organization. TIRF-SIM imaging of CXCR4 relative to F-actin revealed no significant positive or negative Pearson correlation between the two (**Figures 1J,K**), indicating that CXCR4 is not associated with peripheral actin en masse, however this does not exclude the possibility of transient associations within individual receptors or the stable association of CXCR4 with filaments separate from the brightest actin structures that may not be readily detectable with imaging.

CXCR4 Is Actively Recruited to the Center of the Synapse

To assess how CXCR4 becomes enriched within the cSMAC, we examined the dynamic behavior of individual CXCR4 molecules through live TIRFM. Primary $CD4^+$ T cell blasts transfected with low levels (200–2,000 molecules/cell) of CXCR4-HaloTag were imaged on SLBs containing ICAM1 alone or ICAM1 + UCHT1 Fab'. Videos were captured at 50 ms/frame for 15 seconds, then individual spots were identified and tracked over time in TIRFM to allow individual trajectories to be analyzed (**Figure 2A**; **Supplementary Movie 1**). Three forms of behavior were evident within the CXCR4 population: normal, unconstrained diffusion, active diffusion, and confined/subdiffusion. These three forms most likely correspond to receptors moving freely within the membrane (normal diffusion); receptors undergoing active transport through coupling to directional structures, e.g., actin-myosin (active diffusion); and receptors that are either immobile due to stable interactions with underlying structures or whose free diffusion is restricted to a highly confined area (subdiffusion). Under all conditions, the majority of molecules exhibited normal diffusion, however the proportion of receptors undergoing active and subdiffusion increased substantially when cells were activated with UCHT1 Fab' (**Figure 2B**; **Supplementary Movie 2**). Within the normal, active, and sub-diffusion populations there was no clear difference in behavior across different conditions (**Figure 2C**; **Supplementary Figure 1F**), indicating that cellular activation does not alter the characteristic diffusive modes, but simply changes their relative frequencies. The spatial distribution of different modes of diffusion varied markedly; with freely diffusing CXCR4 predominantly in the periphery of the contact, confined receptors more likely to be in the center (possibly in internal or extracellular vesicles), and actively diffusing receptors centrally offset relative to the majority of normally diffusing molecules (**Figures 2D,E**).

We next mapped absolute trajectory positions to regions of the synapse defined by UCHT1- or ICAM1-accumulation, and the IRM signal in single-frame images taken immediately before video acquisition (**Figure 2F**). As expected, the majority of receptors spent some time within the dSMAC, with the cSMAC containing the fewest tracks (**Figure 2G**). Within the dSMAC, the majority of receptors underwent free diffusion, whereas the cSMAC was occupied predominantly by subdiffusing receptors, and the pSMAC contained a substantial population of actively diffusing receptors (**Figure 2H**). On average, actively diffusing CXCR4 moved closer to the center of the synapse during the lifetime of the track, whereas freely diffusing receptors did not (**Figure 2I**). This indicates that CXCR4 actively migrates from the dSMAC toward the cSMAC, whereupon it becomes highly restricted and hence is retained. This is supported by the observation that only the actively diffusing receptors undergo substantial movement between the different areas of the synapse, predominantly from the dSMAC to pSMAC (**Figures 2J,K**). This indicates that the factors involved in the initial segregation of CXCR4 to the dSMAC also act as a barrier to passive CXCR4 entry into the pSMAC, but processes that progressively recruit CXCR4 to the cSMAC may overcome or circumvent this barrier. The fact that the majority of distal CXCR4 molecules undergo free diffusion supports the notion that although actin is a key driver of CXCR4 redistribution, this is not mediated by extensive, stable CXCR4-actin interaction.

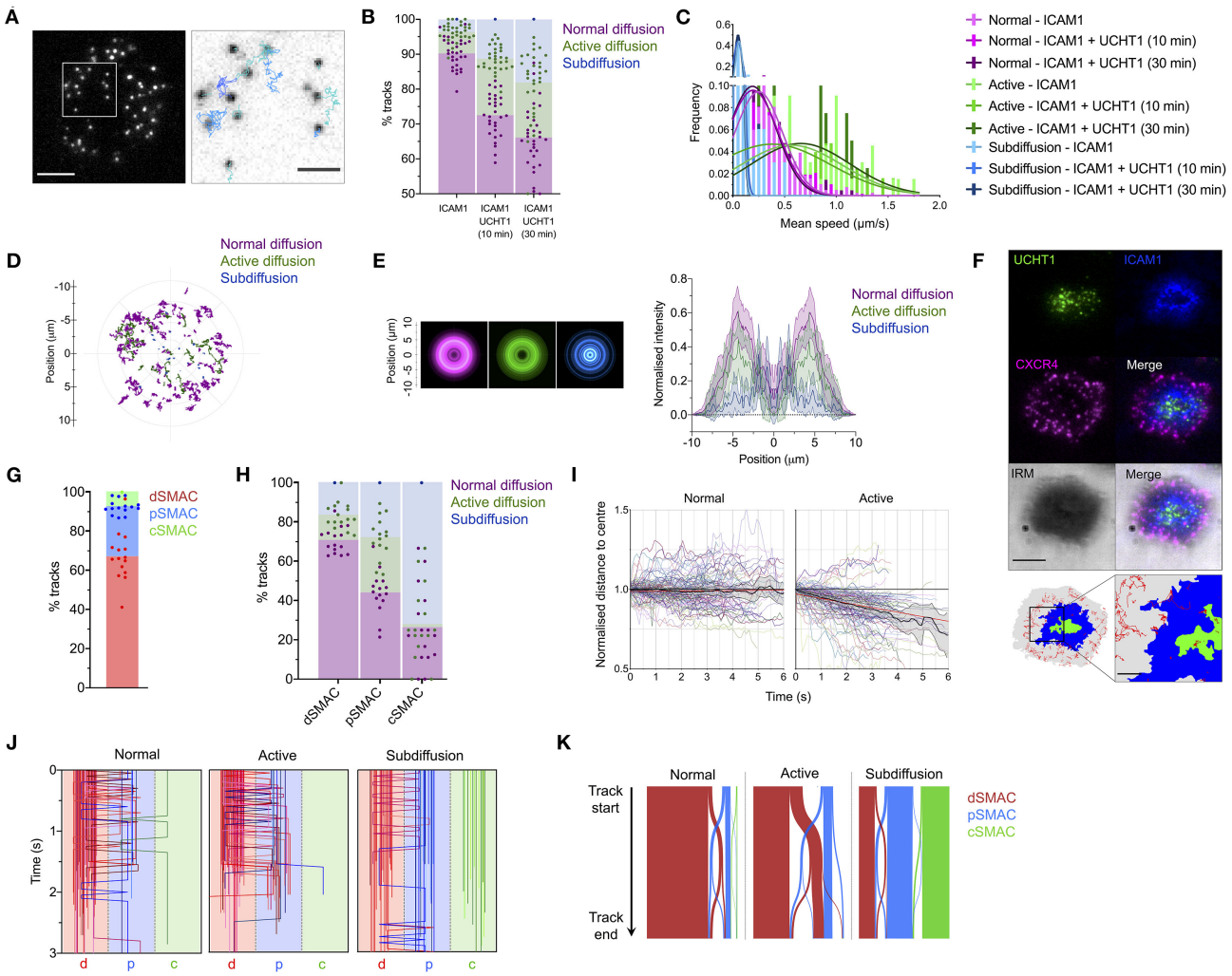

FIGURE 2 | CXCR4 dynamics within the synapse. **(A)** Example of CXCR4-HaloTag single-particle tracking using TIRFM. The xy coordinates of all individual CXCR4-HaloTag spots were recorded every 50 ms and then linked together to derive particle tracks. **(B)** Proportion of single-particle CXCR4-HaloTag tracks exhibiting normal, active, or confined/subdiffusion in $CD4^+$ blasts on inactivating or activating SLBs. Each point represents an individual cell. **(C)** Histogram of mean diffusion speed for tracks with different diffusive properties under activating and non-activating conditions. **(D)** Representative example showing relative location of different tracks within the synapse of a single cell. The entirety of all tracks over 30 frames in length are shown, centered around the approximate center of contact. **(E)** Radial averages of track locations across all cells imaged on activating SLBs (left), and cross-sectional normalized intensity profiles of those averages (right). Plots are mean normalized intensity at each position ± std dev. **(F)** Example of image partitioning based on IRM, ICAM1, and UCHT1 signals into d, p, and cSMAC regions, with single-particle tracks overlaid. **(G)** Proportion of all tracks that spend a minimum of three frames in the indicated regions. **(H)** Proportion of tracks exhibiting normal, active, or confined/subdiffusion in each indicated region. **(I)** Trajectories of 50 representative tracks undergoing normal or active diffusion, and mean ± 95% CL for all such tracks. Trajectories are expressed as normalized distance to center, in which the distance of the starting position of a track from the center of the imaged contact is given a value of one. Red lines indicate linear regression fit of all tracks. **(J)** Region position summaries of 50 example tracks for each diffusion type showing transitions between different regions of the synapse. Tracks are colored according to their starting region: red = dSMAC, blue = pSMAC, green = cSMAC. **(K)** Sankey plot summarizing starting region and transitions between regions for all recorded tracks. Bar width is proportional to the number of associated tracks within that diffusion category. All scale bars are 5 μm except for zoomed inserts (1 μm). All pooled data represent a minimum of $n = 3$ independent donors.

Ligation of CXCR4 Does Not Appreciably Impact Receptor Organization

All of the experiments described thus far were performed in the absence of CXCR4 ligation by chemokine. We therefore set out to determine how the observed organization of CXCR4 is influenced by its cognate ligand CXCL12, both in soluble and surface-presented forms. CXCR4-HaloTag-transfected primary human $CD4^+$ T cell blasts were activated on SLBs as above, with the addition of either soluble CXCL12 at 0.1 μg/ml, or biotinylated CXCL12 attached to the SLB via a streptavidin linker at ∼100 molecules/μm^2. Interestingly, neither form of CXCL12 had any clear impact on CXCR4 distribution in either early or late synapses (**Figure 3A**), even though both promoted greatly increased cell migration on ICAM1-only containing SLBs (**Supplementary Figure 1G**). Single-molecule tracking of CXCR4-HaloTag in the early, mature synapse

(10 min) revealed the same frequency of diffusion types (**Figure 3B**) and track characteristics within each diffusion type (**Supplementary Figure 1H**) regardless of which presentation of CXCL12 was present. This indicates a disconnect between the synaptic behavior of CXCR4 and its ligation state, in stark contrast to the situation within migrating T cells (Martínez-Muñoz et al., 2018).

Due to technical limitations of the SLB system, it was not possible to adequately replicate a scenario of CXCL12 release into the synapse by the APC, nor of potential differences in CXCL12 oligomerisation and/or activity through presentation by glycosaminoglycans and other chemokine-binding molecules on the APC. We therefore visualized CXCR4 distribution within the synapse of direct T cell-APC interactions. In order to permit high-resolution imaging in the x-y axial plane (as opposed to the z-axis orientation achieved through simple coculture), we employed a vertical-capture microfluidics approach (Jang et al., 2015) wherein APCs are first captured in holding pits within the microfluidics chamber and T cells flowed in afterwards to form a vertical conjugate (**Figure 3C**). This allowed confocal imaging of the conjugate synapse following fixation 30 min post T cell introduction. Due to the asynchronous way in which T cell-APC interactions begin in this system, it was not possible to precisely standardize synapse age prior to fixation, and hence imaging was restricted to the late synapse. Jurkat E6.1 cells expressing endogenous CXCR4 genomically fused to HaloTag were used in combination with Raji B cells loaded with Staphylococcal enterotoxin type E (SEE), which cross-links several common Vβ segment containing TCR to MHC class II molecules (Proft and Fraser, 2003). This maximized the likelihood of productive contact formation since all T cells were capable of responding to SEE-loaded B cells, and compensated for the reduced sensitivity of confocal vs. TIRFM as CXCR4-HaloTag expression was higher than in transfected primary cells. CXCR4 in these conjugates exhibited substantial central accumulation (**Figure 3C**) comparable to that observed in primary $CD4^+$ T cells on SLBs, and also in Jurkat E6.1 cells on in the same system (**Figure 3D**). Incubation of Raji B cells with a monensin-containing protein transport inhibitor for 6 h prior to conjugate formation did not impact CXCR4 accumulation

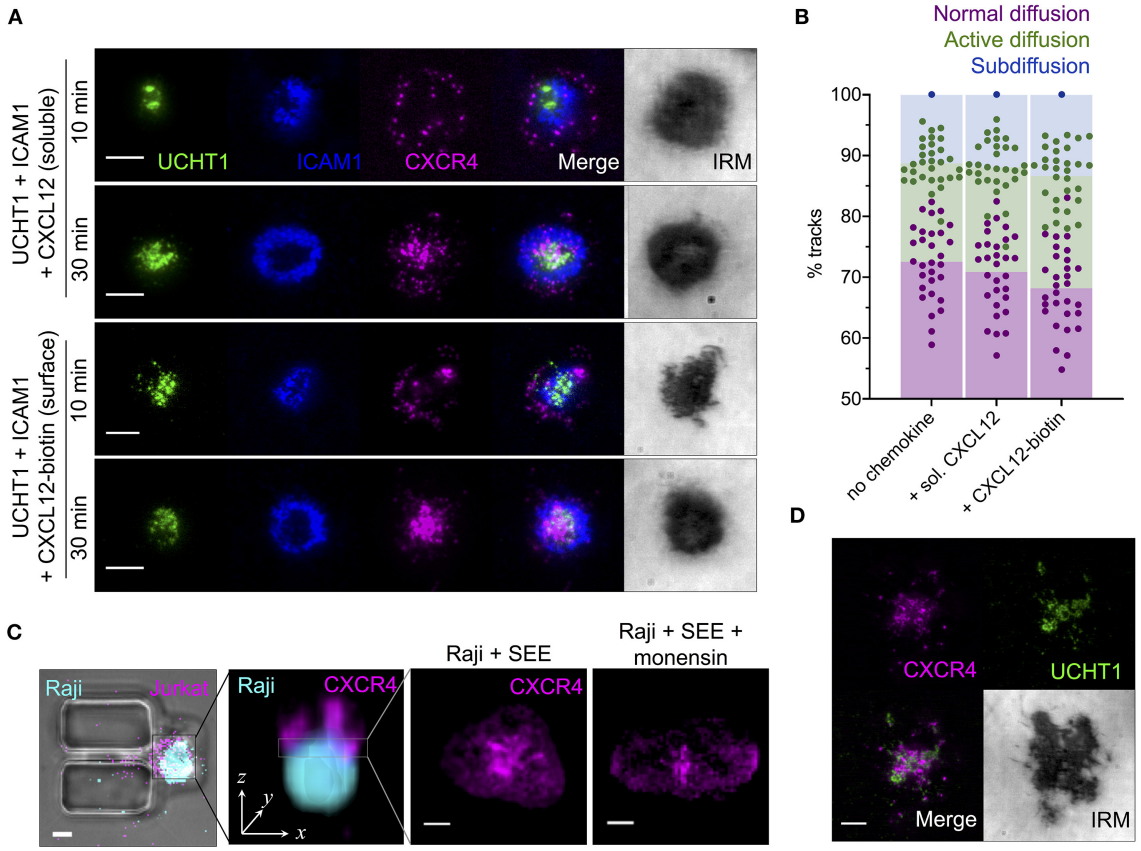

FIGURE 3 | Effects of chemokine ligation upon CXCR4 distribution and dynamics. **(A)** TIRFM examples of CXCR4-HaloTag-expressing $CD4^+$ T cell blasts interacting with activating SLB for 10 or 30 min in the presence of soluble or SLB-presented CXCL12. **(B)** Proportion of single-particle CXCR4-HaloTag tracks exhibiting normal, active, or confined/subdiffusion in cells on activating SLBs ± soluble/surface CXCL12 for 10 min. Each point represents an individual cell. **(C)** Example confocal microscopy image of a Raji-Jurkat conjugate within microfluidic vertical contact chamber (far left) and as three-dimensional z-stack (center left); then representative examples of CXCR4-HaloTag within the Raji-Jurkat interface in the presence or absence of protein transport inhibitor (monensin; right). **(D)** TIRFM example of CXCR4-HaloTag in a Jurkat E6.1 cell on activating SLB for 10 min. All scale bars are 5 μm. All pooled data represent a minimum of $n = 3$ independent donors.

(**Figure 3C**), suggesting that this process is independent of active secretion into the synapse by the APC.

CXCR4 Distribution Is Dependent on Ubiquitination in Its C-Terminal Domain

To investigate the molecular determinants of CXCR4 organization within the synapse, we generated five function-specific C-terminally HaloTagged CXCR4 mutants and transfected them into primary $CD4^+$ T (**Figure 4A**). These were: (1) deficient in G protein-coupling due to Arg-Asn substitution in the conserved DRY motif; (2) deficient in possible Tyr phosphorylation in the DRY motif due to a Try-Phe substitution; (3) deficient in all Ser/Thr phosphorylation in the C-terminal region due to substitution of all Ser/Thr residues with Ala; (4) C-terminally truncated after K314; and (5) deficient in C-terminal ubiquitination due to substitution of all Lys residues in the C-terminal domain with Arg. To avoid complicating factors from dimerisation with endogenous CXCR4, the native *CXCR4* gene was first disrupted in these cells by electroporation of an *in vitro*-generated ribonucleoprotein (RNP) complex consisting of the Cas9 nuclease and *CXCR4*-targetted guide RNA. $CXCR4^{-ve}$ cells were isolated by fluorescence-activated cell sorting prior to transfection with CXCR4 mutants (**Supplementary Figure 2A**).

Importantly, whereas CXCR4 mutants in which G protein-coupling or possible Tyr phosphorylation at the DRY motif were inhibited showed wild type-like synapses (**Figures 4B–E**), truncation of the CXCR4 C-terminal region led to impairment of CXCR4 accumulation at the cSMAC. The latter replicates the observed aberrant CXCR4 accumulation observed on WHIM syndrome-associate truncated CXCR4 (Kallikourdis et al., 2013). Interestingly, this was not replicated by Ala substitution of Ser/Thr residues within the C-terminal region, which should impair phosphorylation by GRKs and interaction with arrestins, but was observed for mutant receptors in which potential sites of Lys ubiquitination were replaced with Arg (**Figures 4B–E**). This indicates a role for CXCR4 ubiquitination in the events orchestrating correct receptor migration within the synapse beyond the endpoint of internalization. Single particle tracking of mutant receptors in $CXCR4^{-ve}$ cells showed diffusion behaviors correlating with this interfacial distribution. All forms of the receptor exhibited wild type-comparable normal, active, and sub diffusions at 10 min post activation except for the truncated and ubiquitin-deficient mutants, which underwent much less detectable active and subdiffusions (**Figure 4F**; **Supplementary Movie 3**).

Correct CXCR4 Organization Is Required for Maximal T Cell Responses to Activation

To determine the impact of impaired CXCR4 organization upon its contribution to T cell activation, we stimulated $CXCR4^{-ve}$ and *CD19*-targetted control primary $CD4^+$ cell blasts with anti-CD3/anti-CD28 beads and assessed expression of CD69, IL2, and IFNγ 6 h post-stimulation using flow cytometry. $CXCR4^{-ve}$ cells exhibited a moderate decrease in the fraction of cells positive for each of the three markers at 6 h (**Figure 4G**; **Supplementary Figure 2C**). Importantly, T cell activation could be partially restored to $CXCR4^{-ve}$ cells through transfection of untagged wild type CXCR4, or of the G protein interaction-deficient, pTyr-deficient, or pSer/pThr-deficient mutants, but not of either C-terminally truncated CXCR4 or the ubquitination-deficient mutant (**Figure 4G**). Activation potential was not fully restored under any circumstances, however this may be due to the reduced expression of the transfected receptors compared to endogenous CXCR4 in wild type cells (**Supplementary Figure 2B**). Effects of mutant receptor expression upon CXCL12-induced chemotaxis were assessed using a transwell migration assay, wherein a gradient was generated between growth media containing 0 and 0.25 μg/ml CXCL12 separated by a 5 μm-pore transwell membrane, and the movement of cells up this gradient in 1 h quantified. All forms of the receptor restored responsiveness to CXCL12 in this assay, with the exception of the G protein interaction-deficient mutant, though none induced substantial migration across transwell inserts coated with UCHT1 (**Figure 4H**), in line with previous observations that CXCL12 does not override TCR signaling (Bromley et al., 2000). C-terminally truncated CXCR4 has previously been reported to sensitize cells to CXCL12 to overcome TCR-derived arrest signals (Kallikourdis et al., 2013), however this may not be replicated here again due to due to the relatively low expression of the transfected receptors.

Gαi2 Undergoes Substantial Exclusion From the Synapse

Our data so far indicate a disconnect between the behavior of CXCR4 within the synapse and its conventional coupling to G proteins of the Gαi family. CXCR4-CCR5 complexes are known to cease signaling via Gαi-dependent pathways upon formation of the synapse (Molon et al., 2005), however the reasons for this are poorly understood. While this inhibits CXCL12-dependent migration, it will also inhibit basal ligand-independent Gαi-coupled signaling exhibited by CXCR4 (Mona et al., 2016). We therefore chose to examine the synaptic distribution of the most abundant T cell-expressed Gαi protein, Gαi2 (Foley et al., 2010). Primary human $CD4^+$ T cell blasts transfected with Gαi2 fused to SNAP-tag were examined with TIRFM on activating SLBs. Within fully formed synapses, Gαi2 underwent substantial redistribution to the dSMAC, with very clear negative correlation with TCR-UCHT1 distribution (**Figures 5A,B**; **Supplementary Figure 3A**). This redistribution of Gαi2 was evident during the early stages of IS formation before the cSMAC had fully coalesced (1-2 min; **Supplementary Movie 4**), indicating that this is not simply a product of molecular crowding, and did not appreciably change over the lifetime of the synapse (**Supplementary Figure 3A**). Using three-dimensional confocal microscopy, we observed that, relative to the rest of the cell, Gαi2 was substantially depleted across all but the extreme periphery of the contact in T cell blasts on activating SLB but not in resting cells on SLB containing ICAM1 alone (**Figures 5C,D**). Interestingly, T cell activation alone was not sufficient to drive maximal exclusion of Gαi2, as cells activated on SLBs containing only UCHT1 Fab' (**Figures 5C,D**) or on glass coated with anti-CD3/anti-CD28 antibodies (**Figure 5D**;

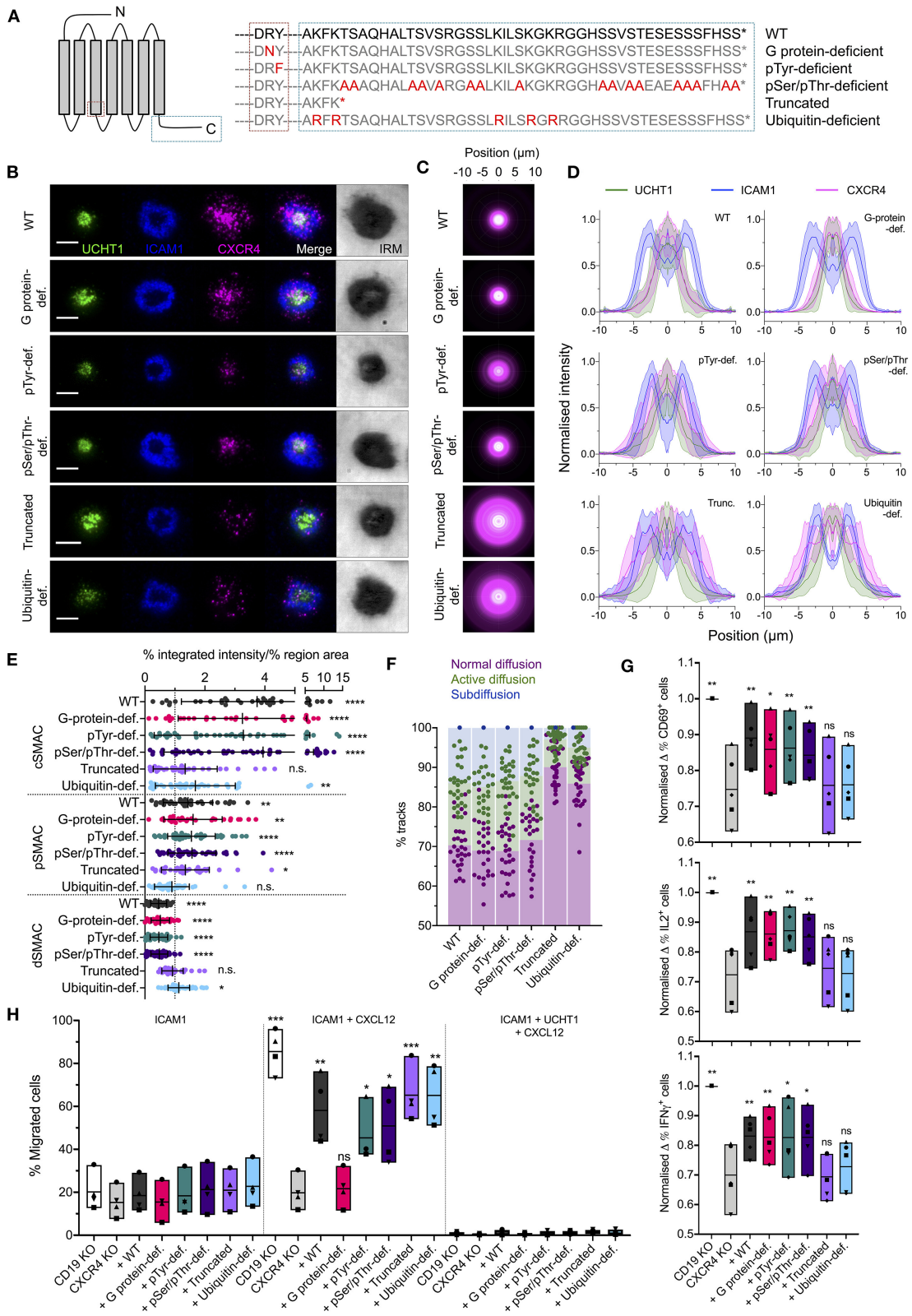

FIGURE 4 | Organization of functional CXCR4 mutants in the synapse. **(A)** Summary of CXCR4 mutants used. **(B)** TIRFM examples of wild-type and mutant CXCR4-HaloTag in $CD4^+$ T cell blasts on activating SLB for 30 min. Scale bars are 5 μm. **(C)** Radial averages of CXCR4-HaloTag intensity from > 30 cells for each mutant. **(D)** Cross-sectional normalized intensity profile of radial averages of all labeled proteins for each indicated condition. Plots are mean normalized intensity at

(Continued)

FIGURE 4 | each position ± std dev. **(E)** Relative enrichment of CXCR4-HaloTag mutant intensity in cSMAC, pSMAC, and dSMAC regions of the synapse on activating SLB. **(F)** Proportion of single-particle wild-type and mutant CXCR4-HaloTag tracks exhibiting normal, active, or confined/subdiffusion in cells on activating SLBs for 10 min. **(G)** Normalized change in CD69- (top), IL2- (middle), or IFNγ- (bottom) expressing cells upon incubation with anti-CD3/CD28 beads for 6 h. Cells are primary $CD4^+$ blasts, KO for either CD19 or CXCR4 and transfected with indicated CXCR4 mutants. Each symbol represents a different T cell donor. Box plots show mean, minimum, and maximum values; significance is relative to untransfected CXCR4 KO cells as assessed by two-tailed *t*-test. **(H)** Percentage of KO, mutant-transfected cells that migrate across transwell inserts coated with ICAM1 or ICAM1+UCHT1 in the presence or absence of CXCL12 in the lower chamber. Each symbol represents a different T cell donor. Significance is relative to untransfected CXCR4 KO cells as assessed by two-tailed t test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., not significant. All pooled data represent a minimum of $n = 3$ independent donors.

Supplementary Figure 3B) exhibited much less Gαi2 depletion. To achieve maximal exclusion, adhesion molecules (either ICAM1 or CD58) and the formation of SMACs were also necessary. Non-specific cell adhesion and activation (Santos et al., 2018) on poly-$_L$-lysine-coated glass did not induce Gαi2 exclusion (**Figure 5D**; **Supplementary Figure 3B**), indicating that both TCR triggering and engagement of either ICAM1 or CD58 are required for Gαi2 redistribution. TIRF-SIM of Gαi2 within the synapse revealed strong nanoscale exclusion from TCR-UCHT1-enriched domains (**Figures 5E,F**; **Supplementary Figure 3C**) even for the minority of residual Gαi2 within the cSMAC.

We next used giant unilamellar vesicles (GUVs) to activate Gαi2-SNAP-tag-transfected $CD4^+$ T cell blasts and observed the distribution of Gαi2 with confocal microscopy. GUVs are analogous to SLBs except that they exist as spherical vesicles 10–100 μm in diameter, which can be loaded with His-tagged proteins via Ni-NTA-functionalised lipids (Jenkins et al., 2018). This allows x-y cross-sectional images to be captured at the equatorial plane of T cell-GUV contacts that is not possible with the SLB approach. As expected, Gαi2 was largely excluded from contacts between T cell blasts and GUVs presenting UCHT1 Fab' and ICAM1 (**Figures 5G,H**). CD45, a classical example of IS-excluded molecules (Dustin, 2014), was also excluded, whereas UCHT1 was enriched in the contact. Disruption of the synapse 15 min after formation by acute addition of inhibitors of Src kinase activity (PP2), or polymerisation of actin (latrunculin A) or microtubules (nocodazole) led to a loss of Gαi2 exclusion from the contact even though CD45 exclusion was still evident (**Figures 5G,H**). 18 h pre-treatment with PTx, which inhibits Gαi activity and coupling to GPCRs, did not impair Gαi2 exclusion. These data indicate that the redistribution of Gαi2 upon formation of the synapse is dependent on active cytoskeletal processes and continuous TCR signaling, and not upon active coupling to GPCRs. This opens the possibility that the inversely directional movement of CXCR4 and Gαi2 may be a deliberate mechanism by T cells to prevent CXCR4-Gαi coupling in response to TCR triggering, and hence to dampen pro-migratory CXCR4 signaling.

Numerous GPCRs Exhibit Modulatory Functions on T Cell Activation

Since Gαi-coupled signaling is a common pathway for many T cell-expressed GPCRs, we questioned whether many such receptors might experience altered signaling within the synapse due to the redistribution of Gαi2. Modulatory function in T cell responses has been reported for several GPCRs (e.g., Contento et al., 2008; Linnemann et al., 2009; Oda et al., 2013; Laufer et al., 2018), however in most cases this has been examined in the context of receptor ligation rather than inherent ligand-independent activity, and no exhaustive screen of GPCR contributions to T cell activation has thus far been performed. We therefore set out to determine which, if any, GPCRs commonly expressed in $CD4^+$ T cells influenced cellular responses to activation in the absence of exogenous receptor ligation. Using publicly available whole genome RNA sequencing (RNA-seq) data from the BluePrint consortium (Expression Atlas: E-MTAB-3827) we identified all GPCRs expressed to a level above five fragments per kilobase exon per million reads mapped (FPKM) in either primary total or effector memory $CD4^+$ T cells. This identified 28 GPCRs, the majority of which were members of the *Rhodopsin* family, with many known to couple to Gαi/o family members (**Supplementary Table 2**). The highest FPKM belonged to CXCR4, however many other receptors also exhibited strong expression. This panel of receptors did not include a number of known influencers of T cell activation, including adenosine (Linnemann et al., 2009) and adrenergic (Fan and Wang, 2009) receptors, most likely because they are not highly abundant at the mRNA level or are inconsistently expressed. Although the ligand-dependent effects of these receptors are well-reported, we chose not to pursue them here as their low copy number reduced the likelihood of inherent ligand-independent effects. We cannot, however, exclude the possibility of ligand-independent effects of low-transcript GPCRs not investigated here.

Using the Cas9 RNP approach described above, the genes encoding candidate receptors, as well as those encoding CD3δ and CD28, were individually disrupted in resting human $CD4^+$ T cells isolated from blood (guide sequences given in **Supplementary Table 2**). These were then divided into two populations, one of which was kept in resting culture without additional IL2, and the other was blasted for 3 days with anti-CD3/anti-CD28 beads and cultured in the presence of 100 U/ml IL2. Seven days post-transfection, all cells were activated either with anti-CD3/anti-CD28 beads or in co-culture with donor-matched APCs loaded with titrated amounts of SEE. For the activation of resting cells (which we consider to be predominantly naïve given extended culture without IL2, selecting against resting effector cells), activated monocyte-derived DCs (moDCs) were used as APCs, whereas for blasted cells B cells were used. Expression of CD69, IL2, and (for blasted cells only) IFNγ 6 h post-activation was assessed using flow cytometry (**Supplementary Figure 4A**) and normalized to the response observed in control cells transfected with RNP

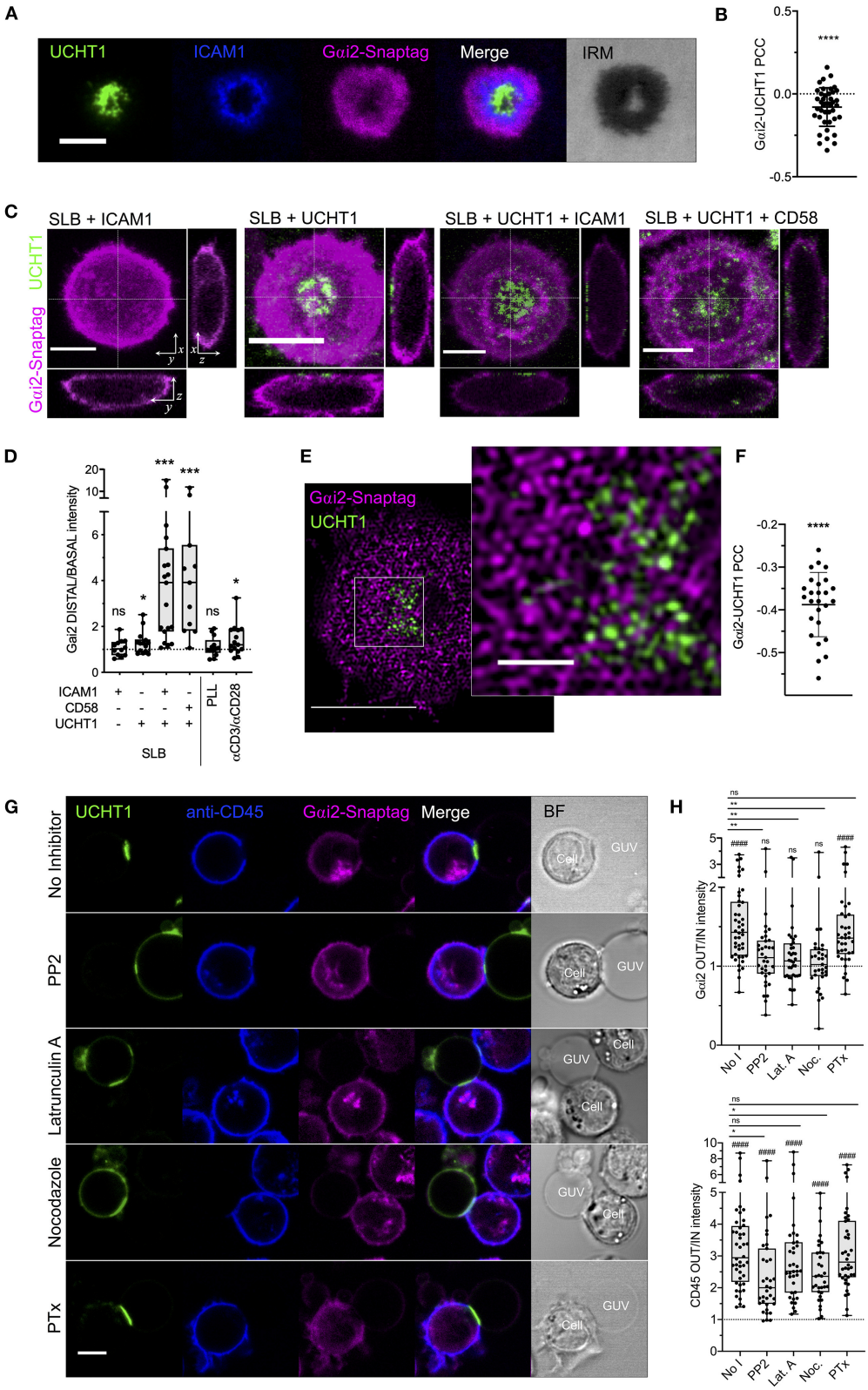

FIGURE 5 | Gαi2 distribution within the synapse. **(A)** TIRFM example of Gαi2-SNAP-tag in a CD4$^+$ T cell blast on activating SLB for 10 min. **(B)** PCC values for Gαi2 vs. UCHT1 in TIRF-imaged cells. Each point represents a single cell. Significance is shown relative to a value of 0 assessed with a one sample, two-tailed *t*-test. **(C)** Max-intensity projections and orthogonal views of confocal microscopy z-stacks of CD4$^+$ T cell blasts on SLB presenting ICAM1, UCHT1, ICAM1 + UCHT1, or CD58 *(Continued)*

FIGURE 5 | + UCHT1. **(D)** Ratio of Gαi2-SNAP-tag intensity at the distal vs. basal membranes for $CD4^+$ T cell blasts on the indicated SLB compositions or glass surfaces coated with PLL or anti-CD3/CD28 antibodies. Box and whiskers show mean, maximum, minimum, upper, and lower quartiles. Significance is shown relative to a value of 1 assessed with a one-sample, two-tailed *t*-test. **(E)** TIRF-SIM example of Gαi2-SNAP-tag in a $CD4^+$ T cell blast on activating SLB for 10 min. **(F)** PCC values for of Gαi2 vs. UCHT1 in TIRF-SIM-imaged cells. Significance is shown relative to a value of 0 assessed with a one sample, two-tailed *t*-test. **(G)** Confocal microscopy images at the equatorial plane of Gαi2-SNAP-tag-expressing, anti-CD45-stained $CD4^+$ T cell blasts interacting with ICAM1 + UCHT1-bearing GUVs in the presence of indicated inhibitors. **(H)** Gαi2-SNAP-tag and CD45 intensities outside vs. inside cell-GUV contact regions. Significance vs. a fixed value of 1 as assessed by a one-sample two-tailed *t*-test is represented with hashes (####$p < 0.0001$). Significance between samples indicated by bars was assessed with a two-tailed *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s., not significant. All scale bars are 5 μm except for zoomed inserts (1 μ m). All pooled data represent a minimum of $n = 3$ independent donors.

complexes targeting *CD19*. Cytokine retention was enhanced by the addition of a monensin-containing protein transport inhibitor 2 h after the start of activation. The efficacy of gene disruption was confirmed through TIDE (Tracking of Indels by DEcomposition) analysis of genomic DNA isolated from blasted cells 7 days post-transfection (Brinkman et al., 2014). This reliably reported approximate disruption efficiency for both the blasted and resting populations (**Supplementary Figures 4B–D**). Cells were not selected for receptor knockout, so the cells used in stimulation experiments represented a population of majority homozygous knockout with a minority of wild type and heterozygous partial knockouts.

Knockout of several GPCRs had a significant effect on T cell responses to activation by SEE-loaded APCs (**Figures 6A,B,E**). This was most strongly evident in the naïve $CD4^+$ population, wherein disruption of 12 GPCR genes significantly altered all measured responses, compared to four in blasted T cells (**Figure 6E**). This is perhaps unsurprising given the increased dependence of naïve T cells for costimulation during activation compared to effector cells. The genes with the greatest effects on responses to activation were typically those with the greatest transcript abundance in the RNA-seq data (**Figure 6E**), most substantially *CXCR4*, *GPR183*, *S1PR1*, *CCR7*, *P2RY8*, *PTGER4*, and *LPAR6*. This correlation was not absolute, however, as disruption of *LPAR2* also exhibited effects on response to activation despite having only a low associated FPKM. Similarly, several genes with relatively high associated FPKM values exhibited no clear effect, including *P2RY10* and *CCR4*.

Although several receptors only appeared to influence responses in naïve cells, this was most striking for GPR183, which had a very significant effect in naïve cells but no clear effect in blasted cells. Indeed, knockout of both GPR183 and S1PR1 had an unexpectedly dramatic impact on naïve T cell responses, with a greater loss of response than for knockout of CD28. The EC50 values relative to SEE concentration for GPR183 and S1PR1 knockouts were 1–2 orders of magnitude greater than the control cells, suggesting a possible central role in signal amplification from the TCR and/or CD28.

Responses to activation with anti-CD3/anti-CD28 beads were typically less sensitive to GPCR knockout than that with SEE-loaded APCs (**Figures 6C,D,E**). In blasted cells, only CXCR4 exhibited a consistent contribution to all three activation markers, with S1PR1 also having a significant effect on CD69 and IL2 responses. Naïve cells again showed greater sensitivity to GPCR knockout, though of the 12 receptors with consistent contributions in APC-mediated activation, five (P2RY8, S1PR4, CD97, PTGER2, and GPR174) failed to exhibit significant effects upon CD69 and/or IL2 responses following bead-mediated activation.

In all cases, the effects observed were not due to altered expression of either TCR or CD28 in the knockout cells, as these were unaffected by GPCR disruption (**Supplementary Figure 4E**). The only evident difference in the resting state of any knockout cells was the basal CD69 expression in S1PR1-deficient cells, which was greatly enhanced relative to all other cells (**Supplementary Figures 4E,F**). This is not unexpected since S1PR1 and CD69 undergo reciprocal negative regulation due to direct physical interactions (Bankovich et al., 2010). The effects of S1PR1-knockout on CD69 responses are therefore more difficult to interpret, however the fact that effects were also observed for IL2 and IFNγ responses increases confidence that these effects are genuine.

To examine the potential impact of GPCR knockouts on T cell effector function, we quantified release of CD40L- and TCRαβ-containing synaptic ectosomes from $CD4^+$ blasts. Following disruption of *CD28*, *CCR7*, *CXCR4*, *GPR183*, *LPAR6*, *PTGER4*, *S1PR1*, or *CD19*, $CD4^+$ blasts were incubated with bead-supported lipid bilayers (BSLBs) presenting ICAM1 at 200 molecules/μm^2, CD40 at 20 molecules/μm^2, and UCHT1 Fab' at titrated densities from 0 to 2,000 molecules/μm^2 (Saliba et al., 2019). These are equivalent to SLBs but formed around silica beads, allowing transferred proteins to be retained and quantified. After 90 min BSLBs were detached from cells, stained for CD40L and TCRαβ and assessed with flow cytometry. Among GPCR and CD28 knockouts, no significant differences were observed in the transfer of synaptic ectosomes containing CD40L and TCRαβ to BSLBs, indicating that these had no participation in the delivery of helping factors by $CD4^+$ T cells (**Figures 6F,G**; **Supplementary Figure 4G**).

GPCR Dynamics Do Not Correlate With Costimulatory Potential

Given the evident effects of several tested GPCRs on T cell responses, we examined the distribution and dynamics of a subset with the aim of identifying any commonalities with CXCR4. We chose two receptors that showed costimulatory function in both blast and naïve cells (CCR7 and S1PR1), one that had an effect only in naïve cells (GPR183), one that had no evident effect (CXCR3), and one that is not typically expressed in conventional T cells (CXCR5—normally restricted to follicular helper T cells). All five receptors were transfected as C-terminal HaloTag fusions into blasted primary $CD4^+$ T cells and assessed by TIRFM on

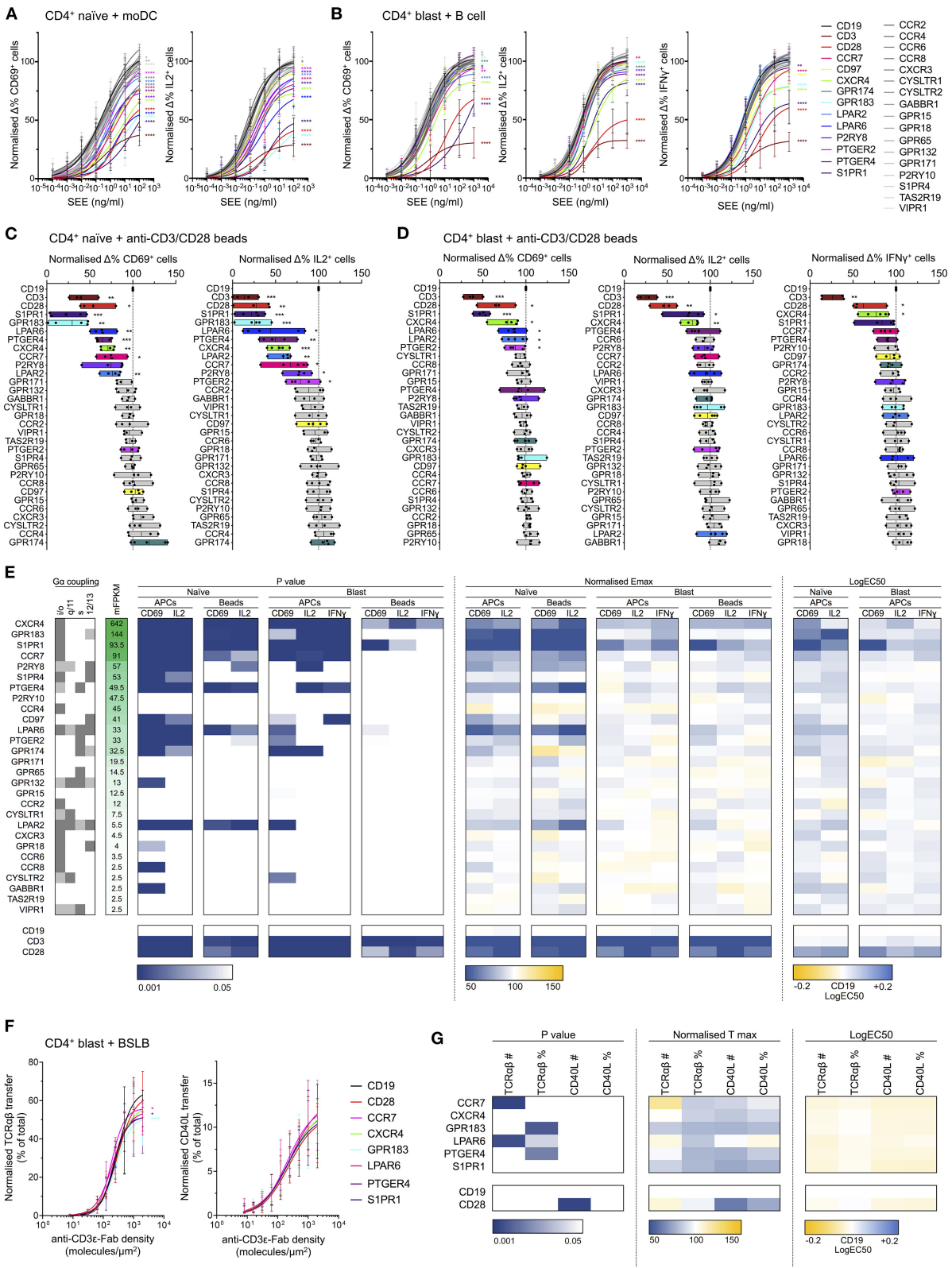

FIGURE 6 | Effects of GPCR knockout in activation of naïve and blasted CD4+ T cells. **(A)** Normalized change in CD69+ (left) and IL2+ (right) naïve CD4+ T cells incubated with moDCs loaded with titrated concentrations of SEE. Plots show mean ± std dev., with best-fit non-linear response curves for each target. Datasets are colored according to target (key far right), with non-gray sets used only for targets exhibiting significant effects in both the CD69 and IL2 responses. Significance

(Continued)

FIGURE 6 | relative to the CD19 target data was assessed using an extra sum-of-squares F test and is indicated for all significant ($p < 0.05$) datasets. **(B)** Normalized change in $CD69^+$ (left), $IL2^+$ (center), and IFNγ (right) blasted $CD4^+$ T cells incubated with B cells loaded with titrated concentrations of SEE. Data are represented as in **A**. **(C)** Normalized change in $CD69^+$ (left) and $IL2^+$ (right) naïve $CD4^+$ T cells incubated with anti-CD3/CD28 beads. Boxes show mean, minimum, and maximum values, significance is shown relative to CD19 as assessed with a two-tailed *t*-test. Non-gray datasets are colored as in **A**. Each point represents a different T cell donor. **(D)** Normalized change in $CD69^+$ (left), $IL2^+$ (center), and IFNγ (right) blasted $CD4^+$ T cells incubated with anti-CD3/CD28 beads. Data are represented as in **C**. **(E)** Summarized GPCR knockout screen data. Calculated F test *p*-values, normalized Emax, and logEC50 for all assays are represented as a heatmap, colored according to the corresponding scales below. For T cell-APC assays, Emax was derived as the value of the fitted response curve at the highest SEE concentration. Reported G protein coupling for each GPCR is shown as dark gray (primary coupling), light gray (secondary coupling), or white (no coupling), as listed in the GPCR database (gpcrdb.org). Receptors are ordered according to mean FPKM (mFPKM) values in RNA-seq from primary total or effector memory $CD4^+$ T cells (E-MTAB-3827). **(F)** Normalized transfer of TCRαβ (left) or CD40L (right) from $CD4^+$ blasts to BSLBs presenting ICAM1, CD40, and titrated densities of UCHT1, as a percentage of total cellular TCRαβ/CD40L. Data are represented as in **A**. **(G)** Summarized BSLB transfer assay data. Calculated F test p values, normalized Tmax, and logEC50 for all assays are represented as a heatmap, colored according to the corresponding scales below. Values are shown for raw amount of protein transferred (TCRαβ# or CD40L#) or as a percentage of total cellular protein (TCRαβ% or CD40L%). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All pooled data represent a minimum of $n = 4$ independent donors.

activation SLB. CCR7, GPR183, and S1PR1 exhibited distribution at 10 min that closely resembled that of CXCR4—with substantial depletion from the central regions of the synapse and enrichment in the dSMAC (**Figures 7A,B**). Conversely, CXCR3 and CXCR5 showed much greater accumulation in the cSMAC at 10 min, but less extensively so than CXCR4 at 30 min. The distribution of all receptors remained broadly unchanged between 10 and 30 min (**Figures 7A,B**), in stark contrast to CXCR4. Interestingly, despite the two different overall distributions across the receptors, when assessed by single-particle tracking all five demonstrated comparable dynamics and spatial distribution of diffusion types (**Figures 7C,D**; **Supplementary Movie 5**). This was highly comparable to that observed for CXCR4, with a majority of freely diffusing tracks that were restricted largely to the distal regions of the synapse; actively diffusing tracks moving centrally; and tracks undergoing subdiffusion predominantly in the cSMAC. When investigated by TIRF-SIM, all five receptors exhibited segregation from regions of TCR enrichment (**Figures 8A,B**), and no detectable nanoscale correlation with F-actin (**Figures 8C,D**), again in line with the organization observed for CXCR4. There was no evident correlation between receptor dynamics or nanoscale organization and reported impact on T activation. Given this common behavior, it seems likely that the observed differences in gross receptor distribution (**Figures 7A,B**) are the result of differences in concurrent receptor trafficking—i.e., internalization from or endocytic deliver to the synapse.

DISCUSSION

In this study we observe that CXCR4, as a key GPCR of interest, undergoes active reorganization within the synapse, characterized by initial exclusion to the periphery followed by active transport toward the center (**Figure 9A**). The correlation of CXCR4 with F-actin-enriched regions is consistent with a previous report of CXCR4-drebrin-actin interactions upon TCR triggering (Pérez-Martínez et al., 2010), however our observation of freely diffusing CXCR4 in these regions indicates that such interactions are likely not sufficiently stable to fully restrict receptor movement. We do not observe nanoscale correlation of CXCR4 (or indeed any GPCR here studied) with the TCR, arguing against the formation of stable CXCR4-TCR complexes. Previous reports of such complexes have been based primarily on resonance energy transfer experiments or diffraction-limited imaging (Kumar et al., 2006; Trampont et al., 2010), which could also be consistent with increased crowding of CXCR4 and TCR in the cSMAC without the need for direct interaction. Nonetheless, we cannot exclude the possibility of short-lived interactions that transiently impact signaling during microcluster migration. We also cannot comment on how CXCR4-TCR distribution may vary according to TCR-pMHC stability, and it is possible that stable complexes may be induced by TCRs of a particular affinity.

Using single-particle tracking, we observed substantial cell activation-dependent changes in CXCR4 dynamics characterized by a large fraction of freely diffusing receptors with smaller populations of actively migrating and subdiffusing molecules. Such behavior is similar to that of the TCR, except that we observed no obvious formation of migrating CXCR4 microclusters, and that the time scale to accumulation in the cSMAC was much slower than that of the TCR. The majority of normally diffusing CXCR4 is in contrast to a previous study reporting only ∼11% of freely diffusing receptors on fibronectin-coated glass (Martínez-Muñoz et al., 2018). This difference could arise from distinct behavior of CXCR4 on immobile fibronectin vs. mobile ICAM1. In both studies the majority of tracked receptors remained mobile, but on fibronectin ∼78% of these remained within 200 nm over the >2 s life of the track. A marginal increase in CXCR4 mobility on ICAM1 may have allowed this large fraction of receptors to exhibit normal diffusion over the >1.5 s track length acquired in this study.

Although comparisons of our single-particle diffusion data across conditions is valid, certain caveats should be considered when directly interpreting frequencies of different species. Due to the nature of diffraction-limited imaging, clustered receptors will be underreported in the tracking data as they will be detected as single spots. In the case of CXCR4, this seems most likely for subdiffusing receptors near the center of the synapse. Similarly, new fast-diffusing spots are more likely to enter the imaging field during the course of image capture, again causing under-representation of slow-moving spots in the data. Conversely, faster moving spots are more likely to leave the imaging field within 30 frames, and are more difficult to accurately connect, which will reduce their representation in the reported tracks. These effects mean the absolute receptor proportions described herein should be interpreted with care.

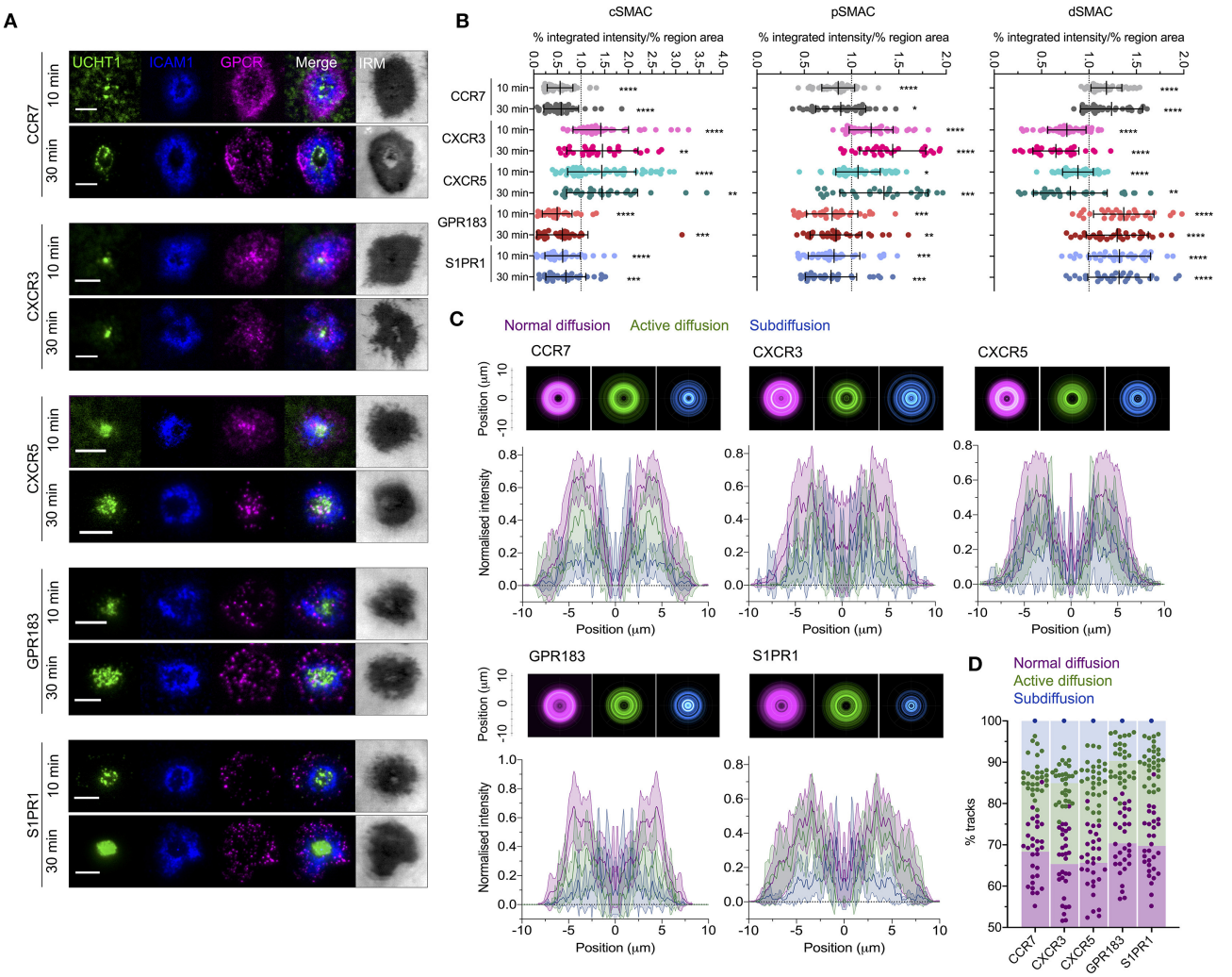

FIGURE 7 | Distribution and dynamics of additional GPCRs within the synapse. **(A)** Representative TIRFM examples of HaloTag-fused GPCRs in transfected CD4$^+$ T cell blasts interacting activating (ICAM1 + UCHT1) SLB for 10 or 30 min. **(B)** Relative enrichment of GPCR-HaloTag intensities in cSMAC, pSMAC, and dSMAC regions of the synapse on activating SLB. Values are expressed as the percentage of total intensity within a region divided by the percentage of the total IRM-defined area that constitutes that region. Significance is shown relative to a value of 1 assessed with a one-sample, two-tailed *t*-test. Each point represents an individual cell; bars are mean ± std dev. **(C)** Radial averages of single-particle track locations for all GPCRs and all diffusion types in CD4$^+$ blasts on activating SLBs for 10 min (top), and cross-sectional normalized intensity profiles of those averages (bottom). Plots are mean normalized intensity at each position ± std dev. **(D)** Proportion of different GPCR-HaloTag tracks exhibiting normal, active, or confined/subdiffusion in CD4$^+$ blasts on activating SLBs for 10 min. All scale bars are 5 μm except for zoomed inserts (1 μ m). All pooled data represent a minimum of $n = 3$ independent donors. $^*p > 0.05$, $^{**}p > 0.01$, $^{***}p > 0.001$, $^{****}p > 0.0001$.

We failed to detect any significant effects of CXCL12 ligation on CXCR4 organization or dynamics. This was surprising given the effects of CXCL12 on CXCR4 ubiquitination (Marchese and Benovic, 2001) and internalization (Haribabu et al., 1997), however it appears that TCR signaling supersedes the ordinary effects of CXCL12, as previously described (Bromley et al., 2000). More unexpected was the possible dependence on CXCR4 ubiquitination. Previous studies have suggested that regulation of CXCR4 behavior in the synapse is mediated by GRK-dependent phosphorylation of the C-terminus (Dinkel et al., 2018) or on association with arrestins (Fernández-Arenas et al., 2014). We failed to see clear effects of C-terminal Ser/Thr-Ala substitution (which will also block arrestin binding) on either CXCR4 dynamics or overall organization, or on its costimulatory potential. CXCR4 distal from the T cell-APC contact has previously been observed to be redirected to the synapse in an arrestin-dependent manner (Fernández-Arenas et al., 2014), which we did not assess, opening the possibility that CXCR4 undergoes first arrestin- then ubiquitin-dependent regulation at different stages of its delivery and organization. This is particularly interesting since CXCR4 ubiquitination is partially dependent on phosphorylation of the C-terminal domain (Marchese and Benovic, 2001), which raises the question of why mutation of phosphorylation sites did not have the same

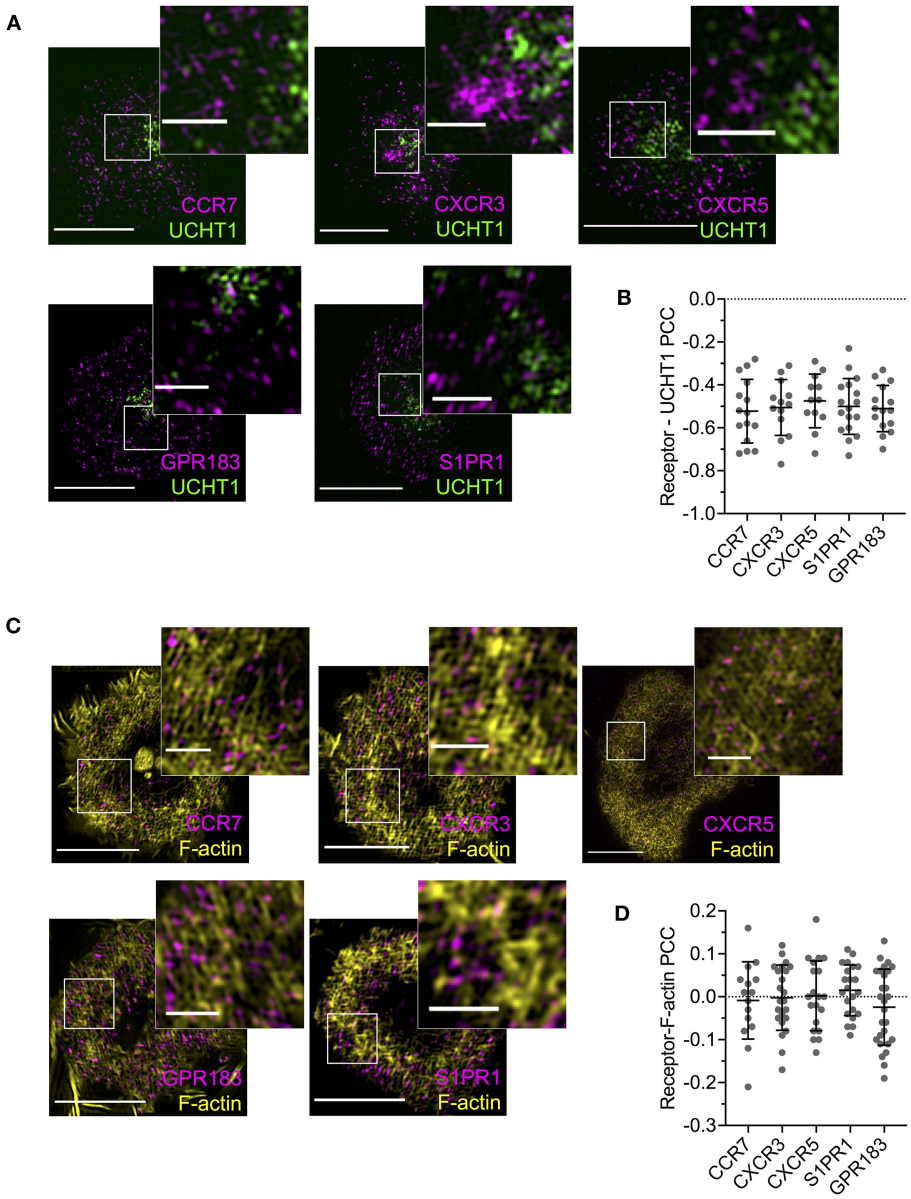

FIGURE 8 | Nanoscale distribution of additional GPCRs. **(A)** TIRF-SIM examples of GPCR-HaloTag-expressing CD4$^+$ T cell blasts on activating SLBs for 10 min. Inserts correspond to white boxes. **(B)** Pearson's correlation coefficient (PCC) values for GPCR-HaloTag constructs vs. UCHT1 in TIRF-SIM-imaged cells. **(C)** TIRF microscopy examples of F-actin and GPCR-HaloTag constructs in CD4+ T cell blasts on activating SLB for 10 min. **(D)** Pearson's correlation coefficient (PCC) values for GPCR-HaloTag constructs vs. F-actin in TIRF-SIM-imaged cells. All scale bars are 5 μm except for zoomed inserts (1 μm). All pooled data represent a minimum of $n = 3$ independent donors.

effect as mutation of ubiquitination sites. It is still possible that such mutation affects CXCR4 behavior in a way that we were not examining. Moreover, it is possible that C-terminal Lys-Arg substitution has impacts independent of ubiquitin. Nonetheless, the ability of both the C-terminally truncated and ubiquitin-deficient mutants to restore migration but not full responsiveness to activation indicates that the reduction of activation in CXCR4^{-ve} cells is not a product of reduced cell mobility.

Ubiquitination of GPCRs is subject to complex, receptor-specific regulation (Kennedy and Marchese, 2015), and although most commonly described in the context of receptor internalization, it is not a pre-requisite for GPCR removal from the plasma membrane (Kang et al., 2014). Ubiquitination of CXCR4 is required for sorting into intraluminal vesicles via the ESCRT (endosomal sorting complex required for transport) pathway (Marchese, 2014), which also regulates the sorting of proteins into synaptic ectosomes (Saliba et al.,

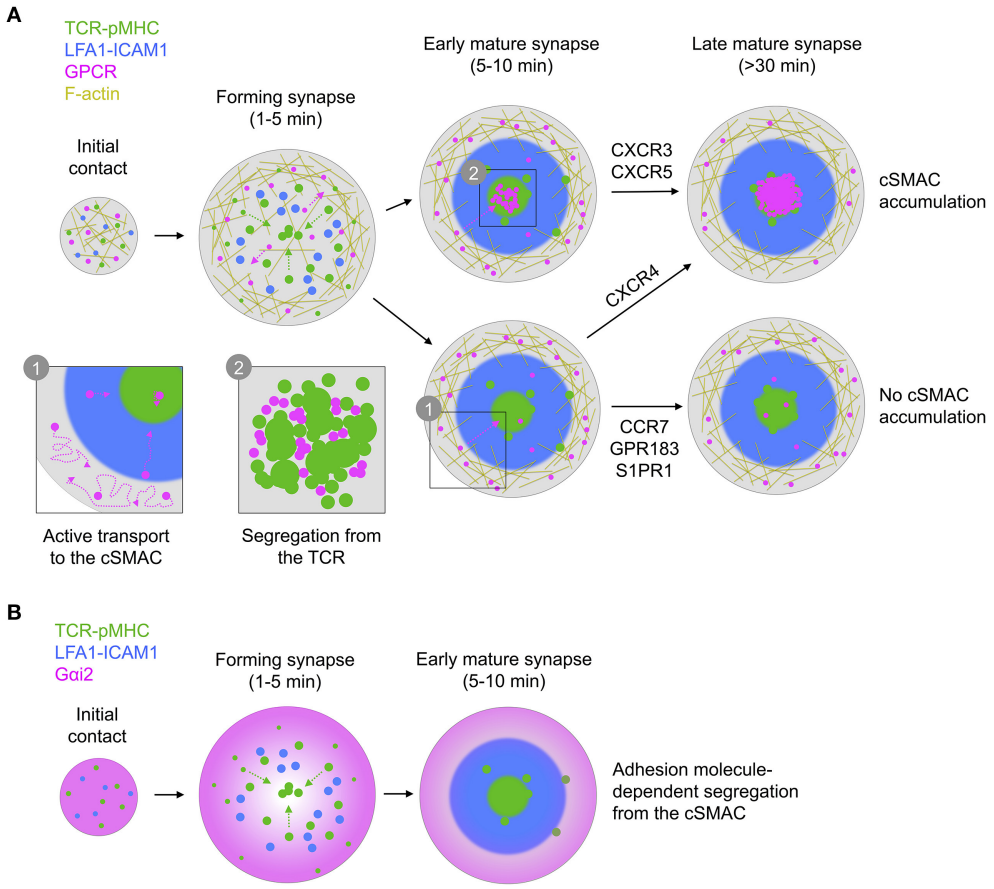

FIGURE 9 | Graphical summary of receptor/G protein distribution and dynamics. **(A)** All GPCRs examined exhibit similar dynamics, characterized by free diffusion at the periphery of the synapse with active transport to the center. This leads to different overall distribution, with either no, early, or late accumulation in the cSMAC, dependent on receptor. In all cases, receptors in the cSMAC are segregated from TCR clusters. **(B)** Gαi2 is depleted from the center of the contact even before full formation of the cSMAC, with this becoming more pronounced during maturation of the synapse.

2019). Irregular receptor accumulation of ubiquitin-deficient and C-terminally truncated CXCR4 could emerge from defects in correct receptor trafficking, through endocytosis and/or incorporation into synaptic vesicles, though it is not clear how this impacts CXCR4 signaling. It is well established that GPCR signaling does not immediately terminate upon endocytosis (Weinberg and Puthenveedu, 2019), and removal of CXCR4 from the synaptic plasma membrane could serve to prevent G protein-mediated signaling while maintaining signaling via arrestins or other partners. Arrestins help coordinate correct TCR trafficking to and from the synapse (Fernández-Arenas et al., 2014) and so endocytosed CXCR4 (or other GPCRs) may contribute to this regulation. Alongside endocytosis, T cells release substantial numbers of synaptic vesicles (Choudhuri et al., 2014), and it is possible that CXCR4 or other GPCRs are incorporated. Previous proteomic analysis of the composition of synaptic vesicles only identified CD97 as the sole GPCR enriched in such vesicles (Saliba et al., 2019), however this does not preclude incorporation of others under different circumstances. GPCRs can be incorporated into extracellular vesicles at cilia (Nager et al., 2017), which share many close similarities with the immunological synapse (Cassioli and Baldari, 2019), so such a process in activated T cells is not implausible. In such a case, CXCR4 release would likely serve to terminate migratory signaling and possibly to act as local scavengers of chemokine ligands. Our observations can be explained without the need for CXCR4 release in vesicles, and the lack of correlation with the TCR may argue against this, however their enrichment at the cSMAC would bring them into close proximity with the vesicular export machinery.

Another key question surrounds the apparent loss of coupling to Gαi following recruitment to the immunological synapse (Molon et al., 2005). We report that the predominant Gαi protein in T cells, Gαi2, is actively excluded from most of the synapse rapidly upon its formation and remains partially depleted throughout its lifetime (**Figure 9B**). Given the active recruitment of CXCR4 and other GPCRs into the synapse, this could be one contributing reason for the loss of Gαi-coupling, however several other factors could also influence this [e.g., Src kinase-mediated phosphorylation of the DRY motif (Hauser

et al., 2016)]. We were unable to achieve reasonable expression of tagged Gαq and so cannot compare the distribution of this or other G proteins with Gαi2. The nature of Gαi2 exclusion is also unclear, although we observe that is dependent on formation of an active synapse with engagement of adhesion molecules, and on both actin and microtubule integrity. G proteins interact with microtubules (Schappi et al., 2014), although Gαi2 distribution consistent with microtubule interactions was not clearly evident in our TIRF-SIM experiments. There are several other possible mechanisms for Gαi2 depletion in the synapse—including localized depalmitoylation (Wedegaertner, 2012), lipid packing-induced segregation (Oh and Schnitzer, 2001), or association with actively excluded partners—however the present data do not provide insight into which may be correct.

The findings that CXCR4 appears to segregate from both TCR and Gαi2, and that costimulatory potential can be recovered by receptor mutants deficient in phosphorylation at the C terminus or DRY motif raise the question of how it might deliver co-stimulatory signals within the IS. We did not examine the relative organization of CXCR4 with other components of the T cell activation process, nor the overall features of the synapse in the presence/absence of costimulation through CXCR4. It is therefore possible that CXCR4 may colocalise with costimulatory receptors (e.g., CD28) or adaptor proteins (e.g., LAT) and increase their activity by corecruitment of Lck; or by affecting global organization of the synapse—e.g., increasing integrin accumulation. Both of these models would be consistent with our observations of greater CXCR4- (and GPCR-)dependence in naïve T cells, which are more reliant on both CD28 costimulation and stable synapse formation. CXCR4 is also known to interact with other GPCRs, such as CCR5 (Contento et al., 2008; Felce et al., 2019), and it is also possible that it is able to influence signaling from these receptors within in the IS. Regardless of mechanism, we observe a clear independence on ligation, and so it seems likely this can be regulated primarily by overall CXCR4 expression.

Through a knockout screen of the 28 GPCRs in primary $CD4^+$ T cells we observed a significant contribution of several receptors upon characteristic responses to activation. In some cases, particularly S1PR1 and GPR183, the magnitude of these effects was unexpectedly large, especially in naïve cells. This is in keeping with the greater need for costimulation in these cells (Dubey et al., 1996), however it could also relate to differences in underlying receptor expression as this was not assessed. It could also be due to differences in their accompanying APCs (moDCs for naïve, B cells for blasts), however given that differences were also observed when both were activated use anti-CD3/CD28 beads this seems unlikely. T cell stimulation by APCs was markedly more sensitive to GPCR knockout than stimulation by beads, with several receptors reporting effects in the former but not the latter. This could be due to stronger activation by beads vs. APCs, thereby masking more subtle contributions from GPCRs. Alternatively, the presence of APC-derived factors, both secreted ligands and cell-surface proteins, may be required for costimulation in some cases. Knockout of CD97, e.g., significantly impacted activation of naïve T cells by moDCs but not by beads, which may be due to its capacity to bind integrins (Wang et al., 2005) not present on beads. For GPCRs exhibiting effects on both APC- and bead-mediated stimulation, the presence of APC-derived ligands seems unlikely to have fully contributed to the observed effects. However, the presence of T cell-endogenous ligands cannot be discounted—particularly in the case of the sphingosine-1-phosphate and lysophosphatidic acid receptors, which are believed to engage their lipid ligands directly from the local membrane (Hanson et al., 2012). Nonetheless, even if endogenous ligation of such GPCRs is required for effects on T cell activation, this can be considered the baseline state of these receptors in these cells, and hence they would still possess intrinsic influence on cellular responses. This may be reflected in the large proportion of identified receptors that recognize lipid or lipid-soluble ligands (LPAR2, LPAR6, GPR174, GPR183, PTGER2, PTGER4, S1PR1, S1PR4), however this is broadly overshadowed by the stronger correlation with expression level. In general, those receptors identified as influencing T cell activation have a range of other known functions. Several classically mediate cell migration—e.g., chemokine (CXCR4, CCR7), sphingolipid (S1PR1, S1PR4), phospholipid (LPAR2, LPAR6), or oxysterol (GPR183) receptors – whereas others mediate sensitivity to proinflammatory (e.g., PTGER2, PTGER4) and/or immunoregulatory (e.g., P2RY8) ligands. All are members of the *Rhodopsin*-family of GPCRs with the sole exception of the *Adhesion*-family receptor CD97, and a large majority are known to couple preferentially to Gαi proteins.

Interpretation of these data must include a number of considerations. Firstly, knockout of each gene was targeted using a single guide RNA, and although each was selected for minimal off-target effects (**Supplementary Table 2**) we cannot fully exclude the possibility of contributions from other affected genes. Secondly, CD28 was engaged in all experiments and so the observed effects could arise from influences on either proximal or downstream signaling from TCR, CD28, or both. CD28 was not engaged when cells were stimulated with BSLBs, which may explain the lack of reported knockout effects on protein transfer. Alternatively, since the effects of GPCR knockout were typically more significant in naïve than blasted T cells, it is possible that ligand-independent GPCR costimulation disproportionately influences T cell activation over effector function. Furthermore, we do not dispute the possibility for ligand-dependent contributions of GPCRs not examined here (described for adenosine (Linnemann et al., 2009), and adrenaline (Fan and Wang, 2009) receptors, among others). Nonetheless, given the overall correlation between transcript abundance and knockout effect, it seems unlikely that GPCRs with very low expression contribute strongly in a ligand-independent manner.

Despite substantial differences in the effects of knockout across different GPCRs, we observed no evident correlation between receptor distribution or dynamics and costimulatory function. All studied receptors exhibited consistent centripetal migration yet no correlation with TCR. There were differences in the extent of central accumulation, however this likely stems from differences in the underlying rate of internal trafficking either to or from the synapse. These commonalities may hint at a possible shared mechanism for GPCR redistribution within the synapse,

with emergent effects on T cell costimulation depending heavily on receptor-specific properties.

Our observations offer new insights into the contributions of GPCRs to T cell activation, and the nature of their organization within the T cell immunological synapse. Nonetheless, many outstanding questions remain, including how active receptor redistribution relates to costimulatory effects; how this is affected by the local distribution of G proteins; and why it appears to be largely disconnected from receptor ligation, at least in the case of CXCR4.

MATERIALS AND METHODS

Primary CD4$^+$ T Cell Isolation

Primary human CD4$^+$ T cells were isolated using the RosetteSep Human CD4$^+$ T Cell Enrichment Cocktail (StemCell Technologies) as per the manufacturer's instructions from leukocyte cones provided by UK National Health Service Blood and Transplant. Isolated cells were cultured in RPMI-1640 supplemented with 10% FCS, 4 mM L-glutamine, 10 mM HEPES, 1% non-essential amino acid solution (Gibco), and 1% penicillin-streptomycin solution (Gibco) at 37°C, 5% CO_2. T cell blasts were generated by stimulating cells between 24 and 72 h after isolation. Cells were diluted to 1×10^6/ml in supplemented RPMI-1640 containing 50 U/ml recombinant IL2 (PeproTech) and anti-human CD3/CD28 Dynabeads (Gibco) at 1×10^6/ml. Cells were cultured for 3 days then beads were removed by magnetic separation and the medium replaced with fresh supplemented RPMI-1640 + 50 U/ml IL2. Cells were cultured for a further 4 days with medium replaced and cells diluted to 1×10^6/ml as required.

HA-restricted clone 40 cells were generated as described previously (Peng et al., 2015). Briefly, peptide-specific T cells were isolated using IFNγ secretion assay and cloned by limiting dilution. Single cells were cultured with feeder cells (irradiated, pooled PBMCs from 2 to 3 healthy donors at a total cell concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated AB human serum and 30 μg/ml of PHA). IL2 was added on day 3 and replaced every 2–3 days. Every 14–16 days, T cell clones were restimulated with feeder cells as mentioned above. Antigen specificity of the T cell clone was assessed with intracellular cytokine staining after each round of expansion.

pGEM Vector Cloning and mRNA Preparation

mRNA for transfection of exogenous proteins was produced *in vitro* from the T7 promoter-containing pGEM vector using the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Genes encoding proteins of interest were directly synthesized as gene strings using the GeneArt service (ThermoFisher) and ligated into pGEM following digestion with *AgeI* and *HindIII*. For HaloTag-fused constructs these were followed by a short sequence encoding a GSGSG flexible linker and then the *HaloTag* gene at the 3′ terminus. For *GNAi2-SNAP-tag*, the *SNAP-tag* gene was inserted between nucleotides 342 and 343, corresponding to residues A114 and E115 in the αB-αC loop of Gαi2, following a short GSG linker. This tagging site has been demonstrated previously to retain Gαi2 activity (van Unen et al., 2016).

mRNA Transfection

Cells were transfected with *in vitro*-prepared mRNA 24 h before imaging. Cells were washed three times with OptiMEM (Gibco) at room temperature and resuspended at 2.5×10^6 cells/100 μl. 2.5−10 μg of the appropriate mRNA stock was added to 2.5×10^6 cells, which were gently mixed, transferred to a Gene Pulser cuvette (BioRad) and pulsed for 2 ms at 300 V in an ECM 830 Square Wave Electroporation System (BTX). Cells were then immediately transferred to supplemented RPMI-1640 at 1×10^6/ml and cultured for 24 h. The amount of mRNA used was optimized for each T cell donor and mRNA preparation by performing multiple transfections with titrated mRNA amounts.

Cas9-Dependent Tagging of Endogenous CXCR4

Endogenous CXCR4 in Jurkat E6.1 cells was genetically fused to HaloTag at the C-terminus through Cas9-targetted homology-directed repair. The pSpCas9(BB)-2A-Puro (pX459) v2.0 vector (Ran et al., 2013) was obtained as a gift from Feng Zhang (Addgene plasmid #62988), into which the sequence 5′-TCTTTTACATCTGTGTTAGC-3′ was inserted to target Cas9 to the 3′ end of the *CXCR4* gene. Homology templates were generated by sequential nested PCRs to generate a fragment consisting of the 1 kb upstream and 1 kb downstream of the genomic cut-site flanking the *HaloTag* gene containing a terminal STOP codon. This was blunt-end ligated into the pJET1.2 shuttle vector (ThermoFisher). Nine μg of pJET1.2 HDR template and 1 μg pX459 were transfected into $2x10^6$ Jurkat E6.1 cells using the 100 μl Neon Transfection System (ThermoFisher) with settings: 1,325 V, 10 ms, three pulses. Cells were transferred to supplemented RPMI-1640 and cultured in the presence of 10 μM SPE7 pyrazine (a NHEJ inhibitor; Sigma-Aldrich) and 10 μM RS-1 (an HDR promoter; Sigma-Aldrich) for 7 days. Cells were stained with JanliaFluor 646 HaloTag ligand (Promega; see "HaloTag and SNAP-tag labeling") and the HaloTag$^+$ population sorted using a FACSAria III cell sorter (BD Biosciences). Correct tagging was confirmed by correlative TIRFM in both HaloTag and anti-CXCR4 channels.

HaloTag and SNAP-Tag Labeling

HaloTag- and SNAP-tag-fused constructs were labeled through incubation with their requisite fluorescent ligand (200 nM JaneliaFluor 646 HaloTag ligand (Promega), or 500 nM SNAP-Cell 647-SiR ligand (New England BioLabs), respectively) in supplemented RPMI-1640 for 30 min at 37°C, washed three times, incubated for a further 30 min then washed once and used immediately for imaging.

SLB Preparation and Use

SLBs were prepared as described previously (Choudhuri et al., 2014). Briefly, micelles of 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids Inc.) supplemented with 12.5% 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)

iminodiacetic acid) succinyl]-Ni (Avanti Polar Lipids Inc.) were flowed onto glass coverslips hydroxylated with piranha solution, plasma cleaned, and affixed with adhesive 6-lane chambers (Ibidi). SLBs were blocked and washed, then incubated with recombinant His-tagged proteins of interest (all produced in-house except HLA-DRB1*09:01-HA, which was obtained from the NIH tetramer facility) at the requisite concentrations to achieve the desired density: 30 molecules/μm^2 for UCHT1-Fab and HLA-DRB1*09:01-HA, 200 molecules/μm^2 for ICAM1. The specific combination of unconjugated proteins or proteins conjugated to different dyes (AlexaFluors 405, 488, 568, and 657) was varied to suit the demands of each experiment. Within 2 h of preparation, SLBs were pre-warmed to 37°C and cells were infused into the SLB chambers at $\sim 5 \times 10^5$/lane. Samples were either imaged live or fixed with warm 4% para-formaldehyde in PBS. During experiments in which soluble CXCL12 was present, recombinant CXCL12 (PeproTech) was added to a final concentration of 0.1 μg/ml in the imaging buffer prior to cell exposure to SLB. In order to present CXCL12 on SLB, 0.005% biotinylated 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids Inc.) was included in the SLB preparation then loaded with 4 μg/ml streptavidin for 20 min. After washing, CXCL12-biotin (Chemotactics) was then added at 100 ng/ml for 20 min to allow capture by the SLB-presented streptavidin at a density of 100 molecules/μm^2.

GUV Preparation and Use

GUVs were prepared using an electro-formation method. One mg/ml lipid mixture (POPC:nickelated lipid, 96:4 molar ratio) was deposited on platinum wire, dried, and dipped into a Teflon-coated chamber filled with 300 mM sucrose. GUV formation was triggered by a 10 Hz AC field for 1 h which was followed by 2 Hz for 30 min. After formation, 100 μL of the GUV suspension was incubated with 1 μg/ml His-tagged protein for 30 min.

UCHT1/ICAM-bearing GUVs were mixed with Gαi2-SNAP-tag-expressing $CD4^+$ T cells in L-15 medium (Sigma-Aldrich) containing 0.1 μg/ml anti-CD45 Fab fragment (Gap8.3 clone) conjugated to AlexaFluor 647. Live cell-GUV contacts were imaged by confocal microscopy after 10–30 min incubation at 37°C. In conditions using selective inhibitors, the relevant compound was added 15 min after cell-GUV mixing, and contacts imaged 15 min later. These were nocodazole (10 μg/ml final concentration; Sigma-Aldrich), latrunculin-A (1 μg/ml final concentration; Sigma-Aldrich), or PP2 (10 μM final concentration; Sigma-Aldrich). The exception was PTx (Tocris Bioscience), which was added to the cells in normal culture medium 18 h before imaging to a final concentration of 2 μg/ml.

Glass Coating for Cell Activation

For activation experiments on glass without SLB, 8-well μ-slide chambers (Ibidi) were coated with either PLL or anti-CD3/CD28 prior to cell loading. PLL was applied by incubation of 250 μl/well 0.01% PLL (Sigma Aldrich) in dH_2O for 15 min followed by 3 washes with 300 μl PBS. For antibody coating, wells were first coated with 250 μl 50 μg/ml polyclonal donkey anti-mouse antibody (ThermoFisher Scientific) in coating buffer (50 mM Na2CO3, 50 mM NaHCO3, pH 9.6, filtered using a 0.22 μm Millex®-GP syringe filter unit) at 4°C overnight, then washed with 3 x 300 μl PBS and incubated with 250 μl mouse anti-CD3 (OKT3; BioLegend) and mouse anti-CD28 (CD28.2; eBioscience) at 5 μg/ml in PBS for 1 h before final 3 × 300 μl PBS washes.

Microfluidic Chamber Preparation and Use

For the formation and imaging of the T cells conjugates we followed the approach detailed in Jang et al. (2015). The device design is the same as previously described but the fabrication technique differs slightly. The device comprised two parts, top and bottom, that were fabricated separately and assembled before use. The top and bottom masters were made using SU8 2015 photoresist (MicroChem) with a height of 30 and 15 μm, respectively. Polydimethylsiloxane (PDMS) soft lithography (SYLGARD®184 kit, Dow Corning) was used to fabricate the microfluidic device with base to curing agent ratio 10:1. For the bottom part a thin layer of PDMS (approximately 100 μm) was spun on the master and on a glass microscopy coverslip (Menzel Gläser) which was then carefully positioned on top of the device before curing on a hot plate at 70°C for 40 min. For the top part the curing was done in an oven at 80°C for 1 h. The two parts were then plasma cleaned and assembled under an inverted microscope with the aid of a drop of methanol to ease positioning. After assembly the device was put under vacuum for bonding. Prior to use, devices were filled with PBS + 5% BSA and left to block overnight, before washing and refilling with supplemented RPMI-1640, taking care to avoid the introduction of bubbles.

For conjugation experiments, 1×10^7 Raji B cells were incubated in 10 ml supplemented RPMI-1640 containing SEE (Toxin Technology) at 1 μg/ml and CellTracker Green CMFDA (ThermoFisher Scientific) at 10 μM for 30 min at 37°C, pelleted at 300 × g for 5 min and washed with 10 ml fresh medium, repeating three times. For experiments with monensin treatment, 7 μl GolgiStop Protein Transport Inhibitor solution (BD Biosciences) was also added to the cells 6 h before SEE incubation. Cells were then resuspended in RPMI-1640 at 1×10^7/ml, filtered with a 70 μm cell strainer (Fisher Scientific) and injected into the microfluidic device using a Legato 100 single syringe pump (WPI) at 5 μl/min for 5–10 min until most chambers were occupied with cells as observed down a white-light microscope. The device was removed from the pump and centrifuged in a swing-bucket centrifuge at 300 x g for 1 min. Jurkat E6.1 cells expressing CXCR4-HaloTag and pre-stained with JaneliaFluor 646 HaloTag ligand were introduced into the device at 1×10^7/ml, 5 μl/min for 10 min, followed by 37°C RPMI-1640 at 5 μl/min for 20 min, during which the device was housed within an incubator at 37°C. Cells were then fixed with PBS + 4% PFA flowed in at 10 μl/min for 10 min, then washed with PBS at 10 μl/min for 20 min.

TIRF, TIRF-SIM, and Confocal Microscopy

Conventional TIRFM was performed on an Olympus cellTIRF-4Line system using a 150× (NA 1.45) oil objective. Confocal images were acquired using a Zeiss 780 LSM using a 40× water objective (NA 1.2). Imaging of live samples was performed

at 37°C, and of fixed samples at room temperature. Super-resolution imaging was performed on a custom-built TIRF-SIM setup based on a ferroelectric spatial light modulator used to generate diffraction patterns and adjust the TIRF angle (Li et al., 2015). The TIRF angle was selected to ensure below 150 nm penetration depth 488, 560, and 640 nm laser lines. Illumination and detection was performed through an Olympus 100× (NA 1.49; UPLAPO100XOHR) oil objective. Raw images were obtained on two Hamamatsu Orca Flash 4.0 cameras, and reconstructed with custom made software (Li et al., 2015). Multi-channel TIRF-SIM images were corrected for chromatic aberrations using the MultiStackReg plugin for ImageJ and 0.1 μm TetraSpeck microspheres (ThermoFisher Scientific) on glass as a reference standard.

Image Analysis and Visualization

All image analysis and visualization was performed using the ImageJ software. Pearson correlation coefficients (PCCs) were calculated using the Coloc 2 plugin to perform pixel intensity correlation between channels. Only above-threshold pixels in either channel were included in the analysis to avoid false positive correlations. Radial averages were generated by using the transform function to rotate the starting image by all angles 1°-359°, then compressing the resultant transformations into a single stack and performing a z-projection of mean intensity. Radial averages from multiple cells were combined and averaged using the z-projection function following intensity normalization.

Three-dimensional z-stacks were visualized using the 3D-projection and orthogonal view functions. Comparisons of basal vs. distal intensity were performed by defining an area of 3 × 3 μm at x-y coordinates corresponding to the center of the synapse in the basal plane, then deriving the mean pixel intensity value within this area across all z positions. The peak intensity at the lower z position was taken as the basal intensity, and that at the higher z position as the distal intensity.

Analysis of intensity inside vs. outside GUV-cell contacts was performed using the multipoint tool function. Using the CD45 and UCHT1 signals to define the plasma membrane of the T cell and the site of the contact, the gray value intensity of individual pixels was measured at regular intervals of 0.5 μm within the contact and either side of the contact to a distance equivalent to 1× the width of the contact. The final intensity values inside and outside the contact were determined as the mean intensity across all measured pixels within that area.

Single-Particle Tracking

Videos used for single-particle tracking were captured at 50 ms/frame for 15 s using TIRFM. Single-particle tracking analysis was performed in ImageJ using the TrackMate plugin (Tinevez et al., 2017), version 3.8.0. Spots were identified through sub-pixel localization using a difference of Gaussians filter with an estimated spot diameter of 0.5 μm, then filtered by quality. Frame-to-frame spot linking was performed using a Linear Assignment Problem tracker with a with a maximum linking distance of 1 μm, a maximum gap-closing distance of 1 μm, and a maximum gap-closing frame gap of one frame. Trajectory coordinates were characterized using the TraJClassifier plugin (Wagner et al., 2017), with a minimum track length of 30 frames, window size of 30 frames, minimum segment length of 30 frames, and resample rate of one. As a result, only tracks of at least 30 frames (1.5 s) were taken forward for characterization. Total numbers of cells imaged and tracks recorded are given in **Supplementary Table 1**. For trajectory analysis in which absolute position was important (i.e., track movement relative to defined cell regions or to synapse center), track x and y coordinates at each time point were compared to coordinate maps of each cell derived from single-frame images of region-defining channels (UCHT1, ICAM1, IRM) taken immediately prior to particle tracking, thereby sorting each frame of each track into one of the defined c, p, or dSMAC regions. Visualization of track positions were generated using GraphPad Prism 8, or the SankeyMATIC software (https://github.com/nowthis/sankeymatic).

Primary B Cell and Monocyte Isolation, Differentiation, and Stimulation

Primary human B cells and monocytes were isolated using the RosetteSep Human B Cell and monocyte Enrichment Cocktails (StemCell Technologies) as per the manufacturer's instructions from leukocyte cones provided by UK National Health Service Blood and Transplant. Isolated cells were cultured in RPMI-1640 supplemented with 10% FCS, 4 mM L-glutamine, 10 mM HEPES, 1% non-essential amino acid solution (Gibco), and 1% penicillin-streptomycin solution (Gibco) at 37°C, 5% CO_2. B cells were also cultured in the presence of 1 mM sodium pyruvate (Gibco), 50 ng/ml IL4 (PeproTech), 25 ng/ml IL2 (PeproTech), 100 ng/ml BAFF (BioLegend), and 100 ng/ml IL21 (BioLegend). Monocytes were differentiated into moDCs by culturing with 50 ng/ml IL4 (PeproTech) and 100 ng/ml GM-CSF (Immunotools) at $1 \times 10^6/cm^2$ in adherent culture for 6 days. Twenty-four h before use in T cell stimulation assays, moDCs were activated by addition of 1 μM prostaglandin E2 (Sigma-Aldrich), 50 ng/ml TNFα (PeproTech), 10 ng/ml IL1β (Bio-Techne), and 20 ng/ml IFNγ (Bio-Techne). Differentiation was confirmed by assessing expression of CD11c and CD86 (see "Flow cytometry").

Cas9 RNP Preparation and Transfection

Gene disruption in primary $CD4^+$ T cells was performed by transfection with *in vitro*-prepared Cas9 ribonucleoprotein (RNP) complexes. For all targets, gene-specific Alt-RCRISPR-Cas9 gRNA was obtained from IDT (sequences given in **Supplementary Table 2**). To generate RNP complexes, 150 pmol Alt-RCRISPR-Cas9 gRNA was incubated with 150 pmol Alt-R tracrRNA (IDT) in nuclease-free duplex buffer (IDT) at 95°C for 5 min and resultant duplex allowed to cool to room temperature. One hundred and fifty pmol of Alt-R S.p Cas9 Nuclease V3 (IDT) and duplexed gRNA were mixed in 8 μl nuclease-free duplex buffer and incubated at 37°C for 15 min. One hundred and fifty pmol Alt-RCas9 Electroporation Enhancer (IDT) was added to the RNP solution, and the whole mix then added to 1.5×10^6 naïve primary $CD4^+$ T cells, which had previously been washed with room-temperature OptiMEM three times and resuspended in 50 μl OptiMEM. The cell-RNP mix was transferred to a Gene Pulser cuvette (BioRad) and pulsed for 2 ms at 300 V in an ECM

830 Square Wave Electroporation System (BTX). Cells were then immediately transferred to 500 μl supplemented RPMI-1640. Hundred μl of cells were removed and blasted for 3 days as described above, while the remaining ∼1.2 × 10^6 cells were left in resting culture until used in T cell stimulation assays.

TIDE Analysis

The efficiency of gene disruption was determined using TIDE analysis (Brinkman et al., 2014). Genomic DNA (gDNA) was isolated from 0.5 × 10^6 transfected $CD4^+$ T cell blasts 7 days after RNP transfection using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) as per the manufacturer's instructions. Isolated gDNA was then used as the template in polymerase chain reactions using the relevant oligonucleotide primers given in **Supplementary Table 2**, to amplify the ∼500 bp region surrounding the predicted genomic cut site for each target. These products were sequenced using reversible terminator sequencing and the resulting chromatograms compared to those derived from PCR products of untransfected cell gDNA using the TIDE algorithm (Desktop Genetics). TIDE analysis outputs are given in **Supplementary Figure 4**.

T Cell Stimulation Assay

Stimulation of knockout cells was performed 7 days post-transfection with RNP complexes. Both naïve and blasted $CD4^+$ T cells were activated with anti-human CD3/CD28 Dynabeads (Gibco) or SEE-loaded antigen-presenting cells (APCs). moDCs were the APCs used for naïve T cells, B cells for blasted T cells. In each case, APCs and T cells were obtained from the same blood donor. Twenty four h before stimulation, T cell blasts were transferred to IL2-free growth medium and B cells were transferred to cytokine-free growth medium. Immediately before stimulation, T cells were centrifuged at 300 × g for 5 min and resuspended in supplemented RPMI-1640 at a density of 5 × 10^4 / 50 μl. APCs were loaded with SEE (Toxin Technology) for 1 h at 37°C at concentrations ranging from 10^{-4} ng/ml to 10^3 ng/ml or with no SEE, then washed four times with growth medium and resuspended at 1 × 10^5 / 50 μl. Fifty μl of T cell suspension was added to either 50 μl APC suspension, 50 μl growth medium containing 1 × 10^5 anti-human CD3/CD28 Dynabeads, or 50 μl growth medium alone in a U-bottomed 96-well plate, which was gently centrifuged at 25 × g for 1 min then returned to culture. After 2 h, 50 μl of growth medium containing 0.1 μl GolgiStop Protein Transport Inhibitor solution (BD Biosciences) was added to cells. After a further 4 h, cells were centrifuged at 300 × g for 5 min then fixed with 4% para-formaldehyde in PBS for 10 min before staining for flow cytometry.

Flow Cytometry and Cell Sorting

Following T cell stimulation assays, samples were permeabilised with 0.1% saponin in PBS for 15 min, quenched with 100 mM glycine in PBS for 20 min, then blocked with 6% bovine serum albumin (BSA) in PBS for 2 h, with 3 PBS washes between each step. Following blocking, cells were incubated for 2 h with 1 μg/ml anti-CD69 (FN50; BioLegend), anti-IL2 (MQ1-17H12; BioLegend), anti-IFNγ (4S.B3; BioLegend), and either anti-CD19 (4G7; BioLegend) in the case of B-T cell conjugates, or anti-CD11c (3.9; BioLegend) for moDC-T cell conjugates, all in PBS + 3% BSA + 0.02% saponin. Cells were washed 3 times with PBS + 0.1% saponin and resuspended in 100 μl PBS + 1 mM EDTA for analysis. Samples were analyzed using the high-throughput 96-well plate sampler of a FACSCanto II Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo version 8.8.7. T cells were gated first by FS/SS and then as the $CD19/CD11c^{-ve}$ population (in the case of APC-T cell conjugates) or as the $PE\text{-}Cy5^{-ve}$ $PE\text{-}Cy7^{-ve}$ population (for bead-T cell conjugates). Thresholds at which cells were defined as positive for CD69, IL2, and IFNγ were determined by reference to cells stained with appropriate isotype controls. Response to stimulation was expressed as normalized $\Delta CD69/IL2/IFN\gamma^+$ cells, which was defined as the difference between the frequency of positive cells in a sample and that in the control sample consisting of T cells + APC with no SEE (for APC-T cell conjugates) or of T cells alone (for bead-T cell conjugates). This was then normalized to the maximum value observed for the $CD19^{-ve}$ control, which was set at 100.

Alongside this, the unstimulated cell condition was stained with anti-TCR (IP26; BioLegend) and anti-CD28 (CD28.2; BioLegend) without prior permeabilisation at 1 μg/ml for 45 min then washed and analyzed in the same manner. For other experiments where surface staining was sufficient, cells were fixed and stained in the same manner as above, using the relevant antibodies in each case; one or more of anti-CXCR4 (12G5; BioLegend). anti-TCR (IP26; BioLegend), or anti-CD28 (CD28.2; BioLegend).

A pure $CXCR4^{-ve}$ population was obtained for mutant CXCR4-HaloTag transfection by fluorescence-activated cell sorting. Cells were stained with 1 μg/ml anti-CXCR4 (12G5; BioLegend) in PBS + 2% FCS on ice for 30 min, then washed 3 times with cold PBS + 2% FCS and the negative population sorted using a FACSAria III cell sorter (BD Biosciences).

Bead Supported Lipid Bilayers (BSLB)

Unfunctionalised silica beads (5.0 μm diameter, Bangs Laboratories, Inc.) were washed extensively with PBS in 1.5 ml conical microcentrifuge tubes. BSLBs were formed by incubation with mixtures of liposomes to generate a final lipid composition of 0.2 mol% Atto-DOPE Atto565; 12.5 mol% DOGS-NTA in DOPC at a total lipid concentration of 0.4 mM. The resultant BSLBs were washed with 1% human serum albumin (HSA)-supplemented HEPES-buffered saline (HBS), subsequently referred to as HBS/HSA. To saturate NTA sites, BLSBs were then blocked with 5% casein 100 μM $NiSO_4$ for 20 min. After two washes, BSLBs were loaded with concentrations of His-tagged proteins required to achieve the indicated molecular densities (see figure legends). Excess proteins were removed by washing with HBS/HSA after 30 min. Primary $CD4^+$ T cell blasts (2.5 × 10^5/well) were incubated with BSLBs at 1:1 ratio in a V-bottomed 96 well plate (Corning) for 90 min at 37°C in 100 μl HBS/HSA. For gentle dissociation of BSLB-cell conjugates, culture plates were gradually cooled down by incubation at RT for 15 min, followed by incubation on ice. After 45 min, cells and BSLBs were pelleted at 300 x g for 5 min prior to resuspension in ice-cold

5% BSA in PBS pH 7.4. The single BSLBs and cells were gently resuspended prior to staining for flow cytometry analysis.

Multicolour Flow Cytometry of BSLBs

Staining with fluorescent dye-conjugated antibodies was performed immediately after dissociation of cells and BSLB conjugates. Staining was performed in ice-cold 5% BSA in PBS pH 7.4 (0.22 μm-filtered) for a minimum of 30 min at 4°C with agitation to avoid BSLB sedimentation (700 rpm in the dark). Cells and BSLBs were then washed three times and acquired immediately using an LSRFortessa X-20 flow cytometer equipped with a high-throughput sampler. For absolute quantification, we used Quantum Molecules of Equivalent Soluble Fluorescent dye (MESF) beads (see below), which were first acquired to set photomultiplier voltages to position all the calibration peaks within an optimal arbitrary fluorescence units' dynamic range (between 10^1 and 2×10^5, and before compensation). Fluorescence spectral overlap compensation was then performed using unlabelled BSLBs and cells, and single color-labeled cells and BSLBs. For markers displaying low surface expression levels unstained and single color stained UltraComp eBeads (Thermo Fisher Scientific Inc.; #01-2222-42) were used for the calculation of compensation matrixes. Resulting compensation matrixes were applied and experimental specimens and Quantum MESF beads were acquired using the same instrument settings. In most experiments acquisition was set up such that a minimum of 5×10^4 single BSLBs were recorded.

Transwell Migration Assay

6.5 mm transwell inserts with 5 μm pore polycarbonate membranes (Scientific Laboratory Supplies) were coated overnight at 4°C with 100 μl PBS containing 50 μg/ml hICAM1 and either anti-CD3 (UCHT1) or mouse IgG1 isotype control at 50 μg/ml. Inserts were then washed 3 times and blocked with PBS + 5% BSA for 2 h at 4°C then washed three times with OptiMEM. Six hundred μl supplemented RPMI-1640 containing 0 or 0.25 μg/ml CXCL12 (PeproTech) was added to wells of a 24-well plate, on top of which the insert was carefully overlaid then filled with 1 × 105 T cells in 100 μl supplemented RPMI-1640. Cells were allowed to migrate for 1 h at 37°C then the total number of cells in the bottom chamber was counted using a FACSCanto II Flow Cytometer (BD Biosciences). Cell numbers were Normalized to wells in which 100 μl cell suspension was added directly to the bottom chamber.

Statistical Analysis

All statistical tests were done with GraphPad Prism 8 software. The appropriate statistical test for each experiment is noted in the figures. The number of independent replicates in each case is provided in the associated figure legend.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JHF and MLD conceived and led the study, secured primary funding, and wrote the manuscript. JHF performed most experimental work and prepared the figures. LP designed and prepared the microfluidic devices, with practical support from MJ, and intellectual and financial support from DA and JF. ES performed the GUV experiments. PFC performed the BSLB transfer experiments. KK assisted with the TIRF-SIM experiments. MF and KK established and maintained the TIRF-SIM system, with MF and MLD securing funding. YP and TD generated and maintained the HA-restricted T cell clone. All authors provided helpful feedback on the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.608484/full#supplementary-material

Supplementary Figure 1 | (A) TIRFM image examples of primary CD4$^+$ T cell blasts expressing CXCR4-HaloTag interacting with UCHT1- and ICAM1-containing SLBs for 5, 10, and 30 min. **(B)** Max-intensity projections and orthogonal views of a confocal microscopy z-stack of CD4$^+$ T cell blast expressing CXCR4-HaloTag on SLB presenting ICAM1 and UCHT1 for 30 min. **(C)** TIRFM image examples of primary CD4$^+$ T cell blasts prestained with anti-CXCR4 mAb interacting with UCHT1- and ICAM1-containing SLBs for 30 min. **(D)** TIRFM image examples of HA-DRB1-specific CD4$^+$ T cells expressing CXCR4-HaloTag and pre-stained with anti-TCR mAb interacting with SLB presenting ICAM1 and pHA-MHC for 10 and 30 min. **(E)** TIRFM images (left), cross-sectional normalized intensity profile (center), and radial averages (right) for CD4$^+$ T cell blasts expressing CXCR4-HaloTag and stained for F-actin with phalloidin, on activating SLB for 10 min and treated with indicated inhibitors. Plots are mean normalized intensity at each position ± std dev. **(F)** Histogram of straightness for single

particle CXCR4-HaloTag trajectories with different diffusive properties under activating and non-activating conditions. **(G)** Histogram of cell displacement over 20 min incubation for $CD4^+$ T cell blasts on ICAM1-containing SLB in the presence of soluble and surface-presented CXCL12, measured by time-lapse IRM. **(H)** Histograms of mean diffusion speed and straightness for single particle CXCR4-HaloTag trajectories with different diffusive properties under activating and conditions in the presence of soluble and surface-presented CXCL12. All scale bars are 5 μm.

Supplementary Figure 2 | (A) Example flow cytometry histogram of anti-CXCR4 staining on wt and $CXCR4^{-ve}$ $CD4^+$ T cell blasts transfected with HaloTag-fused CXCR4 mutants. **(B)** Example flow cytometry histogram of anti-CXCR4 staining on wt and $CXCR4^{-ve}$ $CD4^+$ T cell blasts transfected with untagged CXCR4 mutants. **(C)** Example flow cytometry histograms of anti-CD69, -IL2, and -IFNγ staining on wt and $CXCR4^{-ve}$ $CD4^+$ T cell blasts transfected with untagged CXCR4 mutants and stimulated with anti-CD3/CD28 beads for 6 h.

Supplementary Figure 3 | (A) Representative TIRFM examples of Gαi2-SNAP-tag-expressing $CD4^+$ T cell blasts interacting with or activating SLB for 10 or 30 min. **(B)** Max-intensity projections and orthogonal views of confocal microscopy z-stacks of $CD4^+$ T cell blasts on glass coated with PLL or anti-CD3/CD28 antibodies. **(C)** TIRF-SIM examples of Gαi2-SNAP-tag in $CD4^+$ T cell blasts on activating SLB for 10 min. All scale bars are 5 μm.

Supplementary Figure 4 | (A) Example histograms of flow cytometry data of anti-CD69 staining on wt and $CD3^{-ve}$ $CD4^+$ T cells (blast and naïve) from the same donor following 6 h stimulation with anti-CD3/CD28 beads or APCs loaded with the indicated concentration of SEE. **(B)** Example flow cytometry data for CD3, CD28, or CXCR4 expression in wt $CD4^+$ T cells (blast and naïve) and cells transfected with Cas9 RNP complexes targeting the relevant gene ("KO"). **(C)** Example sequencing chromatograms used for TIDE analysis. *CXCR3* sequences in wt and CXCR3 KO cells were compared to identify regions of aberrant signal (bottom), which then underwent decomposition to determine the relative frequencies of different indel mutations. **(D)** Pooled TIDE data from all Cas9 screen experiments. Values indicate the percentage of the sequencing data that corresponds to fully wild-type sequence. Boxes are mean with min-max; each symbol indicates a different donor. **(E)** Normalized CD3, CD28, and CD69 expression levels as measured by flow cytometry in unstimulated $CD4^+$ T cell blasts following knockout of the indicated genes. Values are normalized to the gMFI value in wt cells. Boxes are mean with min-max; each symbol indicates a different donor. **(F)** Example flow cytometry histograms of anti-CD69, -IL2, and -IFNγ staining on unstimulated wt and individual-gene knockout $CD4^+$ T cells (blast and naïve) from a single donor. **(G)** Absolute transfer of TCRαβ (left) or CD40L (right) from $CD4^+$ blasts to BSLBs presenting ICAM1, CD40, and titrated densities of UCHT1. Plots show mean ± std dev., with best-fit non-linear response curves for each target. $^{***}p < 0.001$.

Supplementary Table 1 | Summary of cell and track numbers for single-particle tracking experiments. All samples were examined in three independent experiments using cells derived from different donors.

Supplementary Table 2 | Summary of Cas9 gRNA and TIDE oligonucleotide sequences used in knockout of all investigated genes. All sequences are given 5′-3′. On- and off-target scores are those provided by the gRNA supplier (IDT).

Supplementary Movie 1 | Example of single-particle tracking of CXCR4-HaloTag in primary $CD4^+$ T cells on SLB. Images are raw acquisition (left), spot detection (center), and frame-to-frame tracking (right).

Supplementary Movie 2 | Example videos of CXCR4-HaloTag in primary $CD4^+$ T cells on resting (left) and activating (center & right) SLB at the indicated time points.

Supplementary Movie 3 | Example videos of mutant CXCR4-HaloTag in $CXCR4^{-ve}$ $CD4^+$ T cells on activating SLB 10 min activation.

Supplementary Movie 4 | Example video of Gαi2-SNAP-tag in primary $CD4^+$ T cells landing on activating SLB. UCHT1 is shown in green, ICAM1 in blue, Gαi2 in red, IRM in gray.

Supplementary Movie 5 | Example videos of HaloTagged GPCRs in primary $CD4^+$ T cells on activating SLB 10 min after activation.

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MINI REVIEW
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Microclusters as T Cell Signaling Hubs: Structure, Kinetics, and Regulation

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When T cell receptors (TCRs) engage with stimulatory ligands, one of the first microscopically visible events is the formation of microclusters at the site of T cell activation. Since the discovery of these structures almost 20 years ago, they have been studied extensively in live cells using confocal and total internal reflection fluorescence (TIRF) microscopy. However, due to limits in image resolution and acquisition speed, the spatial relationships of signaling components within microclusters, the kinetics of their assembly and disassembly, and the role of vesicular trafficking in microcluster formation and maintenance were not finely characterized. In this review, we will summarize how new microscopy techniques have revealed novel insights into the assembly of these structures. The sub-diffraction organization of microclusters as well as the finely dissected kinetics of recruitment and disassociation of molecules from microclusters will be discussed. The role of cell surface molecules in microcluster formation and the kinetics of molecular recruitment via intracellular vesicular trafficking to microclusters is described. Finally, the role of post-translational modifications such as ubiquitination in the downregulation of cell surface signaling molecules is also discussed. These results will be related to the role of these structures and processes in T cell activation.

Keywords: TIRF-SIM, lattice light sheet microscopy, vesicle traffic, microclusters, live cell imaging

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INTRODUCTION

The central event in the initiation of the adaptive immune response to foreign antigen is the interaction of the T cell antigen receptor (TCR) with an antigenic peptide presented by a protein encoded by the major histocompatibility complex (pMHC). The rapid biochemical events that then transpire, defined as T cell activation, have been the subject of extensive research for over three decades. Rapid recruitment and activation of Src family protein tyrosine kinases (PTK) and ZAP-70 lead to phosphorylation of tyrosine residues on the cytosolic regions of the TCR (the CD3 and TCRζ chains), adapter proteins (LAT and SLP-76), and various enzymes (Itk and PLC-γ1). These phosphorylations, in turn, lead to creation of sites for SH2 domain-mediated binding, leading to formation of protein complexes and to the activation of many of the bound enzymes (Weiss and Littman, 1994; Smith-Garvin et al., 2009; Balagopalan et al., 2010; Samelson, 2011; Courtney et al., 2018). These multiple events occur in the first seconds to minutes following TCR-pMHC engagement. Subsequently and dependent on these proximal events, further phosphorylations (primarily due to activation of protein serine kinases) and other enzymatic events lead to activation of transcription and to global cellular changes mediated by cytoskeletal reorganization. The most

dramatic example of the latter is the generation of the immune synapse (IS) that forms between the T cell and an antigen-presenting cell (APC). The initial description of the IS was that of a segregated bulls-eye structure with a centralized TCR (the cSMAC) surrounded by the integrin LFA-1 (the pSMAC), with large molecules such as the phosphatatase CD45 excluded from the central region (Monks et al., 1998; Grakoui et al., 1999). IS formation was observed to take place about 10–30 min after TCR stimulation, while the biochemical events described above occur in seconds, indicating that the IS does not trigger initial TCR signals (Lee et al., 2002).

An early consequence of TCR-pMHC binding is the aggregation of the TCR and many of the above-described signaling molecules in structures known as microclusters. Visualization of these submicron-sized bodies was enabled by using high-speed confocal microscopy (see **Box 1** in **Supplementary Material**) to visualize T cell activation in live cells expressing fluorescently tagged signaling molecules. Microclusters are sites of T cell activation as evidenced by the accumulation of tyrosine-phosphorylated proteins (Bunnell et al., 2002). Extensive analysis revealed that a substantial number of signaling molecules defined biochemically to be involved in T cell activation (as described above) were found within microclusters. Our original studies were performed on cells activated by anti-TCR antibodies on glass, while several subsequent studies by others employed activation by pMHC conjugated to planar lipid bilayers on glass and total internal reflection fluorescence microscopy (TIRFM; see **Box 1** in **Supplementary Material**) (Campi et al., 2005; Yokosuka et al., 2005). More recently, advanced imaging techniques such as lattice light sheet microscopy (LLSM; see **Box 1** in **Supplementary Material**) have enabled the visualization of microclusters at the initiation of T cell contact, thus confirming the role of these structures as signaling units that drive T cell activation (Ritter et al., 2015).

In this minireview, we aim to summarize recent insights into the organization and formation of microclusters and discuss the regulation of these structures via endocytic and exocytic mechanisms. Along the way, we will highlight the new imaging methodologies that have enabled these novel insights.

SPATIAL ORGANIZATION OF MICROCLUSTERS

Since the discovery of microclusters, the spatial organization of signaling molecules in these structures has been extensively studied. In the initial description of microclusters, the exclusion of large glycoproteins, CD43 and the phosphatase CD45, from microclusters, similar to their exclusion from the cSMAC of the IS, was described (Bunnell et al., 2002). More recently, the accumulation of LFA-1 surrounding the TCR microcluster to form an "adhesion ring" in microscale during the initiation of T cell activation was observed, reminiscent of the bulls-eye organization of the IS (Hashimoto-Tane et al., 2016). Though microclusters are thought of as T cell activation units, assemblies of receptor and signaling proteins can be detected in the membrane of resting T cells, suggesting that smaller preformed "nanoclusters" may pre-segregate into specialized membrane domains prior to TCR triggering (Lillemeier et al., 2006, 2010; Crites et al., 2014). Despite considerable ambiguity on spatial distribution, structural organization, and nomenclature of these structures, most investigators believe that upon TCR ligation, these "nanoclusters" undergo concatenation, remixing, and aggregation to form larger TCR microclusters (Lillemeier et al., 2010; Sherman et al., 2011; Hu et al., 2016).

Multiple super-resolution microscopy techniques, including single-molecule localization microscopy (SMLM; see **Box 1** in **Supplementary Material**), have been developed that allow more detailed studies of the structure of signaling complexes. These investigations have revealed nanoscale organization of the TCR and other important components of the signal transduction pathway. Early studies using photo-activation localization microscopy (PALM; see **Box 1** in **Supplementary Material**) showed that the TCR and LAT are clustered in both unactivated and activated cells and that the extent of clustering increased after TCR activation (Lillemeier et al., 2010; Sherman et al., 2011). Also, the TCR and LAT clusters tend to be segregated from each other, with some overlap at "hotspots" (Sherman et al., 2011). The two studies detected different sizes of clusters, with the Lillemeier study finding significantly larger clusters. These differences are likely due to differences in their analytical approach as described in **Table 1** below.

Another study using STED showed that the clusters are smaller than STED resolution, in the range of 50–70 nm (Balagopalan et al., 2015). Despite the discrepancy in cluster size, these studies agreed that the TCR and LAT were found in clusters that increased in size with T cell activation. Since these early reports, there has been increasing interest in developing methods to analyze clustering. These range from stand-alone programs such as DBScan (Ester et al., 1996) to the use of machine learning

TABLE 1 | Fundamental differences in analytical approaches employed by Lillemeier et al. and Sherman et al. to analyze SMLM data.

Parameters	Lillemeier et al.	Sherman et al.	Implication
Statistical method for cluster analysis	Ripley's K-function analysis	Pair correlation function (PCF)	Smaller clusters are under-reported in Ripley's functions, which should be used mainly to report separation distances rather than cluster size. PCF shows results uniformly across all length scales and is more appropriate for detecting small-scale clusters.
Poisson model	Standard Poisson null model	Heterogeneous Poisson model	Effects of plasma membrane heterogeneity are considered in the heterogeneous Poisson process, while the standard Poisson model can report membrane ruffles as clusters.
Intensity-based thresholding	Yes	No	Many molecules are gated as background and small clusters may be removed from analysis.

(Williamson et al., 2020). Both the principles of SMLM and data analyses have been reviewed recently (Wu et al., 2020).

The organization of other molecules including Lck, ZAP-70, Grb2, and SLP-76 in microclusters has also been studied (Lillemeier et al., 2010; Purbhoo et al., 2010; Hsu and Baumgart, 2011; Sherman et al., 2011; Rossy et al., 2013; Neve-Oz et al., 2015). ZAP-70 kinase mixes uniformly with TCR but shows only partial mixing with LAT. LAT clusters recruited Grb2 regardless of size, indicating that even small nanoclusters contain phosphorylated LAT and participate in T cell activation. Interestingly, LAT and SLP-76 were reported to form nanostructures with LAT tending to be in the center and SLP-76 distributed on the outside (Sherman et al., 2011). Further investigation showed that this LAT-SLP nanostructure develops during the spreading process (Barr et al., 2016), suggesting that the nanostructure of signaling complexes is dynamic and changes with time. This research has also revealed the difficulty in analyzing the patterns of multiple proteins. Methods such as the bivariate PCF can evaluate the interactions of two molecules, but it is difficult to determine how larger numbers of proteins interact. One study, which extended the analysis to three proteins, demonstrated concentric arrangements of molecules at LAT clusters, with VAV1 and PLCγ1 near LAT at the center, while SLP-76 was found at the periphery and actin was seen surrounding the clusters (Sherman et al., 2016). The analysis also examined the recruitment of subsets of proteins to LAT clusters. SLP-76 recruitment was promoted by interactions with PLCγ1 and actin. However, both PLCγ1 and actin associations with LAT clusters were independent of SLP-76. At this time, good statistical methods are not available to determine the organization of multiple proteins within the signaling complexes.

Despite the high precision reported by localization algorithms, visualization of proteins within complex structures has been hampered by several issues, including the accurate determination of the actual location of single molecules and limitations in the alignment of multi-color images. The density of the label also affects the accuracy of the image (Patterson et al., 2010). Current SMLM techniques rarely give true counts of the number of molecules; both overcounting and undercounting errors are common (Krizek et al., 2011). In particular, SMLM methods tend to produce multiple localizations from the same molecule. The difficulty in properly assigning these localizations to the correct molecule or the grouping of localizations remains one of the most stubborn problems (Erdelyi et al., 2015). Without this crucial correction, it is impossible to perform a detailed molecular analysis of microclusters and the IS. A recent technique, madSTORM (see **Box 1** in **Supplementary Material**), addressed some of these issues and allowed the visualization of multiple targets at high resolution in a single sample. This method was able to produce high-resolution images of samples containing up to 20 different proteins (Yi et al., 2016). However, even in this scenario, only fixed samples could be used; each final image required capturing thousands of frames, and the process required several days to gather all the data. For now, SMLM is not able to determine microcluster structure in live cells.

More recent studies in live T cells using high-speed super-resolution microscopy techniques such as total internal reflection fluorescence structured illumination microscopy (TIRF-SIM; see **Box 1** in **Supplementary Material**) have brought more clarity to the spatial organization TCR microclusters and their kinetics of assembly upon T cell activation (Yi et al., 2019). Two spatially segregated domains were identified within microclusters. TCR and ZAP-70 colocalized and marked the "receptor domain," while LAT with its associated adaptor (GRB2, GADS, and SLP-76) and signaling proteins (ADAP, NCK, PLCγ, and VAV1) constituted the "signaling domain" of TCR microclusters. Sub-diffraction resolution images generated by TIRF-SIM showed that LAT was situated adjacent to the receptor domain proteins (TCRζ and ZAP-70) but did not colocalize with the latter. Likewise, adaptor and signaling proteins colocalized with each other and were positioned adjacent to and yet segregated from the receptor domain (**Figure 1**). The presence of such distinct domain organization within TCR microclusters might explain previously observed spatially segregated proteins islands of LAT

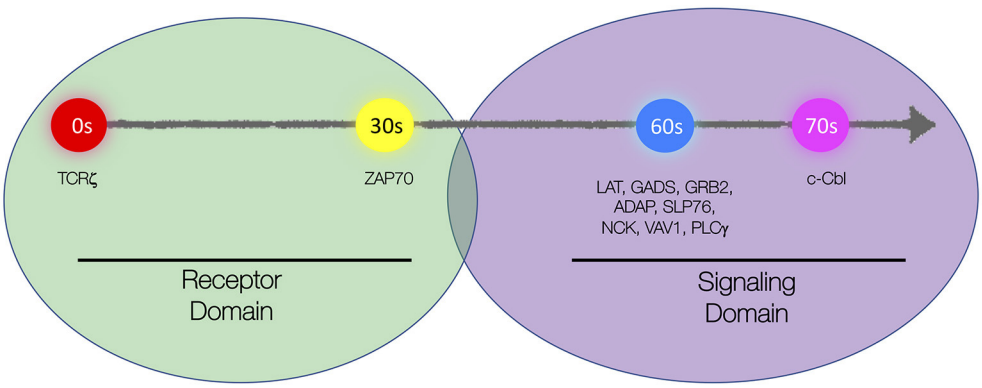

FIGURE 1 | Schematic representation of spatial and kinetic organization of microclusters. Microclusters are organized into the "receptor domain" containing TCRζ and ZAP70 and the "signaling domain" containing several signaling proteins including LAT, GADS, GRB2, ADAP, SLP76, NCK, VAV, PLCγ1, and c-Cbl. Molecules are sequentially recruited to the microcluster with TCRζ being recruited first, ZAP70 recruited 30 s after TCRζ, LAT, and LAT-associated signaling proteins recruited simultaneously 30 s after ZAP70, and c-Cbl recruited 10 s after LAT.

and TCRζ at single-molecule resolution. Colocalization of TCRζ and ZAP-70 in the receptor domain echoes earlier observations of extensive mixing of ZAP-70 in TCRζ nanoclusters as originally observed by Sherman et al. With the discovery of such distinct domain organization of microclusters, questions arise on how the constituents of these two domains are brought in close proximity and if there are additional molecules (e.g., Shb, Lck, or others) that would be required to hold these complexes together (Welsh et al., 1998; Lindholm et al., 1999, 2002; Lo et al., 2018).

The idea of domains in the PM is not new. The lipid composition of the PM is not homogeneous, and it contains liquid-disordered and liquid-ordered domains. The liquid-ordered phase is enriched in cholesterol and sphingolipids and has long been studied as "lipid rafts," where signaling proteins including TCR, Lck, and LAT segregate upon activation (Brdicka et al., 1998; Montixi et al., 1998; Zhang et al., 1998). Early evidence for the existence and functional relevance of "lipid rafts" came from resistance to detergent extraction, the effects of cholesterol depletion, and mutants that failed to localize to these domains (Munro, 2003). However, these methods do not identify these microdomains as they exist in the PM of cells. The visualization of lipid microdomains has been difficult because they are of a size below the resolution of conventional microscopy (Zacharias et al., 2002; Shaw, 2006). Phase-sensitive membrane probes and new imaging methodologies have allowed the direct visualization of membrane order in T cells. The IS has been shown to contain ordered membrane domains (Gaus et al., 2005; Owen et al., 2010). However, the presence of lipid order in microclusters is unclear. A study using FRET reported that several lipid raft markers do not accumulate in microclusters, suggesting that TCR microclusters form independently of lipid rafts (Hashimoto-Tane et al., 2010). However direct visualization of lipid order in activated T cells showed that the TCR resides in ordered plasma membrane domains that aggregate upon TCR engagement (Dinic et al., 2015). The role of lipid ordered domains in T cell signaling should still be considered in models of T cell activation (Courtney et al., 2018).

In addition to lipid-mediated phase separation, multivalent protein interactions lead to phase transitions within microclusters. LAT serves as an important scaffolding protein by virtue of its multiple interactions with other adapters and enzymes. Oligomerization of LAT mediated by multivalent interactions between LAT, LAT-bound adaptors, and adaptor-bound enzymes drives microcluster formation and has important functional outcomes (Houtman et al., 2006; Kortum et al., 2011; Coussens et al., 2013). *In vitro* reconstitution studies have demonstrated that LAT microclusters form due to a biophysical phase separation mediated by protein oligomerization (Su et al., 2016). Thus, both lipid and protein-mediated phase separation can create distinct physical compartments that facilitate signaling.

Most of the studies discussed above were performed using stimulatory surfaces such as antibody-coated cover glass or lipid bilayer systems. This raises the concern of whether such systems can accurately represent three-dimensional (3D) membrane dynamics that would naturally occur in a conjugate system of APC and T cell. With the advent of newer and sophisticated imaging techniques such as LLSM, some have started to capture the 3D membrane dynamics of T cell with unprecedented speed and resolution. There is growing evidence that indicates that dynamic membrane protrusions of T cells, called microvilli, play a critical role in T cell activation. High-resolution lattice lightsheet microscopy showed how microvilli play a crucial role in actively scanning the surface of APC for antigens (Cai et al., 2017). An approach using variable angle TIRF (VA-TIRF; see **Box 1** in **Supplementary Material**) and super-resolution microscopy revealed the localization of fluorescently labeled TCR and signaling molecules nano-clustered at the tips of the microvilli (Jung et al., 2016; Ghosh et al., 2020). All these results generate a unified concept that preexisting nanoclusters or protein islands can be enriched in specialized membrane domains within dynamic microvilli. These nanoclusters can undergo intermixing and reorganization after TCR ligation thereby bringing receptor, adaptor, and signaling proteins into close proximity to generate intracellular signaling events.

MICROCLUSTERS ARE ASSEMBLED IN DISCRETE KINETIC STEPS

In contrast to the prediction of stochastic recruitment according to the "Protein Island" model, Yi *et al.* showed that individual proteins were recruited into the microcluster in a non-stochastic and stepwise sequential manner. Live-cell TIRF-SIM and TIRF microscopy approaches showed that, following TCR engagement, ZAP-70 was first recruited to TCR microclusters, followed by simultaneous recruitment of signaling and adaptor domain proteins (LAT, SLP-76, GRB2, ADAP, VAV1, NCK, and PLCγ). The simultaneous recruitment of LAT with its associated adaptors, signaling, and enzyme proteins is compatible with previous results, which established highly cooperative protein–protein interactions and stochastic cross-linking of multiprotein complexes (Houtman et al., 2006; Coussens et al., 2013). Recruitment of signaling domain proteins also leads to intracellular calcium flux, which indicates initiation of active signaling at the microclusters (**Figure 1**).

Distinct kinetic lags were established between recruitment of individual proteins in the microcluster. The assembly phase was followed by a disassembly or signal attenuation phase marked by recruitment of the E3 ubiquitin ligases, c-Cbl, which is involved in internalization and degradation of LAT signaling complexes (Balagopalan et al., 2007, 2011). LAT-bound signaling domain proteins showed a bimodal dissociation behavior from the microcluster. GRB2 and PLCγ showed slower dissociation kinetics, while GADS and SLP-76 showed rapid dissociation. Multiple mechanisms can be postulated for different kinetics of dissociation, such as inherently different affinities; different rates of dephosphorylation, ubiquitination, and endocytosis; and distinct pulling forces from the actin network on these structures (Barda-Saad et al., 2005; Yi et al., 2012; Kumari et al., 2015).

The kinetics of molecular recruitment to microclusters were shown to be sensitive to temperature and intracellular calcium

levels. The kinetic lag between TCRζ and ZAP-70 showed a linear inverse relationship with temperature. However, the kinetic lag between ZAP-70 and GRB2 turned temperature-independent above 30°C. Because GRB2 recruitment depends on LAT clustering, the temperature independence of the ZAP-70 and GRB2 kinetic lag at this temperature could be attributed to the effect of temperature on membrane lipids, which can in turn influence LAT clustering (Tanimura et al., 2003; Su et al., 2016). Intracellular calcium was also found to have an impact on these kinetic lags, with calcium flux coincident with longer kinetic lags. Early microclusters that formed before calcium flux occurred showed negligible kinetic lags, and kinetic lags increased with time. A dose-dependent response in kinetic lags was observed by varying calcium concentration in the medium (Yi et al., 2019). This is consistent with an inhibitory role for calcium in the recruitment kinetics of proteins. In support of calcium flux dampening T cell signaling, our previous study reported that calcium chelation led to increased phosphorylation of signaling proteins and increased microcluster size (Balagopalan et al., 2018). Interestingly, an increase in intracellular calcium concentration led to reduced TCR mobility and promoted actin polymerization (Dushek et al., 2008), suggesting that calcium flux may regulate signaling protein kinetics via multiple pathways.

Advanced understanding of the kinetics of recruitment of molecules at the TCR microcluster have identified new "control nodes" in the kinetic proof-reading model of TCR signaling. According to this model, TCR signaling is a multi-step kinetic process in which progression to a subsequent step is contingent on achieving a "signaling threshold" or "signaling competent state" at the preceding kinetic step. Therefore, the duration (kinetic lag) and dissociation constant of each kinetic step is a major determinant of its progression to the next step (McKeithan, 1995; Lever et al., 2014). The kinetic lags observed by Yi *et al.* would directly feed into the kinetic parameters of a proof-reading model. Kinetic lags are also drastically altered after calcium flux. Therefore, TCR activation threshold and kinetic parameters of the proof-reading model will also be different before and after calcium flux. Calcium-dependent increases in kinetic lags can also act as a negative feedback mechanism to limit TCR signaling after a certain threshold. Kinetic lags between recruitment of signaling domain proteins and c-Cbl are also important. Recruitment of c-Cbl marks the dissociation of the signaling complexes in the microclusters and contributes to the window for active signaling at the microcluster. Thus, calcium-dependent stepwise assembly of microcluster components followed by bimodal dissociation of signaling proteins from microclusters represent new modes of T cell signal regulation.

REGULATION OF SIGNALING FROM MICROCLUSTERS VIA ENDOCYTOSIS AND RECYCLING

Imaging of live T cells in real time during activation has revealed the changing signaling components and dynamics of microclusters. Soon after microclusters form, molecular mechanisms are activated to disassemble them and regulate the extent of signaling. These include recruitment of inhibitory receptors or adapters that either compete for binding with ligand or recruit phosphatases that allow for dephosphorylation of tyrosine residues and stochastic release of SH2 domain-containing proteins (Acuto et al., 2008; Yokosuka et al., 2010, 2012; Kong et al., 2019). Activation-induced protein endocytosis at microclusters is another effective way to regulate signaling duration by rapidly altering the subcellular locations of signaling proteins (Balagopalan et al., 2009). The recruitment of the E3 ubiquitin ligase c-Cbl coincides with microcluster disassembly and endocytosis of signaling molecules (Bunnell et al., 2002; Yokosuka et al., 2005; Yi et al., 2019). Dynamic disassembly of microcluster components indicates signal termination because reducing the dissociation of microclusters results in increased T cell signaling (Mossman et al., 2005; Barr et al., 2006; Balagopalan et al., 2007; Nguyen et al., 2008; Hashimoto-Tane et al., 2010; Lasserre et al., 2010; Vardhana et al., 2010).

Several studies have collectively shown that following T cell activation, increased receptor endocytosis, diminished recycling, and an increase in degradation causes a reduction in the number of TCR molecules at the plasma membrane (Alcover and Alarcon, 2000; Geisler, 2004). In a spatial context, signal initiation is thought to occur at the periphery of the IS and terminate at the cSMAC where TCRs are centrally accumulated and then internalized (Lee et al., 2003; Varma et al., 2006). A recent study using photoactivation to follow endocytosed TCR in real time reported that TCR endocytosis increased upon T cell stimulation and internalized TCR sorted into an endosomal compartment marked by flotillins that control recycling of TCR to the immunological synapse (Compeer et al., 2018). The strength of TCR signal plays a role in signal termination, with both weak and strong stimuli causing recruitment of signaling microclusters in the pSMAC and cSMAC, but strong ligands inducing TCR internalization from the cSMAC (Cemerski et al., 2008). Surprisingly, a study using Correlative *L*ight and Electron Microscopy (CLEM; see **Box 1** in **Supplementary Material**) revealed that the majority of the centrally accumulated TCRs (in an IS formed on a lipid bilayer) are located on extracellular microvesicles (Choudhuri et al., 2014) that may serve as a channel for cell-to-cell communication with the APC (Mittelbrunn et al., 2011). Studies from our laboratory revealed that the dissipation of LAT and SLP-76 molecules away from early sites of microcluster formation are endocytic events (Barr et al., 2006; Balagopalan et al., 2007). While SLP-76 is endocytosed in a clathrin-independent mechanism, LAT is endocytosed via multiple pathways. After internalization from the PM, a portion of LAT undergoes retrograde trafficking to the Golgi (Carpier et al., 2018) and is delivered back to the synapse in an anterograde trafficking pathway regulated by golgin molecules (Zucchetti et al., 2019). Interestingly, in the case of both TCR and the adapters SLP-76 and LAT, internal pools of signaling-competent endosomes have been detected (Barr et al., 2006; Yudushkin and Vale, 2010; Evnouchidou et al., 2020), indicating that at least some of the endocytosed molecules are still active. Signals emanating from complexes located in endosomes might be qualitatively and/or quantitatively

different from signals generated from complexes located at the plasma membrane.

As indicated by the recruitment of the E3 ligase c-Cbl to microclusters, an important molecular mechanism that determines the cellular fate of endocytosed signaling molecules is ubiquitination. Inhibition of cellular ubiquitination increased microcluster lifetime and signal persistence (Barr et al., 2006; Vardhana et al., 2010). The Cbl family of ubiquitin ligases promote the ubiquitination and degradation of ZAP-70, Lck, LAT, SLP-76, Vav1, and WASP (Rao et al., 2000, 2002; Miura-Shimura et al., 2003; Barr et al., 2006; Balagopalan et al., 2007; Reicher et al., 2012). We have shown previously that the endocytosis of microclusters containing LAT and SLP-76 is regulated by c-Cbl mediated ubiquitination, and inhibition of c-Cbl function increases microcluster lifetime (Barr et al., 2006; Balagopalan et al., 2007). Ubiquitin is a sorting signal that regulates trafficking events within the endocytic pathway (Piper et al., 2014), and ubiquitin-binding ESCRT-I protein Tsg101 recognizes ubiquitinated chains of signaling proteins to be transported to lysosomes (Vardhana et al., 2010). Another important negative feedback mechanism is the phosphorylation of the adapters SLP-76 by the serine–threonine kinase HPK1 (hematopoietic progenitor kinase 1). Phosphorylation of SLP-76 on serine promotes 14-3-3 binding (Di Bartolo et al., 2007; Lasserre et al., 2011), resulting in SLP-76 ubiquitination and degradation (Wang et al., 2012). Thus, multiple endocytic feedback loops operate to regulate the extent of signaling from microclusters at the IS (**Figure 2**).

Consistent with a role for protein ubiquitination in signal termination, a LAT mutant that cannot be ubiquitinated (LAT 2KR) displayed enhanced signaling (Balagopalan et al., 2011; Kunii et al., 2013; Rodriguez-Pena et al., 2015). In a recent study, we examined the correlation between LAT ubiquitination and LAT cellular trafficking by comparing the cellular route of 2KR and wild-type LAT. Though internalization of LAT is Cbl and ubiquitin-dependent, ubiquitin-resistant 2KR LAT and wild-type LAT were internalized at comparable rates, indicating that LAT ubiquitination itself is not necessary for internalization of LAT (Balagopalan et al., 2020). LAT is predominantly monoubiquitinated (Balagopalan et al., 2011) and though a single Ub is perhaps an insufficient endocytic signal, the aggregate effect of multiple Ubs on multiple microcluster proteins may trigger endocytosis (Piper et al., 2014). Critically, LAT ubiquitination served as a signal for lysosomal trafficking and degradation, thus preventing LAT recycling to the cell surface. In 2KR LAT molecules that cannot be ubiquitinated, mutant LAT continues to recycle back to the cell surface, thus increasing the protein lifetime of LAT and providing a cellular trafficking correlate for the enhanced function of 2KR LAT (Balagopalan et al., 2020).

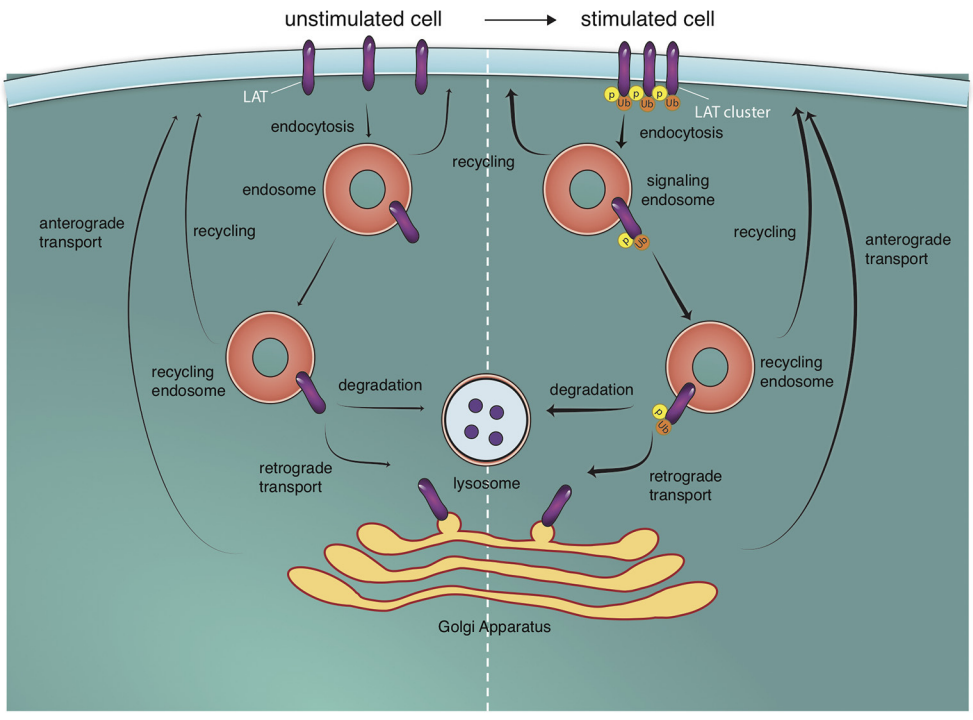

FIGURE 2 | Endosomal trafficking pathways to and off the T cell surface. T cell stimulation triggers the formation of microclusters. LAT molecules (and other signaling molecules) in microclusters are phosphorylated (p) and ubiquitylated (u). These molecules are internalized into endosomes from which they can potentially signal. They can then proceed to the lysosome where they are degraded or be recycled back to the cell surface. Recycling back to the cell surface can occur directly or via retrograde trafficking to the golgi apparatus and anterograde trafficking from the golgi apparatus to the plasma membrane. These trafficking pathways also exist in unstimulated cells but are increased upon T cell activation (indicated by thicker arrows).

RECRUITMENT OF VESICLES CONTAINING SIGNALING MOLECULES TO THE IS

The IS is a site of bi-directional membrane trafficking. In addition to endocytic events described above, polarized traffic of exocytic vesicles to the IS is crucial for T cell function. During the formation of the IS, the movement of the microtubule organizing center (MTOC) toward the APC results in the directed secretion of cytokines in helper T cells (Kupfer et al., 1991) and secretory granules in cytotoxic T cells (Stinchcombe et al., 2006). Several recent studies have described the vesicular delivery of signaling molecules important in early activation of T cells. Signaling molecules critical for T cell activation, such as TCRζ, Lck, and LAT, are not present at the plasma membrane exclusively. They also reside in distinct, non-overlapping intracellular compartments (Soares et al., 2013) that are rapidly polarized toward the IS upon T cell activation (Ehrlich et al., 2002; Bonello et al., 2004; Das et al., 2004; Purbhoo et al., 2010). Signaling proteins in vesicular pools are delivered to the IS differentially via specific subpopulations of endocytic and exocytic machinery. Multiple regulatory proteins, such as Rab GTPases (Patino-Lopez et al., 2008; Carpier et al., 2018); t-SNAREs, SNAP-23, and syntaxin 4, and v-SNARES, VAMP-3, and VAMP-7 (Das et al., 2004; Larghi et al., 2013; Soares et al., 2013; Carpier et al., 2018); IFT system protein IFT20 (Finetti et al., 2009, 2015); Sorting Nexins (Osborne et al., 2015); and the ARP 2/3 activating WASH complex (Piotrowski et al., 2013), have been shown to play a significant role in the trafficking of TCRζ and proximal signaling proteins to the IS. The functional role of vesicular pools of signaling proteins were revealed in studies in which perturbations of the regulatory proteins involved in membrane trafficking interfered with T cell activation and function. Inhibition of SNARE-mediated fusion by tetanus toxin (Das et al., 2004), overexpression of dominant negative proteins (Patino-Lopez et al., 2008), and siRNA-mediated silencing of trafficking regulators (Finetti et al., 2009, 2015; Larghi et al., 2013; Soares et al., 2013; Carpier et al., 2018) have all clearly confirmed the importance of vesicular trafficking in T cell function. More recently, capture assays (Zucchetti et al., 2019) and optogenetic aggregation methods (Redpath et al., 2019) have emphasized the importance of the precise spatial organization of endocytic regulators in T cell activation.

Though the critical role of vesicular traffic of signaling proteins in T cell activation has been clearly demonstrated, how and when the vesicular pools of signaling molecules regulate T cell activation remains less defined. The relative roles of vesicular vs. cell surface LAT pools for phosphorylation of LAT and TCR signal transduction has been controversial. While some studies proposed that plasma membrane LAT is the predominantly phosphorylated pool of LAT (Lillemeier et al., 2010; Sherman et al., 2011; Balagopalan et al., 2013), others proposed that docking or fusion of LAT vesicles at the IS is critical for LAT phosphorylation (Williamson et al., 2011; Larghi et al., 2013; Soares et al., 2013). Clues to the spatial and temporal contribution of vesicular signaling proteins came from fast 4D imaging using LLSM. LLSM enabled simultaneous imaging of surface and vesicular pools at the initiation of T cell activation, and revealed a role for both cellular pools. Early T cell activation was observed to occur in two phases: a first phase when recruitment of predominantly cell surface proteins formed microclusters, and a second phase, when the large pool of vesicles associated with the MTOC are recruited to the synapse (**Figure 3**) (Ritter et al., 2015; Balagopalan et al., 2018). In the second phase, directed movement of vesicles between microclusters on microtubules was observed. Vesicles displayed decreased speed and increased contact times at microclusters. Microclusters displayed fluorescence oscillations with an increase in fluorescence of LAT and signaling molecules coincident with when vesicles interacted with microclusters (Balagopalan et al., 2018). The observed oscillations indicate that vesicles sustain T cell signaling via delivery of a second wave of signaling molecules.

Once trafficked to the microcluster, LAT on vesicles could be either trans-phosphorylated by ZAP-70 localized at the PM or cis-phosphorylated once they fuse with the PM. There are contradictory reports about whether LAT vesicles undergo fusion with the PM. A flow cytometry approach to detect cell surface recruitment of LAT did not detect an accumulation of fused LAT at the PM (Larghi et al., 2013), leading to the conclusion that LAT vesicles dock close to but do not fuse with the PM. In contrast, interference with calcium-dependent vesicular fusion either by chelation of calcium or silencing of the calcium sensor synaptotagmin7 impeded microcluster formation, leading to a model in which calcium-dependent exocytosis of vesicles drives T cell signaling (Soares et al., 2013). Live-cell imaging using LLSM to directly visualize this process captured increases in LAT fluorescence when vesicles approached the IS. While the increases in fluorescence could be representative of vesicle fusion, a complete collapse of the vesicle was not observed (Balagopalan et al., 2018). This leads to the possibility that either vesicles dock transiently at microclusters or they undergo "kiss and run" exocytosis (Alabi and Tsien, 2013). It should be noted that the temporal acquisition speed of LLSM (4 s/frame) is too slow to allow for capture of exocytic events that occur very rapidly. A decrease in LAT signal from the vesicle after the flare is indicative of vesicular LAT delivery to the PM. In addition, no detectable LAT phosphorylation in subcortical vesicles was observed (Purbhoo et al., 2010), lending support to the model that LAT vesicles fuse with the synaptic membrane where LAT phosphorylation occurs (**Figure 3**). Polarized vesicle transport may also regulate the lipid composition at microclusters. A study of lipid order of sub-synaptic vesicles showed that they are not a homogenous population and vesicles display different degrees of membrane lipid order. Interestingly, LAT segregates into higher membrane order vesicles as it does on the PM (Ashdown et al., 2018). Thus lipid order-based sorting and delivery of cargo could contribute to maintaining lipid composition in the microcluster vicinity (Gagnon et al., 2012; Dinic et al., 2015).

Vesicle movement at the IS appears to be demarcated by microcluster location (Purbhoo et al., 2010; Balagopalan et al., 2018). Given the organization of the TCR and LAT

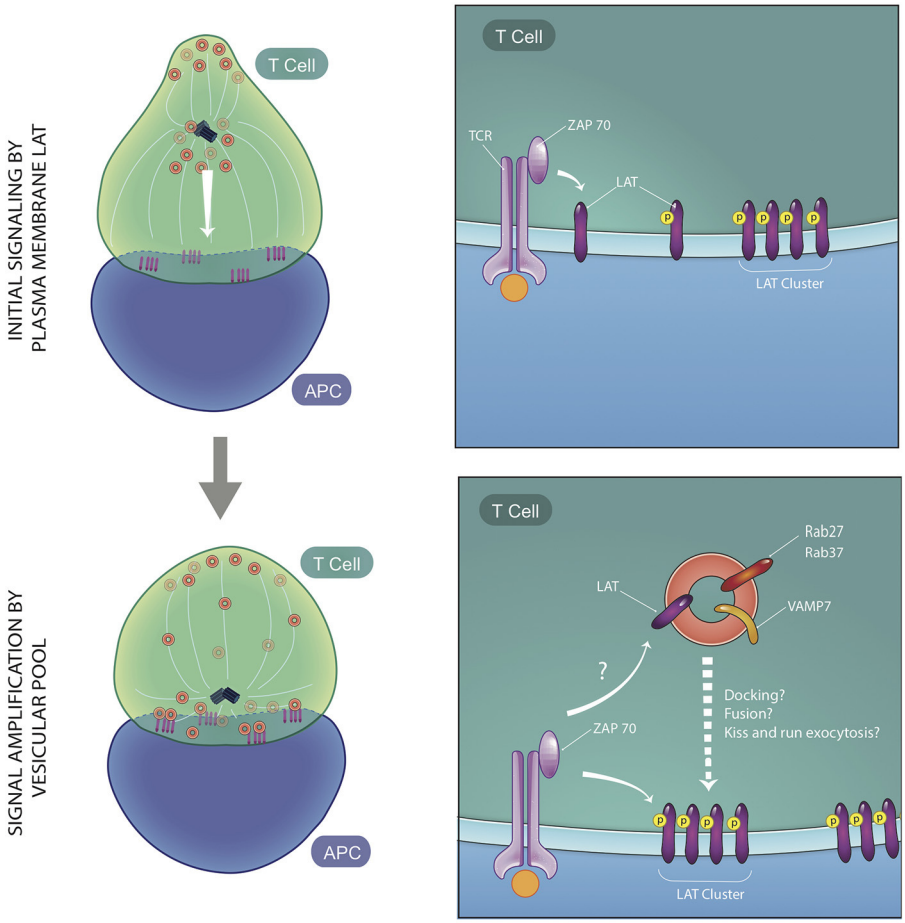

FIGURE 3 | Two phases of early T cell activation. At early time points, vesicles containing signaling molecules are several microns away from the immune synapse, while plasma membrane-resident LAT is phosphorylated by ZAP70 and moves laterally to be recruited to microclusters. Soon after, vesicles expressing LAT, VAMP7, and Rabs are recruited to the immune synapse. These vesicles maintain and amplify signaling at the microclusters and interact dynamically with microclusters via either docking, fusion, or kiss and run exocytosis at microcluster sites. ZAP70 may trans-phosphorylate LAT on vesicles or cis-phosphorylate LAT at the PM, once vesicular fusion occurs (figure adapted from Balagopalan et al., *Nature Communications* 2018).

in adjacent spatial domains, vesicular trafficking could be directed precisely to distinct nanoterritories at the IS. Spatial confinement of exocytosis to specialized plasma membrane regions has been reported in several biological systems (Yuan et al., 2015), and an important next step will be to investigate whether localized docking and/or exocytosis of vesicles occur at microcluster "hotspots." Vesicle docking and fusion machinery such as SNARES (Chang et al., 2017) and exocyst components (Saez et al., 2019) may serve to mark microclusters as active docking or fusion zones. Microdomains enriched in intracellular calcium (Wei et al., 2009) could locally target calcium-dependent vesicle fusion. Just as differential usage of membrane trafficking regulators enables orchestration of endosome trafficking, defined spatial organization of fusion molecules could allow for targeting of distinct signaling molecules to discrete adjacent plasma membrane territories. Precise localization of fusion machinery and visualization of their accumulation kinetics are important next steps in uncovering the highly synchronized process of exocytosis and endocytosis at the IS.

FUTURE GOALS

Increases in spatial and kinetic resolution in imaging technologies will certainly allow for novel insights into the interplay between the recruitment of molecules to the IS, compartmentalization of signaling components, vesicle movement, and location of signaling activity. The ability to combine super-resolution microscopy with readouts of function could provide insights into how signaling molecule organization at the nanoscale correlates with T cell activation and immune function. Multiplexing of biophysical measurements, high-throughput readouts and super-resolution imaging will be powerful next steps in uncovering novel insights to further understand immune cell signaling at the nanoscale. Such

advances could potentially be used to manipulate T cell function in future immunotherapy.

AUTHOR CONTRIBUTIONS

LB and LS conceptualized, wrote, and edited the review, KR wrote sections of the review. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.608530/full#supplementary-material

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REVIEW
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How the Discovery of the CD4/CD8-p56^{lck} Complexes Changed Immunology and Immunotherapy

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The past 25 years have seen enormous progress in uncovering the receptors and signaling mechanisms on T-cells that activate their various effecter functions. Until the late 1980s, most studies on T-cells had focused on the influx of calcium and the levels of cAMP/GMP in T-cells. My laboratory then uncovered the interaction of CD4 and CD8 co-receptors with the protein-tyrosine kinase p56^{lck} which are now widely accepted as the initiators of the tyrosine phosphorylation cascade leading to T-cell activation. The finding explained how immune recognition receptors expressed by many immune cells, which lack intrinsic catalytic activity, can transduce activation signals via non-covalent association with non-receptor tyrosine kinases. The discovery also established the concept that a protein tyrosine phosphorylation cascade operated in T-cells. In this vein, we and others then showed that the CD4- and CD8-p56^{lck} complexes phosphorylate the TCR complexes which led to the identification of other protein-tyrosine kinases such as ZAP-70 and an array of substrates that are now central to studies in T-cell immunity. Other receptors such as B-cell receptor, Fc receptors and others were also subsequently found to use *src* kinases to control cell growth. In T-cells, p56^{lck} driven phosphorylation targets include co-receptors such as CD28 and CTLA-4 and immune cell-specific adaptor proteins such as LAT and SLP-76 which act to integrate signals proximal to surface receptors. CD4/CD8-p56^{lck} regulated events in T-cells include intracellular calcium mobilization, integrin activation and the induction of transcription factors for gene expression. Lastly, the identification of the targets of p56^{lck} in the TCR and CD28 provided the framework for the development of chimeric antigen receptor (CAR) therapy in the treatment of cancer. In this review, I outline a history of the development of events that led to the development of the "TCR signaling paradigm" and its implications to immunology and immunotherapy.

Keywords: p56^{lck} tyrosine kinase, CD4, CD8, tyrosine phosphorylation, chimeric antigen receptor, immunotherapy, T-cell signaling paradigm, protein-tyrosine activation cascade

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INTRODUCTION

The last decades have witnessed major advances in the identification of the receptors and signaling pathways that control the activation and differentiation of T-cells. Early work in understanding the key signaling events involved the demonstration that anti-CD3 antibodies could increase intracellular calcium (Ca^{2+}) levels as detected by the Indo-1 indicator dye (Tsien et al., 1982). Other pathways involved the identification of oscillations in cAMP/cGMP, the activation of phospholipase C (PLC) which was known to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP2) into the Ca^{2+}-mobilizing second messenger, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Imboden and Stobo, 1985). DAG is a physiological activator of protein kinase C (PKC). Oscillations in calcium were later shown to be essential to the activation of the transcription factor nuclear factor of activated T-cells (NFAT) (Shaw et al., 1988; Jain et al., 1992).

CD4/CD8-p56lck AND THE INITIATION OF TCR SIGNALING

Despite this important work, a critical missing area was the possible involvement of protein tyrosine phosphorylation in T-cells. Emerging data had underscored the importance of this type of phosphorylation in regulating multiple events in other mammalian cells. Most phosphorylation occurs on serine and threonine with <1% on tyrosine residues. Tony Hunter had described phosphorylation on tyrosine residues in the late 1970s, working on middle T-antigen (Eckhart et al., 1979). Transmembrane receptors such as the platelet-derived growth factor receptor (PDGF-R) and the insulin receptor were then found to have intrinsic protein-tyrosine kinase domains in their cytoplasmic tails (Rudd, 1990; Hunter, 2007). However, another family of soluble protein-tyrosine kinases had also been defined with the prototype $pp60^{src}$. Notably, a truncated form of the kinase termed $pp60^{v-src}$ had been identified in the *Rous sarcoma virus* which acted as an oncogene (Parker et al., 1981). Michael Bishop and Harold Varmus had won the 1989 Nobel Prize for showing that the oncogene in the virus was an altered version of a gene derived from the normal cellular gene of normal cells. However, the cellular homolog $pp60^{src}$ had no apparent function in mammalian cells. A role for *src* family members in normal cell function had been unclear. The *src* family of non-receptor tyrosine kinases (SFKs) include Src, Fyn, Yes, Lck, Hck, Blk, Fgr, Lyn, and Yrk (Neet and Hunter, 1996; Serfas and Tyner, 2003). Src, Yes, Lyn, and Fyn are widely expressed in cells, while Blk, Fgr, Hck, and Lck are expressed primarily in hematopoietic cells (Thomas and Brugge, 1997). T cells express predominantly Lck and Fyn that include an alternatively spliced isoform of Fyn termed Fyn^{T}.

In immunology, there was a major gap in knowing whether protein-tyrosine kinases, or a potential phosphorylation cascade operated in T-cells and other immune cells. There were no known surface receptors with endogenous protein-kinase domains connected to the antigen-receptor (TCR/CD3 complex) and little evidence of tyrosine phosphorylation in immune cells. The main evidence came from studies on LSTRA cells, T-cell lymphoma transformed by the Moloney Murine Leukemia Virus that showed elevated tyrosine phosphorylation of intracellular proteins (Casnellie et al., 1982; Gacon et al., 1982; Voronova et al., 1984). However, it was unclear whether this was an anomaly and whether receptors on normal T-cells engage tyrosine kinases to evoke a phosphorylation cascade. The lab of Larry Samelson and Richard Klausner provided some of the first hints by showing that a p21 chain associated with the T cell antigen receptor underwent tyrosine phosphorylation of 294 hybridoma T-cells (Samelson et al., 1986b).

The central problem was that neither the TCR itself nor its associated CD3 γ/ε, δ/ε, or ζ chains showed sequence homology with known protein-tyrosine kinases. Given this situation, it seemed a reasonable possibility to us that the TCR might be coupled to an unidentified transmembrane tyrosine kinase receptor, an activator of a kinase protein tyrosine kinase, or in some unusual manner, might bind to a protein-tyrosine kinase. Our initial studies initially showed little endogenous kinase activity co-precipitated with the anti-CD3 precipitated TCR complex in auto-phosphorylation kinase assays. This observation shifted our attention to the co-receptors CD4 and CD8, which had recently been shown to bind to non-polymorphic regions of the major histocompatibility complex (MHC) (Meuer et al., 1982). For example, the α chain of the CD8 complex binds to HLA's α2 and α3 domains of MHC class 1 antigens (Gao et al., 1997). We envisioned that a situation where a kinase associated with CD4 and CD8 might be brought into physical proximity with the TCR complex for its phosphorylation.

From the outset of our work in 1986, we found that immune precipitates of CD4 and CD8 possessed an unusually high level of endogenous tyrosine kinase activity that was not observed in the precipitates of other receptors. Further, in addition to the phosphorylation of the exogenously added substrate, enolase, we observed a well-labeled band in the 56–65 Kd range in anti-CD4 and CD8 precipitates that was labeled on tyrosine residues (Rudd et al., 1988; Barber et al., 1989). Two other bands in the 30–35 Kd and 75–80 Kd range were also labeled in the anti-CD4 and CD8 precipitates (Rudd et al., 1988; Barber et al., 1989). None of these bands corresponded to CD4 or CD8 indicating that the co-receptors themselves were unlikely to be substrates of the endogenous co-precipitated kinase.

Independent work on $pp60^{src}$ had shown that *src*-related kinases could phosphorylate themselves in a process termed auto-phosphorylation. This occurs when a kinase's active site catalyzes its own phosphorylation (cis autophosphorylation), or when a kinase provides the active site of an adjacent kinase (trans autophosphorylation). It did not escape our notice that the band at 55–65 kd was of a similar size as $pp60^{c-src}$, although src was poorly expressed in T-cells. Perhaps a related kinase might be phosphorylating itself in precipitates, and perhaps it was immune cell-specific mirroring the cell-specific nature of receptors on the surface of immune cells. It may seem self-evident now, with the available information, but at the time this was a rather grand conceptional jump. In this context, a protein at 56 Kd, originally

termed LSTRA protein-tyrosine kinase had been seen in LSTRA lymphoma T-cells by the labs of Bart Sefton and Edwin Krebs (Casnellie et al., 1982; Gacon et al., 1982; Voronova et al., 1984). The kinase was subsequently cloned by Jamey Marth in the lab of Roger Perlmutter [encoded by a genetic locus defined as lsk^{T}] and found to be a T-cell-specific member of the pp60^{src} family, LCK or p56^{lck} (Marth et al., 1985). However, as in the case of the parental kinase pp60^{src}, no function for p56^{lck} had been identified in normal T-cells. The idea that *src* kinases could in some manner interact with surface receptors, rather than interacting solely with intracellular components such as middle T-antigen, had not been established.

Using an anti-p56^{lck} sera from Jim Trevillyan at the University of Texas, we showed that it reacted with our 56Kd protein that had been labeled *in vitro* kinase assays using a combination of blotting and re-precipitation analysis (Rudd et al., 1988; Barber et al., 1989). This clearly showed that the CD4 and CD8 receptors interacted with the *src* family member called p56^{lck}. In our original paper, we stated: "the association appears to represent the only known case of an association between a receptor on the surface of T cells and a member of a family of intracellular mediators with an established ability to activate and transform cells." The fact that both CD4 and CD8 bound to p56^{lck} was consistent with their similar, but complementary roles in binding to non-polymorphic regions of MHC class II and class 1 antigens, respectively. CD4 binds to p56^{lck} in a monomeric form, although in certain contexts, the receptor may form dimers or multimers (Lynch et al., 1999; Matthias et al., 2002; **Figure 1A**). By contrast, CD8 exists as a α/β heterodimer or a α/α homodimer within which the p56^{lck} binds to the CD8α subunit. The homodimer can recruit two p56^{lck} molecules, while the CD8α/β heterodimer binds a single p56^{lck} (**Figure 1A**).

The CD4 and CD8-p56^{lck} complexes were the first examples of a protein-tyrosine kinase to associate with a surface receptor. They were also the first case of an interaction with an SFK and explained how receptors that lack intrinsic catalytic activity could transduce activation signals. The interaction provided a mechanism by which the antigen receptor could induce a possible tyrosine phosphorylation cascade in T-cells and put the focus on p56^{lck} as the central player of T-cell activation, some of which is receptor associated and the rest of which exists in a receptor-free form in cells.

Our original submitted paper languished for over a year with Nature from 1986 to 1987, at which time we decided to re-submit to PNAS for publication and to file patents, which were filed and granted several years later (Nos. 5,250,431, 1993, US5432076; EP0347143A2, 1988). I also began to discuss our unpublished findings openly with colleagues at the Dana-Farber Cancer Institute which led to a contact from Andre Veillette in the lab of Joseph Bolen at the National Institutes of Health. After some discussion, they agreed to collaborate showing the presence of the CD4 and CD8-p56^{lck} complexes in mouse cells (Veillette et al., 1988). This collaborative work was very important and helpful to us, given that, at the time, my group was comprised of a young technician and myself, without an established reputation in the field of protein-tyrosine kinases. The work in our first paper was supported by the Cancer Research Institute (NY), an organization whose founding was based on the work of Dr. William B. Coley in the late 1800s to treat cancer patients with immunotherapy. We were gratified that our CD4 and CD8-p56^{lck} complexes as initiators of the activation cascade in human T-cells are the same signal mediators that stimulate T-cells to react and kill tumors in immunotherapy. Our first paper was recognized as "*Pillars of Immunology*" paper by the American Association of Immunologists together with a paper from our collaborators in the Bolen lab (Rudd et al., 2010; Veillette et al., 2010).

CD4 and CD8-p56^{lck} complexes became models for how other immune receptors employ SFKs in immune cell activation. Lyn and Fyn were subsequently found to associate with the Igα/Igβ heterodimer subunits of the B cell receptor in B-cells (Gauld and Cambier, 2004), Src and Lyn to the Fc receptor (FCR) (Wu et al., 2001) and Fyn and Lyn to the glycoprotein VI (GPVI)-FcR gamma-chain complex, a key receptor for collagen on platelets (Suzuki-Inoue et al., 2002). In fact, a single Lyn single molecule may be sufficient to initiate phosphorylation of multiple aggregated high-affinity IgE receptors (Wofsy et al., 1999). Further, pp60^{Src} is activated by binding the integrin β cytoplasmic domain (Arias-Salgado et al., 2003), while in T-cells, p59^{fyn}, and p56^{lck} associates, albeit with lower stoichiometry, with the CD3 subunits of the TCR receptor (Hartl et al., 2020). p56^{lck} was also been found to associate with the co-receptor CD28 by using its SH2 domain to bind to a phospho-specific site (Kong et al., 2011).

With an emphasis placed on p56^{lck}, it was subsequently ablated in mice and found to be needed for the early and late stages of thymic differentiation (using proximal and distal Lck promoters) (Teh et al., 1991), naive T cell survival (Seddon and Zamoyska, 2002), and T-cell activation. Lck/Fyn double deficient mice show a 3 stage (DN3) block in the thymus which requires pre-TCR signaling (Liao et al., 1997). Similarly, B-cells require Lyn kinase activity for B-cell receptor phosphorylation and function (Fujimoto et al., 1999). Likewise, macrophages lacking the Hck and Lyn are defective in IgG-mediated phagocytosis (Fitzer-Attas et al., 2000). Other examples exist.

In the field of cancer biology, as mentioned, previous seminal work had documented how truncated forms of pp60^{v-src} transformed cells; however, a role for non-oncogenic src-related kinases had been missing. Other non-lymphoid surface receptors, such as the platelet-derived growth factor receptor (PDGF-R) were eventually also shown to bind and generate signals via SFKs (Thomas and Brugge, 1997; Rudd, 1999).

Lastly, our studies impinged on the field of acquired immunodeficiency syndrome (AIDS) and the human immunodeficiency virus (HIV-1), being the first example of a mediator to associate with the HIV-1 receptor, CD4 (Rudd et al., 1988). p56^{lck} and its binding to CD4 were later shown to provide signals that regulate HIV-1 transcription in T-cells (Tremblay et al., 1994). HIV-1 induced apoptosis is accelerated by interaction of CD4 with p56^{lck} (Corbeil et al., 1996).

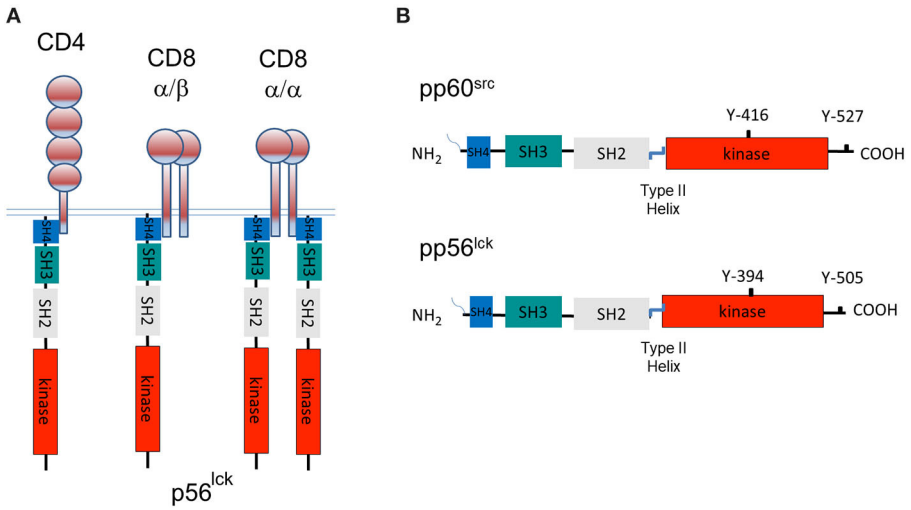

FIGURE 1 | A tale of three CD4 and CD8-p56^{lck} complexes and the structure of pp60^{src} and p56^{lck}. **(A)** The model of three CD4 and CD8-p56^{lck} complexes in T-cells. CD8 is expressed as a CD8α homodimer as well as a CD8α/β heterodimer. p56^{lck} binds to the α subunit but not the β subunit. CD8α homodimer has two p56^{lck} bound molecules and the CD8α/β heterodimer has a single p56^{lck} bound. CD4 binds to p56^{lck} in a monomeric form. **(B)** Structure of pp60^{src} and p56^{lck}. p56^{lck} is an immune cell-enriched member of the pp60^{src} family of protein-tyrosine kinases. p56^{lck} is myristoylated and palmitoylated at the N-terminus, while Src lacks palmitoylation sites. This region is followed by poorly conserved unique SH4 region which in the case of p56lck binds to the cytoplasmic tails of CD4 and CD8, an SH3 domain that binds to proline-rich residues, an SH2 domain that binds to specific sites that are tyrosine phosphorylated, an SH2-kinase linker region, an SH1 kinase domain followed by a C-terminal negative regulatory region. The C-terminal tail has an inhibitory Y-527 site when phosphorylated, in the case of pp60^{src} and a Y-505 site in p56^{lck}. pp60^{src} and p56^{lck} also possess an auto-phosphorylation site in the kinase domain of each kinase corresponding to Y-416 in the case of pp60^{src} and Y-394 in the case of p56^{lck}.

CD4/CD8-p56^{lck} AND PHOSPHORYLATION OF THE TCR COMPLEX

The CD4/CD8-p56^{lck} complexes serve as the initiators of the protein tyrosine phosphorylation cascade in T-cells. As we stated: "an association between the T4 (CD4) receptor and the PTK within the cell would introduce a specific pathway by which T-cells become activated. The T4 (CD4)-associated kinase could act to phosphorylate various intracellular candidates. An obvious and important candidate would be the subunits of the T3-Ti antigen receptor complex." We envisioned this to occur during antigen-presentation by dendritic cells due to CD4 or CD8 and the TCR coordinate binding to MHC antigens. This event would bring p56^{lck} into close physical proximity where trans-phosphorylation could occur. In agreement with this model, using substrate phosphorylation assays, we showed that CD4 and CD8-p56^{lck} could trans-phosphorylate the TCR-ζ and the CD3γ, δ, ε chains on tyrosine residues (Barber et al., 1989). We even observed that anti-CD4 co-precipitated TCR-ζ and all CD3 chains phosphorylated on tyrosine residues (Burgess et al., 1991). Antibody cross-linking of CD4 was also found result in the zeta chain phosphorylation on tyrosine residues (Veillette et al., 1989). Together, these observations fit nicely with the finding of tyrosine phosphorylation of the human TCRζ chain in hybridomas (Weissman et al., 1988) and in T-cells from patients with lymphoproliferative disorders (Samelson et al., 1986a), but additionally, implicated the CD3 subunits as targets of p56^{lck}. Subsequent imaging studies underscored the importance of the spatial distribution of TCR and p56^{lck} in the initiation of T-cell signaling (Purbhoo et al., 2010; Rossy et al., 2012). Antigen-engaged TCRs may scan for co-receptors coupled to p56^{lck} as a rate-limiting step in T-cell activation (Stepanek et al., 2014).

Subsequent work showed that p56^{lck} binding to CD4 also masks a key dileucine motif required for clathrin-mediated endocytosis of CD4 is masked by p56^{lck} (Kim et al., 2003). Although not well-publicized, this observation suggests a second function for p56^{lck} binding to CD4 in increasing the lifespan of CD4 on the surface of T-cells for the generation of activation signals. Following T cell activation, p56^{lck} dissociates from CD4 allowing the coreceptor to be internalized (Pelchen-Matthews et al., 1992, 1993).

Shortly after the 1988 papers, Michael Reth identified a consensus sequence (D/E)xxYxx(I/L)x6–8Yxx(I/L) in the TCR associated chains (Reth, 1989), motifs that eventually became known as the immuno-receptor tyrosine-based activation motifs (ITAMs) (Cambier, 1995). TCR-CD3ζ homodimer possesses six ITAMs while ITAMs existed in the CD3 subunits, each carrying one ITAM. The presence of the ITAMs in both the CD3 and zeta subunits fit nicely into our observations that the various chains were all phosphorylated by p56^{lck}. ITAMs were found also in the CD79-alpha and -beta chains of the B cell receptor complex, certain Fc receptors and other receptors (Zettlmeissl et al., 1990).

A major question that persists today is why are there so many ITAMs within a single receptor complex as targeted by p56^{lck}? Is it a case of evolutionary redundancy, dosage compensation or do different ITAM send unique signals? Several groups heroically

attempted to define a precise order of phosphorylation of the CD3ζ tyrosine residues (Kersh et al., 1998; Housden et al., 2003). ^{1}H-NMR studies of recombinant zeta chain have shown p56^{lck} sequential phosphorylation of the TCRζ N-terminal tyrosine (N1) first followed by 3N >3C >2N >1C >2C (Housden et al., 2003). The efficacy of ITAM phosphorylation also depends on the accessibility of the cytoplasmic tails. The CD3 subunits and zeta chains lie attached at the inner layer of the plasma membrane due to electrostatic interactions with phosphoserine (PS) (Shi et al., 2013). This feature protects ITAMs from spontaneous phosphorylation (Xu et al., 2008; Ma et al., 2017) and accessibility to p56^{lck} (Gil et al., 2002). In this model, increased intracellular calcium and its binding to negatively charged PS may free the CD3-zeta subunits cytoplasmic tails for CD4 and CD8-p56^{lck} access and phosphorylation. It remains uncertain whether the sequential phosphorylation by p56^{lck} of ITAMs has a physiological role in regulating T-cell immunity.

Nevertheless, increasing phospho-ITAMs has been reported to correlate with distinct T cell responses, such as activation, anergy, or apoptosis (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Combadiere et al., 1996; Kersh et al., 1998). Others have documented a linear correlation between the number of wild-type CD3 ITAMs and T cell proliferation, but not in terms of cytokine production (Holst et al., 2008). A low number of TCR-CD3 ITAMs suffices to support cytokine secretion (Guy et al., 2013). However, despite this effort, a seminal paper from the from the lab of Marie and Bernard Malissen showed that the crippling of zeta ITAMs did not impair T cell receptor signaling and only marginally affected T-cell responses to antigen *in vivo* (Ardouin et al., 1999). It, therefore, appeared that the ITAMs in the remaining CD3 subunits sufficed to generate signals needed for *in vivo* responses to antigen. It may, therefore, be possible that the multiplicity of ITAMs regulates proliferation to antigens of low affinity or abundance. From another direction, an interesting study from the lab of Dario Vignali documented a role for multiple ITAMs in thymic selection which discriminates self-antigen on the basis of affinity. Mice with fewer than seven wild type TCR ITAMs developed a lethal, multiorgan autoimmune disease due to defective central tolerance (Holst et al., 2008).

Whether access to glycosphingolipid enriched microdomains (GEMs) or rafts is needed is an open question (Pizzo and Viola, 2003). Rafts are enriched with SFKs (Bunnell et al., 2002) where in the case of p56^{lck}, lipidation targets the kinase to lipid rafts (Rodgers et al., 1994). TCR and CD4/CD8 also move into rafts during the TCR ligation process. The activating complexes in rafts facilitates p56^{lck} phosphorylation CD3 phosphorylation and activation (Arcaro et al., 2001), although others have reported that the kinase in these domains has low activity due to the action of the CBP/PAG/CSK inhibitory complex (Kabouridis, 2006). On the other hand, expression of a mutant construct of p56^{lck} with a transmembrane domain that is excluded from rafts was unable to phosphorylate the TCR (Kabouridis et al., 1997). Due to the fact that the TCR is not raft-associated in resting T cells, these microdomains are likely to play greater roles in maintaining rather than initiating TCR signaling. It is worth noting that cholesterol-rich rafts are also modulated by co-receptors CD28 which promote and CTLA-4 which disassemble the domains (Martin et al., 2001).

REGULATION OF THE CD4/CD8-p56^{lck} COMPLEX

While the regulation of signaling via receptors with intrinsic domains such as the PDGF-R involves dimerization and is well-understood, the mechanism underlying the function of the CD4 and CD8-p56^{lck} complexes is complex and still unresolved. Certain models involve cross-regulation by transmembrane and intracellular phosphatases and kinases, while other models involve the simple dimerization independent of phospho-regulation (Cooper and Qian, 2008). The crosslinking of CD4 with antibody can increase p56^{lck} activity; however, it is unclear that CD4 actually dimerizes during antigen-presentation (Veillette et al., 1989). Similarly, while CD4 and CD8-p56^{lck} complexes aggregate in microdomains and at the immunological synapse (IS), it is unclear whether this is mimics the close proximity of receptors induced by antibody crosslinking. Further, microdomains include the aggregation of numerous other immunoglobulin family members that could complete, or sterically interfere with potential CD4 and CD8 inter-molecular receptor interactions. Although enhanced p56^{lck} activities has been seen in membranes expressing CD4 or CD8 (Liaunardy-Jopeace et al., 2017), the lab of Oreste Acuto found that some 40 per cent of total p56^{lck} in naive T cells is constitutively active (Nika et al., 2010). Intriguingly, TCR and coreceptor engagement did not change the levels of activate p56^{lck} even though TCR ζ phosphorylation was observed (Nika et al., 2010). Overall, it remains an open question whether an increase in p56^{lck} catalytic activity is needed for the function of the CD4 and CD8-p56^{lck} complexes, or whether the simple localization of constitutively active p56^{lck} next to key substrates such as the ITAMs of TCRζ and CD3 chains is sufficient to initiate the activation cascade, as we originally proposed (Rudd et al., 1988; Barber et al., 1989; Rudd, 1990).

p56^{lck} has a classic structure involving an N-terminal src homology domain (SH4) that is myristoylated at Gly2 and palmitoylated at Cys3 and Cys5 (Kabouridis et al., 1997). The latter modification is needed for membrane binding and p56^{lck} diffusion to the IS (Yurchak and Sefton, 1995). Interestingly, all SFKs have palmitoylate linkages except Src and Blk. This region is followed by poorly conserved unique region, an SH3 domain that binds to proline-rich residues, an SH2 domain that binds to phospho-tyrosine motifs, a linker region, the SH1 kinase domain followed by a C-terminal negative regulatory region (**Figure 1B**). Within the kinase, there is an autophosphorylation site within the activation loop of the catalytic domain at residue Y-416 for pp60^{src} and Y-394 for p56^{lck}. At the C-terminus, there is a key negative regulatory residue at Y-527 for pp60^{src} and Y-505 for p56^{lck} (Martin, 2001). p56^{lck} is distinguished by an N-terminal CxxC motif in the SH4 domain that coordinates Zn^{2+} binding in a zinc clasp with CD4 and CD8 (Huse et al., 1998; Lin et al., 1998; Kim et al., 2003). Our initial comparison of the cytoplasmic

tails of CD4 and CD8 identified homologous motifs, Thr-Cys-Gln-Cys-Pro-His in CD4 and Val-Cys-Lys-Cys-Pro-Arg in CD8 for $p56^{lck}$ binding (Barber et al., 1989). It was evident that the β chain of CD8 did not have the motif (Barber et al., 1989). A more refined analysis identified conserved cysteines within a CxCP motif of CD4 and CD8α (Rudd et al., 1989; Shaw et al., 1990; Turner et al., 1990).

In an inactive conformation, $p56^{lck}$ is folded in upon itself as mediated by intra-molecular binding of the SH2 domain to the C-terminal inhibitory Y-505, an interaction aided by SH3 domain binding to the linker region (Xu et al., 1995). These interactions hold the structure in a closed inactive conformation (**Figure 2A**). Dephosphorylation at Y-505 is sufficient to unfold the kinase, holding the kinase in a primed conformation which requires autophosphorylation at Y-394 for full kinase activity.

C-terminal phosphorylation is regulated by inhibitory kinases and a stimulatory phosphatase. The kinases, C-terminal Src kinase (CSK) and the related CSK-homologous kinase (Chk) phosphorylate the C-terminal tyrosine, thereby inhibiting $p56^{lck}$ (Bergman et al., 1992; **Figure 2A**). Key to CSK function is the transmembrane adaptor termed CSK-binding protein (CBP/PAG). When phosphorylated, CBP/PAG recruits CSK to the membrane for its activation and access to SFKs. The dephosphorylation of PAG causes a loss of CSK from the vicinity of the TCR (Horejsi, 2004). CSK lacks N-terminal acylation sites, an autophosphorylation site and C-terminal regulatory sites found in $p56^{lck}$. The C-terminal tyrosine of SFKs may be the only substrate of CSK (Brown and Cooper, 1996). Unlike SFKs, the SH2 and SH3 binding pockets of CSK appear oriented outwards (Ogawa et al., 2002). They inhibit SFKs due to phosphorylation but also possibly by direct binding (Chong et al., 2005). CSK itself is phosphorylated and positively regulated by cAMP-dependent protein kinase (PKA) (Vang et al., 2001). In one model, CSK is activated by CBP/PAG in glycosphingolipid enriched microdomains (GEMs) (or rafts). Overall, the CBP/PAG-CSK complex is likely to maintain T-cells in a quiescent state until there is a requirement for activation signals.

Another key regulator of $p56^{lck}$ is the transmembrane phosphatase (PTPase) CD45 (Mustelin et al., 2002). First identified by the lab of Alan Williams in Oxford, and termed leucocyte common antigen (L-CA), it is an immune specific and unusually abundant protein on T-cells (Barclay et al., 1988; **Figure 2B**). It is highly conserved, comprising as much as 10% of protein on the surface of cells (Barclay et al., 1988). Structurally, it contains an extended extracellular domain, and two tandem intracytoplasmic catalytic PTPase domains (Tonks et al., 1990). We and others showed that CD45 is also processed into different isoforms (Rudd et al., 1987; Takeuchi et al., 1989), which define different subsets of T-cells (Wallace and Beverley, 1990). Naive T lymphocytes are positive for CD45RA with only the A protein region of the differentially spliced protein. By contrast, activated and memory T lymphocytes express CD45RO, the shortest isoform lacking all three of the A, B, and C regions.

Despite its clear importance, the nature of CD45 function and the relevance of the different isoforms continues to confound investigators since it appears to act as a positive and negative regulator (Charbonneau et al., 1989; Mustelin et al., 1989; McNeill et al., 2007; Courtney et al., 2019). Early studies showed that CD45 dephosphorylates Y-505 and activates $p56^{lck}$ (Mustelin et al., 1989), while the Ashwell lab showed that it also acts on the autophosphorylation site Y394 to inhibit full $p56^{lck}$ activity (Ashwell and D'Oro, 1999; **Figure 2B**). As evidence in support of a positive function, certain CD45-negative T cells fail to respond to TCR stimulation and increased CD45 expression correlates with increased sensitivity to TCR ligation (Koretzky et al., 1990; Cahir McFarland et al., 1993). However, others have found that with the inhibition of CSK, CD45 suppresses ζ-chain phosphorylation and alters the pool of active $p56^{lck}$ (Courtney et al., 2019). The kinetic-segregation model of TCR triggering excludes CD45 with its large ectodomain from ligated TCRs (Shaw and Dustin, 1997; Davis et al., 2003). CD45 may have different functions which depend on expression levels, adjacent regulatory molecules and the temporal stage of T-cell activation. In one model, the transient appearance of CD45 in rafts lead to p56lck dephosphorylation and activation. The field is further complicated by its dephosphorylation JAK (Janus kinase) kinases and its negative regulation of cytokine receptor signaling as well as in the negative regulation of other cells such as monocytic and erythroid differentiation (Irie-Sasaki et al., 2001). Further, CD45 seems to have different effects on different SFKs (Roach et al., 1997). Added to the mix, the cytoplasmic phosphatase SHP-1 also dephosphorylates at Y-394 to limit T-cell activation (Chiang and Sefton, 2001; Nagaishi et al., 2006).

CD4/CD8-lck INITIATE THE T-CELL TYROSINE PHOSPHORYLATION CASCADE

Aside from ITAMs, a second major substrate of $p56^{lck}$ is the protein-tyrosine kinase, zeta-chain associated protein kinase 70 (ZAP-70). We originally found that CD4-lck precipitated two other bands that were labeled on tyrosine residues at 38–40 Kd and 70–80 Kd in *in vitro* kinase assays (Rudd et al., 1988). Our initial precipitates showed that anti-SYK (spleen tyrosine kinase) was able to precipitate the 75 Kd protein; however, due to the limited quantity of the antisera available at the time, the results were considered unreliable. SYK had been described in B-cells as a novel protein tyrosine kinase with two tandem SH2 domains separated by a long linker (linker B) from a C-terminal kinase domain. Instead, a major seminal advance came from the lab of Art Weiss with the cloning of the 70 Kd band corresponding to Zeta-chain-associated protein kinase 70 (ZAP-70) (Chan et al., 1992). Similar to $p56^{lck}$, ZAP-70 is primarily expressed in T- and natural killer cells; however, it is structurally homologous to SYK with two SH2 domains that bind to two tandem tyrosines in each ITAM. $p56^{lck}$ phosphorylates both ITAMs needed for ZAP-70 recruitment and sites within ZAP-70 needed for its activation (Iwashima et al., 1994; Chan et al., 1995; **Figure 2C**).

Importantly, in the context of the tyrosine phosphorylation cascade, the range of substrates of $p56^{lck}$ and ZAP-70 are profoundly different. As will be reviewed, while $p56^{lck}$ and related SFKs phosphorylate a broad spectrum of substrates needed for the phosphorylation cascade, ZAP-70 phosphorylates only a few known candidates to date, such as LAT (linker of activated T

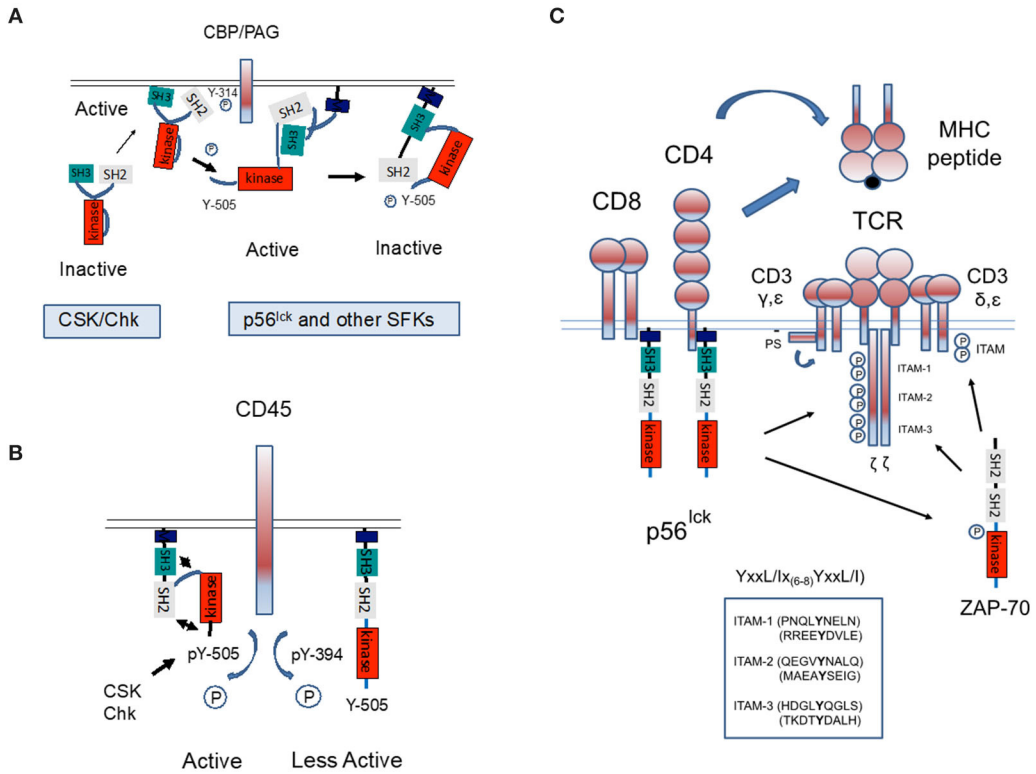

FIGURE 2 | Regulation of p56lck and phosphorylation of the TCR complex. **(A)** Regulation of p56lck kinase activity. The SH2 domain of p56lck binds to the C-terminal inhibitory Y-505, an interaction aided by SH3 domain binding to proline residues. These interactions hold the structure in a closed inactive conformation. Phosphorylation of the C-terminal Y-505 is inhibitory, while the dephosphorylation at Y-505 unfolds the kinase, unleashing its full catalytic activity accompanied by auto-phosphorylation of Y-384 within the catalytic domain. In this context, C-terminal protein kinase and related CSK-homologous kinase (Chk) bind to the anchoring protein CBP/PAG and inactivates p56lck by phosphorylation on Y-505. **(B)** The transmembrane protein phosphatase CD45 counterbalances the effect of CSK by preferentially dephosphorylating the inhibitory Y-505 tyrosine. However, CD45 can also dephosphorylates Y-394 to dampen kinase activity. The relative effects on Y-505 and Y-394 may be temporally regulated. **(C)** Model whereby CD4 and CD8-p56lck phosphorylate ITAMs on the TCRζ and CD3γ, δ, ε chains. During antigen-presentation by antigen-presenting cells (i.e., dendritic cells), coordinate binding of CD4/CD8 and the TCR to MHC antigens would bring p56lck into proximity where trans-phosphorylation would occur. p56lck also phosphorylates and activates ZAP-70.

cells) and SLP-76 (SH2-domain-containing leukocyte protein of 76 kD). This fits with the notion that the p56lck is responsible for the main wave of tyrosine phosphorylation cascade of numerous substrates that includes ZAP-70 with a more specialized function in phosphorylating a limited additional number of key substrates needed for specific functions such as calcium mobilization.

Part of the overall cascade includes immune cell-specific adaptors, proteins that lack enzymatic activities, and instead are made up of domains or sites that mediate complex formation (Rudd, 1999). They are considered types of molecular switches which integrate proximal signaling with downstream events. Key examples include LAT, SLP-76, ADAP (adhesion and degranulation-promoting adapter protein, also known as Fyn-binding protein [Fyb] or SLP-76 associated protein of 130 kD [SLAP-130]) and SKAP1 (or SKAP-55, Src kinase-associated phosphoprotein of 55 kDa; **Figure 3A**).

LAT, as first identified by the lab of Larry Samelson at the NIH, is a transmembrane adaptor with multiple tyrosine residues that binding SH2 domain carrying mediators, phospholipase Cγ1 (PLCγ1) (Y-132) and the small adaptors, Growth factor receptor-bound protein 2 (GRB-2) (Y-171, 191, and 226), and GRB2-related adapter protein 2 (GADs) (Y-171 and 191) (Zhang et al., 1998, 2000). ZAP-70 phosphorylates LAT at all sites needed for recruitment (Bunnell et al., 2000; Zhang et al., 2000). Mutation of individual sites does not prevent GRB2 binding, while the double mutation of Y-171 and Y-191 abolishes GADs binding. Overall, there is cooperativity in the binding of different molecules, including PLCγ1 (Cho et al., 2004). Significantly, LAT deficient Jurkat cells show normal phosphorylation of the TCR complex and ZAP-70 activation, but are defective downstream in the activation of PLCγ1, extracellular-signal-regulated kinases (ERKs) as well as interleukin 2 transcription (Finco et al., 1998). Further, *Lat*$^{-/-}$ mice showed defects in thymic differentiation with a block at the double negative 3 stage (Samelson et al., 1999). The GADs SH3 domain binds to SLP-76 with an unusually high avidity (Berry et al., 2002), bringing the complex with SLP-76 into the LAT signalosome (**Figure 4**).

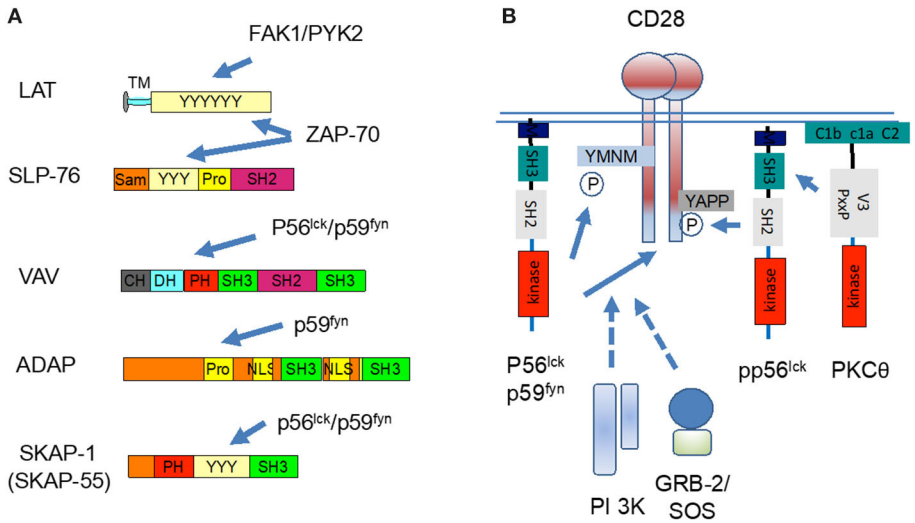

FIGURE 3 | p56lck regulates the function of immune adaptors and CD28 co-stimulation. **(A)** p56lck and related p59fyn phosphorylate immune specific adaptors or molecular scaffolds. These include LAT, SLP-76, VAV, ADAP, and SKAP1. The C-terminal SH2 domain SLP-76 binds to the ADAP, while ADAP binds to SKAP1. **(B)** p56lck and p59fyn phosphorylate the cytoplasmic tail of CD28 YxxM site for the binding of PI 3K and GRB-2/ SOS. CD28 also have a more distal YAPP site which when phosphorylated, binds to the SH2 domain of p56lck. The V3 domain of PKC-θ, in turn, binds to CD28 via binding to Lck. CD28 and PKCθ co-localize and act as markers for the c-SMAC.

Recently, we uncovered an unexpected connection between integrin signaling and LAT phosphorylation (Raab et al., 2017). LFA-1 ligation and crosslinking activated the protein-tyrosine kinases FAK1 and PYK-2 to phosphorylate LAT at a single site at Y-171. The specificity and fidelity of phosphorylation was remarkable as it was seen in *in vitro* and *in vivo* assays. Further, the specificity of FAK1 and PYK-2 contrasts with ZAP-70 phosphorylation of the three LAT sites. It appeared to compete with the action of ZAP-70 acting mostly in the actin-rich periphery of the contact area of T-cells and recruited GRB-2-SKAP1 in the control of adhesion (Raab et al., 2017).

SLP-76, first identified by Jackman et al. (1995), has an N-terminal sterile-α motif (SAM) and a carboxy-terminal SH2 domain that binds to ADAP (da Silva et al., 1997a; Musci et al., 1997) and the hematopoietic progenitor kinase-1 (HPK-1) (Di Bartolo et al., 2007). SLP-76 is needed for phospholipase Cγ1 (PLCγ1) activation, calcium mobilization and thymic differentiation (Jordan et al., 2003). We and others showed that ZAP-70 also phosphorylates SLP-76 at two specific sites (Y113 and Y128) (Bubeck Wardenburg et al., 1996; Raab et al., 1997). p59fyn was also found to phosphorylate the adaptor with unclear consequences (Raab et al., 1997). Lastly, in an unexpected manner, following TCR ligation, we have found that SLP-76 interacts with RanGAP1 of the nuclear pore complex where it promotes NFAT and Nfkb entry into the nucleus (Liu et al., 2015).

A key hallmark consequence of LAT phosphorylation is the phospho-activation phospholipase Cγ-1 (PLCγ1) (Samelson et al., 1995). PLCγ1 phosphorylation is regulated by protein tyrosine kinase-mediated phosphorylation induced by TCR ligation (Mustelin et al., 1990), however, the molecular steps involved had been unclear for decades. Early studies had shown that the loss of SLP-76 was associated with a selective loss of PLCγ1 and calcium mobilization in T-cells (Yablonski et al., 1998). It was then shown that LAT docking of PLCγ1 and SLP-76 facilitates the binding of another kinase, IL-2-inducible T-cell kinase (ITK), which phosphorylates PLCγ1 for activation (Berg et al., 2005). ITK-related resting lymphocyte kinase (RLK) also contributes (Sommers et al., 1999; Schneider et al., 2000). In fact, the deletion of both ITK and RLK eliminates PLCγ1 activity accompanied by defects in calcium flux following TCR engagement (Schaeffer et al., 1999). These discoveries unraveled a longstanding puzzle in T-cell signaling. Activation of PLCγ1 results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 3,4,5-triphosphate (IP3). DAG activates protein kinase C (PKC-θ) and RAS guanyl nucleotide-releasing protein (RASGRP) for activation of the $p21^{ras}$ and ERK pathways (**Figure 4**). IP_3 binds to calcium permeable ion channel receptors (IP3R) in the endoplasmic reticulum (ER) which releases the ion into the cytoplasm. The ER also detects intracellular Ca^{2+} through stromal interaction molecule (STIM). Intracellular Ca^{2+} depletion triggers an influx from outside the cells as mediated by calcium-release activated calcium (CRAC) channel. Increased intracellular Ca^{2+} activates the phosphatase, calcineurin, which in turn dephosphorylates the nuclear factor of activated T cells (NFAT) for entry into the nucleus (Jain et al., 1992). Overall, CD4/CD8-p56lck phosphorylation of the TCR/CD3 subunits sets in motion a cascade where ZAP-70 is recruited leading to the phosphorylation of LAT for PLCγ activation, the mobilization of calcium and the translocation of NFAT into the nucleus of T-cells.

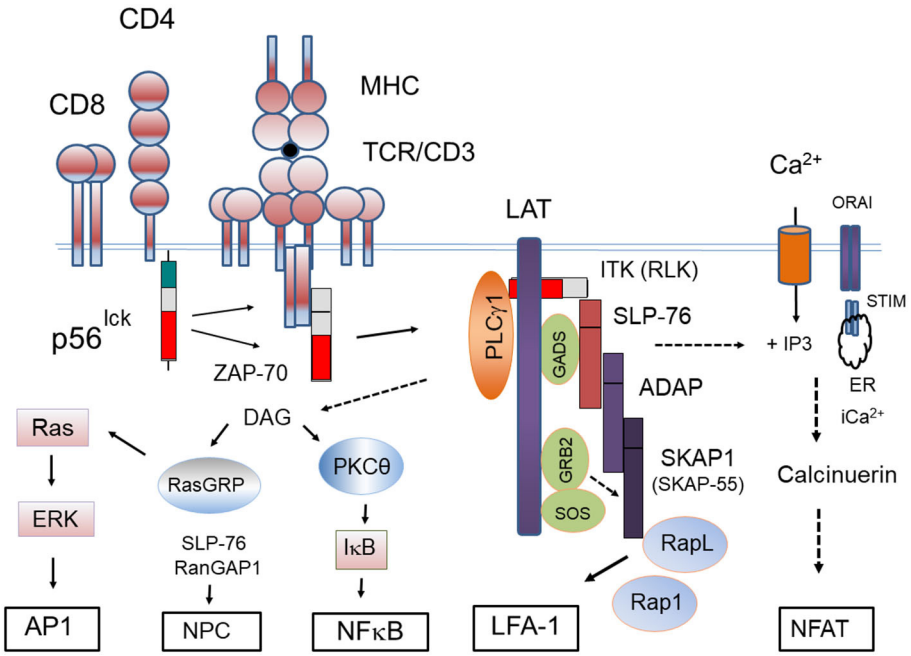

FIGURE 4 | Proximal signaling complexes and downstream responses initiated by the CD4/CD8-p56^{lck} complexes. Model outlining CD4/CD8-p56^{lck} initiation of the protein-tyrosine activation cascade. CD4/CD8-p56^{lck} phosphorylation of TCR ITAMs leads to the recruitment and activation of ZAP-70 followed by its phosphorylation and formation of the LAT signalosome. pLAT recruits several SH2-domain-containing proteins, including phospholipase Cγ-1 (PLCγ1) growth factor receptor-bound protein 2 (GRB2) and GRB2-related adaptor protein (GADS). Through its constitutive association with GADS, SLP-76 constitutively associates with LAT. Associated IL-2-inducible T-cell kinase (ITK) and resting lymphocyte kinase (RLK) phospho-activate PLCγ1 resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP_3 production leads to increases of intracellular free Ca^{2+} concentration, whereas DAG can activate both protein kinase C- (PKC-θ) and RAS guanyl nucleotide-releasing protein (RASGRP). IP_3 generated from PIP_2 binds to the Ca^{2+-}permeable ion channel receptors (IP3R) in the endoplasmic reticulum (ER) releasing Ca^{2+} from within ER stores to the cytoplasm. The ER senses intracellular Ca^{2+} levels through stromal interaction molecule (STIM). Depletion of intracellular Ca^{2+} triggers an Ca^{2+}influx from Orai1 type plasma membrane calcium-release activated calcium (CRAC) channel. Increased intracellular Ca^{2+} activates a protein phosphatase, calcineurin, that dephosphorylates the nuclear factor of activated T cells (NFAT) for its nuclear translocation. pLAT also recruits the SH2 domain of GRB2 and GRB2-associated RAS guanosine nucleotide-exchange factor (GEF), son-of-sevenless (SOS) to activate $p21^{RAS}$. Tyrosine-phosphorylated SLP-76 also associates with the immune cell adaptors ADAP and SKAP1. SKAP1 controls the formation of the Rap1-RapL complex needed for LFA-1 activation. SLP-76 also interacts with RanGAP1 in the nuclear complex for the increased transport of transcription factors NFAT and NFkb into the nucleus.

Further, calcium may bind and neutralize PS facilitating the release of the cytoplasmic CD3 and zeta chains from the inner leaflet of the plasma membrane (Shi et al., 2013). The association normally protects ITAMs from spontaneous phosphorylation (Xu et al., 2008; Ma et al., 2017). However, with activation, antigen-receptor ligation would render ITAMs more accessible to p56^{lck} (Gil et al., 2002).

We and others have shown that ZAP-70 phosphorylates SLP-76 at residues Y-113 and Y-128 for binding to the guanine nucleotide exchange factor (GEF), VAV-1 and another adaptor NCK (Bubeck Wardenburg et al., 1996; Raab et al., 1997, 2001; Michel et al., 1998; Rudd and Raab, 2003). VAV-1 is a member of the Dbl GEF family with activity against for the Rho family of GTP binding proteins. GEFs activate by catalyzing the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). Effectors of Vav1 include RhoA, Rac1, and Cdc42 which play central roles in cytoskeleton organization, cell polarity and movement. p59^{fyn}, p56^{lck}, and ZAP-70 phospho-activate VAV-1 activity (Michel et al., 1998). Vav cooperates with CD28 to induce NF-kB activation via a pathway involving Rac-1 and mitogen-activated kinase (Marinari et al., 2002). The activation of protein kinase B (PKB/AKT) and glycogen synthase kinase-3 (GSK-3) operates independently of VAV-1 (Wood et al., 2006).

Further along the cascade, my lab and others showed that SLP-76 binds to the immune cell adaptor ADAP which, in turn, binds to another immune cell adaptor, SKAP1 (or SKAP55) (da Silva et al., 1997a; Wang et al., 2004; Kliche et al., 2006). SKAP1 had a unique N terminus, a PH domain and a C terminal SH3 domain (Marie-Cardine et al., 1997). The C-terminal SH2 domain SLP-76 binds to the ADAP (da Silva et al., 1997a; Musci et al., 1997; Liu et al., 1998; Veale et al., 1999), while ADAP binds to SKAP1 (Marie-Cardine et al., 1997; Liu et al., 1998). SKAP1 SH3 domain binds to proline residues in ADAP, while the ADAP-SH3-like domain binds to SKAP1 (Kang et al., 2000; Kliche et al., 2006). SKAP1 is an effector in the pathway such that the Rap1-RapL complex fails to form in *skap1*$^{-/-}$ T cells, which correlates with reduced LFA-1 binding to ICAM-1 and T-cell adhesion to dendritic cells (DCs) (Wang et al., 2003; Raab et al., 2010, 2011,

2018). Rap1 also interacts with Rap1-GTP-interacting adaptor molecule (RIAM) which controls recruitment of the cytoskeletal protein and integrin-binding protein, talin, to the membrane (Lafuente and Boussiotis, 2006). In this manner, SKAP1 and RIAM couples the TCR to the activation of the integrin, LFA-1 which is needed to promote the binding of T-cells to antigen-presenting cells (Wang et al., 2003, 2004, 2009; Menasche et al., 2007).

OTHER SUBSTRATES

$p56^{lck}$ and ZAP-70 differ in their phosphorylation specificities. $p56^{lck}$ phosphorylates a wide range of downstream targets that regulates functions as diverse as cell movement, cell cycle, metabolism, cell to cell interactions, morphology, protein synthesis, and gene expression. The main problem in identifying SFK substrates has been the reliance on the use of oncogenic forms of *src* kinases. These versions of the kinases are likely unreliable since their constitutive kinase activities allow for the phosphorylation of secondary targets not engaged by the non-oncogenic forms of the kinase. To this end, elegant add-back experiments have been conducted with c-src (Amanchy et al., 2009; Ferrando et al., 2012). With the qualifier that *c-src* is not palmitoylated, these studies are likely to give an idea of the range of substrates engaged by $p56^{lck}$ since the kinase domains of $pp60^{c-src}$ and $p56^{lck}$ are highly conserved. As seen in **Table 1**, c-src substrates include epidermal growth factor receptor substrate 15 (Eps15) with a role in the assembly of clathrin-coated pits, Tripartite motif protein 28 (TRIM28) involved in transcriptional regulation, cellular differentiation and proliferation, DNA damage repair and apoptosis, Xanthine dehydrogenase (XDH) involved in the oxidative metabolism of purines, Seryl-aminoacyl-tRNA synthetase 1, Guanine monophosphate synthetase eEF 2, and Threonyl-tRNA synthetase involved in protein translation, the protease Calpain 2 and Unc-84 homolog, a nuclear envelope protein. Others include Heat shock protein 9A and Stress-induced phosphoprotein 1 and Heat shock protein 1 (chaperonin) (Amanchy et al., 2009; see **Table 1**). Further, others include cytidine 5-triphosphate (CTP) synthase phosphorylation on multiple sites (Huang and Graves, 2003), pyruvate kinase 3 (type M2) (Eigenbrodt et al., 1992), and valosin containing protein (VCP) which involved in the proteolytic degradation of misfolded proteins (Song et al., 2008). Further, there are phospho-targets involved in adhesion such as Talin, Tensin1-2, FAK, and p130Cas and others involved in actin remodeling as well as others, such as filamin B, ABLIM1, and PARD3 that regulate cell polarity. C3G is a guanine nucleotide exchange factor for the small Ras-related G-proteins Rap1, Rap2, and R-Ras (Ferrando et al., 2012; Sasi Kumar et al., 2015). Rap1 is a small G-protein of the Ras family that antagonizes Ras in some cells (but not T-cells) (Sebzda et al., 2002), and has been implicated in SKAP1 activation of integrin adhesion in T-cells (Raab et al., 2010). CasL, DOK1, and GAB1 are also putative targets. Overall, SFKs intersect in the regulation of FAK, integrin, PAK and PTEN signaling, amongst others (Ferrando et al., 2012). Although targets will vary depending on the localization of each kinase, this approach provides a hint of the array of substrates in the CD4 and CD8-$p56^{lck}$ initiated phosphorylation cascade, linked to functions as diverse as translation, gene expression and metabolism in T-cells.

CD8α/α vs. CD8α/β

As mentioned, the CD8 coreceptor is expressed as an α/α homodimer and an α/β heterodimer. It is the α chain of the CD8 complex that binds to major histocompatibility complex leukocyte antigens (Gao et al., 1997) and non-classical MHC antigens such as the human histocompatibility leukocyte antigen G found on trophoblast cells (Sanders et al., 1991). With two chains to bind to $p56^{lck}$, CD8α/α has the potential to be hyper-stimulatory; however, paradoxically, we and others have found less kinase activity associated with this form of the co-receptor. The molecular basis for this is not known but might involve conformational or trans-phosphorylation issues. Trans-phosphorylation occurs between separate receptors, but within the same covalently linked receptor complex, autophosphorylation might become disordered in some manner.

Similar to other activation antigens such as CTLA-4, CD8α expression is induced by TCR ligation proportional to the strength of signal. In the case of CD8α/β, it is expressed at higher levels in T-cell lines sensitive to TCR engagement (Cawthon et al., 2001) and down-regulated in response to an altered peptide ligand (Barnden et al., 1997). Further, CD8β couples the TCR/CD3 complex to rafts (Arcaro et al., 2001). By contrast, the expression of CD8α/α decreases the functional avidity of TCRs and reduces activation (van Oers et al., 1993). Furthermore, unlike in the case of activation-induced co-internationalization of TCR and the CD8α/β complex, CD8α/α is excluded from lipid rafts (Pang et al., 2007). In one model, CD8α/α sequesters $p56^{lck}$ from rafts leading to a reduction in the TCR phosphorylation. Collectively, this has led to the hypothesis that CD8α/α may act an inhibitory receptor, possibly antagonizing the function of CD8α/β in promoting activation (Cheroutre and Lambolez, 2008). The antagonism may promote the differentiation of activated lymphocytes into memory CD8 T cells (Madakamutil et al., 2004).

$p56^{lck}$ AND CD28 MEDIATED CO-STIMULATION

Although initially discovered in the context of TCR signaling, subsequent work implicated the $p56^{lck}$ and related $p59^{fyn}$ in later stages of the activation process. T-cells are activated by the antigen receptor followed by a "second signal" provided by the co-receptor CD28 and others (June et al., 1994; Rudd, 1996). In this vein, we showed that $p56^{lck}$ and $p59^{fyn}$ phosphorylate the cytoplasmic tails of CD28 and CTLA-4 (Rudd and Schneider, 2003; Rudd et al., 2009; **Figure 3B**). They phosphorylate the YxxM sites of both receptors, an event needed for the binding of lipid kinase, phosphoinositide 3-kinases (or phosphatidylinositol 3 kinases; PI 3K), and in the case of CD28, the adaptor complex, GRB-2/Son of Sevenless (SOS) (Prasad et al., 1994; Raab et al., 1995; Schneider et al., 1995a,b). PI 3K, in turn, catalyzes the production of PI-3P from PI and PI 3,4-P2 from PI 4P,

TABLE 1 | p56lck predicted substrates.

Adhesion	Kinases	Cellular functions	Adaptors	Functions
Talin	Hck	Eps15	GAB1	Cell movement
KIRREL1	ERK1/2	Tripartite motif protein 28 UAP1 like-1 Xanthine dehydrogenase	Cas-L	Cell cycle
PCDH19	ICK	Seryl-aminoacyl-tRNA synthetase 1	PZR	Metabolism
Tensin1-2	PIK3R2	Calpain 2	DOK1	Cell to cell interactions
MAGI1	ARG	Unc-84 homolog	ABI1/2	Cell morphology
PXN		Heat shock protein 9A Threonyl-tRNA synthetase	IRS1	Protein synthesis
FAK		Stress-induced phosphoprotein 1	ANKS1	Gene expression
p130Cas		Guanine monophosphate synthetase eEF 2	CRKL	
		Calnexin	ZO-1	
		ATP citrate lyase	RaspL1	
		Heat shock protein 1	HGS	
		Cytidine 5-triphosphate (CTP) synthase	LPP	
		Pyruvate kinase 3 Valosin	SHC1	
			Shc1	
			LAT	
			SLP-76	
	PTPase			
Actin re-modeling and polarity	PTPRA			
Filamin B	**Others**			
ABLIM1	TTYH2			
PARD3	TMEM106B			
PARD3B	ZDHHC8			
	P53			
	ST5			
	Tenacin			
	PGAM1			
	RPL15			
GEF/GAP				**Other kinases**
GIT1/2				FAK signaling
ARHGAP32				Integrin signaling
C3G				Ephrin signaling
				ERL signaling
				PAK signaling
				PTEN signaling

Adapted from Amanchy et al. (2009) and Ferrando et al. (2012).

a phospholipid that recruits plextrin homology (PH) domain carrying proteins to the plasma membranes. Mutations that affect the levels of PI 3K binding also influences the efficacy of CD28 internalization and removal from the cell surface (Cefai et al., 1998). In this manner, PI 3K is needed for many cellular functions including cell proliferation, endocytosis, differentiation, survival and motility. The p56lck SH3 domain also binds to the p85 subunit of PI 3K thereby bridging of protein tyrosine and lipid kinase pathways in T-cells (Prasad et al., 1993a,b; Kapeller et al., 1994).

The promotion of GRB-2/SOS binding to CD28 by p56lck creates a further link to the p21ras pathway. SOS is a GEF that activates p21ras which, in turn, activates the ERK pathway (Drosten and Barbacid, 2020). p21ras is mutated resulting in a constitutive active protein in 50% of colorectal tumors. In T-cells, to date, GRB-2/SOS complex has been found associated with LAT and CD28. In the case of LAT, it is mediated by ZAP-70 and FAK/PYK2, while the binding to CD28 is mediated by p56lck and p59fyn. p56lck and p59fyn, therefore, orchestrate the second co-stimulatory step of T-cell activation. This step is followed by CD28 de-phosphorylation needed for the binding of clathrin-linked AP2 complex and endocytosis (Schneider et al., 1999).

Further, CD28 also possesses a more distal key tyrosine which in a phosphorylated form binds to the SH2 domain of p56lck (Kong et al., 2011). The lab of Amnon Altman elegantly showed that the V3 domain of PKC-θ, in turn, binds to CD28 via binding to p56lck. Classically, the PKC-θ co-localize and acts as a marker for the central supramolecular signaling cluster (cSMAC) at the center of the interface of T-cells activated with antigen-presenting cells (Shaw and Dustin, 1997; Monks et al., 1998; Freiberg et al., 2002). This pathway implicates CD28 in PKC-θ mediated downstream signaling and the differentiation of T helper type 2 cells (Th2 cells) and interleukin 17-producing helper T cells (Th17 cells), but not of T helper type 1 cells (Th1 cells) (Kong et al., 2011).

p56lck AND CELL ADHESION

Another area involved in the protein-tyrosine phosphorylation cascade involves the "inside-out" pathway by which the antigen-receptor activates integrin adhesion. Adhesion is mediated by LFA-1 and other integrins and is of central importance to T-cell

responses. It controls migration within lymph nodes and to sites of infection and mediates binding to antigen-presenting dendritic cells. In this regard, mice with ablated SKAP1 or its binding partner ADAP have normal numbers of T and B-cells, but they are defective in integrin-mediated adhesion (Griffiths et al., 2001; Peterson et al., 2001; Wang et al., 2007, 2009). In the adhesion pathway, SKAP1 is the effector due to its regulation of RapL-Rap1 complex formation (Raab et al., 2010, 2011, 2018). This pathway accounts for some 40–50% of LFA-1 adhesion and contributes to the "slowing" of T-cells for stable interactions with dendritic cells (Wang and Rudd, 2008; Raab et al., 2010).

p56^{lck} DIFFERS FROM p59^{fyn}

Despite similarities, it is noteworthy that differences exist in the substrates targeted by different p56^{lck} and other SFKs in immune cells. Specifically, p56^{lck} and p59^{fyn} have overlapping and distinct functions. p59^{fyn} can partially substitute for p56^{lck} in T lymphocyte development (Groves et al., 1996) and effector function (Filby et al., 2007); however, p59^{fyn} promotes signals induced by TCR antagonists (Tang et al., 2002) and can inhibit cytokine production and proliferation. Indeed, *p59$^{fyn-/-}$* T-cells are more readily activated, produce more cytokines, and undergo more cell divisions than wild-type T-cells (Filby et al., 2007). Further, unlike p56^{lck}, p59^{fyn} only weakly affects Ca^{2+} mobilization, although it can stimulate the ERK/MAPK pathway (Lovatt et al., 2006).

It is not clear how this might be operating, however, importantly, the work from several groups has shown that the two kinases preferentially phosphorylate different substrates. We initially identified ADAP as a preferred substrate and binding partner of p59^{fyn} (hence, it's origin name FYB for Fyn binding protein) (da Silva et al., 1997a,b; Musci et al., 1997; Veale et al., 1999). Kliche and Schraven found that it's binding partner SKAP1 was also preferentially phosphorylated by p59^{fyn} (Marie-Cardine et al., 1997). As mentioned, SKAP1 and ADAP couple the TCR to the activation of integrins (Griffiths et al., 2001; Peterson et al., 2001), while ADAP has an additional role in the activation of the proinflammatory transcription factor, Nfκb (Medeiros et al., 2007). In fact, a mutant of ADAP defective in binding SLP-76 blocks Nfκb driven HIV-1 transcription and cell-cell viral spread (Wei et al., 2013). Lastly, we showed that SKAP1 acts a scaffold for Polo-like kinase 1 (PLK1) for the optimal cell cycling of T-cells (Raab et al., 2019). Whether the differences in p56^{lck} and p59^{fyn} phospho-targets is due to a distinct structural tropism of the kinase domain for different substrates, or simply reflects difference in intracellular localization is unclear. It, therefore, may be that TCR signals bifurcate into a p56^{lck} driven pathways that primarily regulate proliferation and another, p59^{fyn} pathway which preferentially activates integrin mediated adhesion.

OTHER MECHANISMS FOR p56^{lck} FUNCTION

Despite its importance in signaling in most T-cells, there exists a subset of peripheral T-cell lacking CD4 and CD8 which can be activated via the TCR (D'Acquisto and Crompton, 2011). This begs the question of whether the TCR can also bind to p56^{lck} and whether receptor-free p56^{lck} also plays in role in activation. The unique domain of p56^{lck} has been reported to interact with the CD3ε subunit in the TCR-CD3 complex (Li et al., 2017), while Hartl et al. have reported that non-canonical binding of the lck SH3 domain to the (RK) motif in the CD3ε cytoplasmic tail (Hartl et al., 2020). The RK motif becomes accessible upon TCR ligation, presumably free from interactions with PS molecules in the inner face of the lipid bilayer leading to lck recruitment. This has been reported to increase p56^{lck} activity, CD3 phosphorylation, thymocyte development, and T cell activation (Hartl et al., 2020).

In another model, p56^{lck} unbound to receptors has been found also to play roles in in signaling. Free p56^{lck} was reported by the lab of Nick Gascoigne to be more active than co-receptor bound (Wei et al., 2020). Interestingly, imaging studies showed that free p56^{lck} was recruited to the TCR complex and triggered TCR signaling earlier than the co-receptor-bound p56^{lck} (Nika et al., 2010). The exact temporal nature of involvement of free p56^{lck} relative to co-receptor-bound p56^{lck} in responses of different cells to different affinity ligands needs to be clarified. It may be that some free kinase tweaks the system to then allow CD4 and CD8-p56^{lck} to drive the cascade due to their coordinate interactions with the TCR with the MHC antigens.

OTHER PROTEIN TYROSINE KINASES

The notion of a T-cell protein-tyrosine kinase driven phosphorylation cascade led to a flurry of activity to discover other tyrosine kinases and downstream targets in T-cells. It also led to a major effort by pharmaceutical companies to develop kinase specific inhibitors for the treatment of autoimmunity and inflammatory conditions. Aside from the previously mentioned ZAP-70, a second family protein tyrosine kinases termed TEC kinases were uncovered, interleukin 2 inducible T-cell kinase (ITK) and resting lymphocyte kinase (RLK). ITK modulates the development, function and differentiation of conventional T-cells and non-conventional NKT-cells (Schwartzberg et al., 2005). When APCs activate TCR, phosphorylation events lead to the production of D3 lipids and recruitment of ITK to the cell membrane, where it is phosphorylated by p56^{lck}. By contrast, unlike p56^{lck}, ITK is not needed for CD28 signaling (Li and Berg, 2005). As mentioned, once it is activated, ITK phosphorylates PLCg1 and the mobilization of calcium. ITK operates at later stages of the cascade (Berg et al., 2005) where *Itk$^{-/-}$* mice fail to mount responses to T_H2-cell-inducing pathogens. By contrast, mice overexpressing RLK skew differentiation toward the T_H1-cell lineage. Several studies have also implicated ITK in actin reorganization and cell polarization (Schwartzberg et al., 2005).

Another key family of downstream protein tyrosine kinase includes FAK1 (Focal Adhesion Kinase 1) and PYK2 (proline-rich tyrosine kinase-2). FAKs are comprised of an N-terminal FERM (band 4.1, ezrin, radixin, moesin homology) domain, a linker region, a kinase domain, a large proline-rich region, and a C-terminal focal adhesion targeting domain (Lietha et al., 2007).

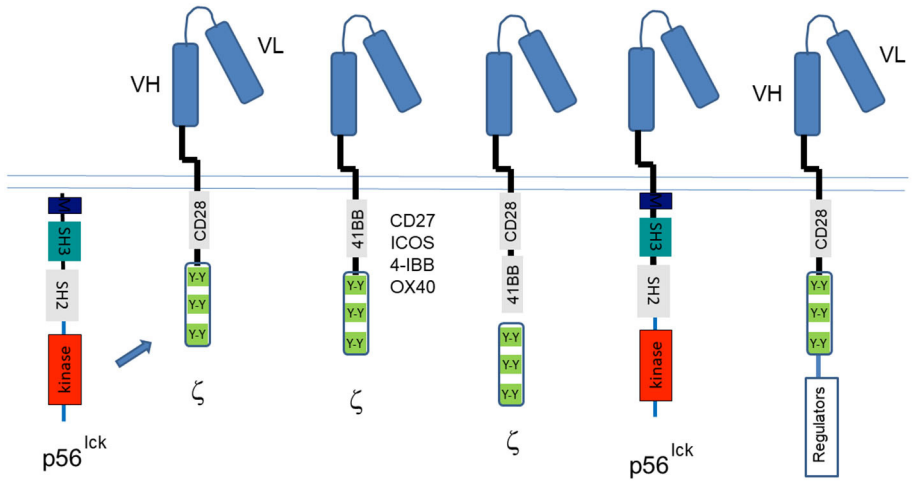

FIGURE 5 | Chimeric antigen receptor (CARs) designed by use of the targets of CD4/CD8-p56lck. The discovery of the CD4/CD8-p56lck initiated tyrosine phosphorylation cascade led to the identification of ITAMs and CD28/ICOS/CTLA-4 motifs needed for the activation of T-cells and the preservation of cell survival. The past years have seen many iterations of CARs that began with the Ig ectodomain linked to TCRζ or CD3 ITAMs followed by the inclusion of CD28 cytoplasmic tails (and partial ectodomains). Both the TCRζ and CD3 ITAMs and the CD28 tyrosines are phosphorylated by p56lck. New iterations have included CD27, ICOS, 41-BB, and OX40 motifs in conjunction with ITAMs, dual CD28, and 41BB motifs with ITAMs, the direct coupling to p56lck and the bicistronic inclusion of CD28-ITAMs with the expression of intracellular regulators of metabolism in the tumor microenvironment and in other events in T-cells.

FAK auto-phosphorylation at the Tyr-397 site is needed for kinase activation and binds to the SH2-domain of p60Src kinase (Arnold et al., 2005). The FERM and kinase domains form an auto-inhibitory interaction (Lietha et al., 2007) which is released in focal adhesions (Arnold et al., 2005). In this context, focal adhesion kinases regulate focal adhesion contacts, motility, and cell survival (Schaller et al., 1992). In T-cells, TCR engagement promotes FAK and PYK2 phosphorylation and translocation to the IS (Sancho et al., 2002; Ostergaard and Lysechko, 2005; Collins et al., 2010). As mentioned, we also recently found that FAK1 and PYK-2 phosphorylate a single specific site on the adaptor LAT for GRB-2 binding and T-cell adhesion (Raab et al., 2017). Non-lymphoid cells from FAK-deficient mice show enhanced focal adhesion contact formation and reduced cell motility (Lietha et al., 2007).

p56lck AND CHIMERIC ANTIGEN RECEPTORS (CARs)

Aside from T-cell activation, the discovery of CD4/CD8-p56lck and its phospho-targets such as ITAMs and CD28 motifs led to the application of this knowledge to the design of chimeric antigen receptors (CARs) (Abate-Daga and Davila, 2016; Kawalekar et al., 2016; Maus and June, 2016). Originally called "T bodies," almost 30 years ago, by Gross et al. (1989), CARs use antigen-recognition domains derived from an antibody or other proteins that are linked to a transmembrane domain and a intracellular cytoplasmic tail that contains the ITAMs from CD3 or TCR-zeta cytoplasmic tails (**Figure 5**). The function of these ITAMs is regulated by p56lck; however, T-cells expressing first-generation CARs with only ITAMs proved to be short-lived. Instead, additional CD28 "co-signals" were needed to enhance cell survival and *in* anti-tumor killing (June et al., 1994; Rudd, 1996; Finney et al., 1998). As originally seen in the nerve growth factor receptor (Yao and Cooper, 1995), PI-3K to CD28 and CTLA-4 generates survival signals for T-cells (Okkenhaug et al., 2001; Schneider et al., 2008; Rudd et al., 2009). Subsequent variations of CARs contain 4-1BB–derived (Tammana et al., 2010), CD27-derived (Song et al., 2012), OX40-derived (Hombach et al., 2012), or ICOS-derived (Shen et al., 2013) costimulatory sequences. T cells engineered to express CARs with tumor specificity have been remarkable in treating patients with hematologic malignancies in combination with adoptive cell therapy. Their therapeutic success is limited in the case of solid tumors requiring new approaches to address the biology within the tumor microenvironment (TME). To this end, next generation CAR-Ts include bycistronic vectors expressing modulators of the TME. Others have used different exodomain spacers and hinge regions (Watanabe et al., 2016), where the length of the CAR endo-domains determine their ability to interact with endogenous signaling molecules (Ramello et al., 2019). Carl June, a frequent attendee at our signal transduction meetings, has pioneered the use of many CAR-Ts in the treatment of patients (Posey et al., 2016). Some new CAR-Ts are being developed with simultaneous triple genome editing by adding the disruption of PD1 to enhance *in vivo* antitumor activity of the gene-disrupted CAR T cells (Ren et al., 2017). Others have used dual- specific T cells, expressing a CAR specific for tumor antigens, and TCR specific for a strong, tumor-unrelated immunogen (Chan et al., 2020).

Since CARs do not recognize MHC molecules, their reactivity of CAR-Ts is depends on active p56lck to phosphorylate ITAMs

and the tyrosine-based motifs within the CD28 co-receptor cytoplasmic tails. However, others have found that the optimal antigen response is dependent upon the incorporation of the receptor in endogenous TCR/CD3 complexes (Bridgeman et al., 2010). These novel approaches may eventually utilize CD4 and CD8 coupled p56lck in addition to free p56lck to promote CAR-T efficacy. Overall, the CAR field developed as a result of fundamental studies that led to the discovery of the TCR complex and the signaling motifs activated by p56lck and which are needed to activate T-cells.

SUMMARY

The discovery of the CD4 and CD8-p56lck complexes opened a window in understanding the nature of signals that control the immune response against antigens. This fundamental mechanism controls the T-cell response in the areas of vaccines, transplantation, autoimmunity, and cancer. They were the first examples of a receptor binding to protein-tyrosine kinase and showed how immune recognition receptors which lack intrinsic catalytic activity can transduce activation signals via non-covalent association with non-receptor tyrosine kinases. Sometimes called the TCR signaling paradigm, the discovery established that the concept that a protein tyrosine phosphorylation cascade operated in T-cells and opened the door to the identification of other protein-tyrosine kinases such as ZAP-70 and an array of substrates such as immune cell adaptors that are now central to studies in T-cell immunity. Other receptors such as B-cell receptor, Fc receptors and others were also subsequently found to use *src* kinases to control cell growth. Moreover, the discovery of CD4/CD8-p56lck and its targets ITAMs and CD28 has led to the application of this knowledge in the design on CARs presently in use in cancer immunotherapy.

AUTHOR CONTRIBUTIONS

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

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